THÈSE PRÉSENTÉE

POUR OBTENIR LE GRADE DE

DOCTEUR DE

L'UNIVERSITÉ DE BORDEAUX

ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTÉ

SPÉCIALITÉ NEUROSCIENCES

Par Margaux TEIL

Etude des mécanismes et cibles thérapeutiques des synucleinopathies

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Soutenue le 25 Novembre 2021

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"We are such stuff as dreams are made on."

For always making me reach for the stars, Dad, this is for you.

Acknowledgments – Remerciements

First, I would like to thank the members of my jury, Dr. Laura Parkkinen, Prof. Francois Tison, and Dr. Tiago Outeiro for taking the time to evaluate this PhD, I am truly honored that you accepted to read and report on my work over the last few years. I would also like to thank Dr. Parkkinen and Dr. Outeiro for traveling all this way for my thesis and for your contributions to this field of work. I would also like to thank the Ministère de la Recherche for the funding to complete this thesis.

Je souhaiterai tout particulièrement remercier Dr. Benjamin Dehay d'avoir été le directeur que tous les étudiants espèrent pendant leur thèse. Ben, ces trois dernières années ont été remplies d'obstacles, aussi bien scientifiques que personnels, et tu as toujours su me guider et m'accompagner sur cette route. Merci d'avoir été là pour ces moments de questionnement scientifique, de frustration quand tout ne fonctionnait pas comme prévu, de rires, et de soutien général. Je pense que personne ne mérite de réussir plus que toi. Tu es intelligent, rigoureux et modeste et il me tarde de voir ce que te réserve les prochaines années. Merci aussi de m'avoir fait découvrir une autre facette de ta personne, le fan du gossip !

Je souhaiterai aussi vous remercier, Dr. Erwan Bézard, pour votre accueil au sein de l'IMN, et plus particulièrement au sein de l'équipe PSP, dès ma deuxième année de Master et pour cette thèse. Merci pour votre soutien dans les moments difficiles et votre accompagnement.

Maintenant, je voudrais remercier les autres personnes au sein de l'IMN qui m'ont accompagnée plus personnellement pendant cette thèse. Tout d'abord, les « meilleurs du labo », la team gériatrie : Marie-Laure, Evelyne, Marie-Laure, Audrey et Alain. Ces instants à la cafétéria avec vous, nos instants de pauses et nos fameux pique-niques ont apporté de la joie, des rires, et surtout un peu de légèreté dans ces dernières années.

Marie-Laure et Evelyne, mes anges gardiens, vous m'avez protégée et soutenue pendant ces plus de trois ans et je ne pourrais jamais assez vous en remercier. Madame Thiolat, merci de m'avoir laissée être ton parasite préféré pendant ces dernières années (pas que je compte arrêter d'être ton parasite, ne t'inquiète pas !). Tu as toujours su m'écouter, me soutenir et me conseiller. Merci pour ta patience quand je n'en avais plus, ta gentillesse quand j'étais à bout, et surtout ton honnêteté en toutes circonstances. Ces moments passés ensemble tous les trois avec Alain dans votre bureau resteront avec moi à jamais.

Evelyne, comme une maman, tu t'es occupée de moi et tu as veillé sur moi en permanence pendant cette thèse. Tu as toujours su quand j'avais besoin de parler un peu pour me libérer l'esprit. Comme vous le dites si bien avec Madame Thiolat, on voit tout dans mon regard, il a tendance à me trahir parfois ! Ces discussions où j'ai pu me livrer à toi et nos petits moments de complicité m'ont aidé à tenir le coup au long de cette thèse. On peut tous admettre que cette aventure a été pleine d'obstacles et tu as toujours su prendre de ton temps pour m'épauler. Merci de m'avoir fait rire avec tes moments d'absences quotidiennes et d'avoir animé les pauses-café.

Marie-Laure, ML, déjà merci d'avoir tenu le coup pendant que j'étais petite stagiaire et tout au long de cette thèse ! Catherine et Liliane ont su se faire remarquer à l'IMN (bon, pas toujours dans le bon sens mais ça ne serait pas drôle sinon !). Merci d'avoir été ma voisine dans tous les bureaux, de m'avoir toujours encouragée et de m'avoir donné un bel exemple pour cette thèse. Plus qu'au bureau, je voulais aussi te remercier pour ton soutien à l'extérieur. Merci pour les pauses à 16h pour se changer les idées, pour les verres en terrasse, les petits messages laissés sur le bureau pour m'encourager, et bien plus. Je finirai juste en disant : non aux tomates, vive les buenos !

Un grand merci à toi Audrey pour tous ces moments ensemble à discuter manip ou à discuter de choses plus personnelles. Tu es exceptionnelle et on ne pourra jamais assez te le dire. Tes filles ont une chance incroyable de t'avoir, et nous avons eu une chance incroyable de t'avoir parmi nous. Je sais que tu vas briller dans ta nouvelle aventure et j'espère que tu y trouveras tout ce que tu espères. Tu es la plus courageuse d'entre nous, ne l'oublie pas.

Alain, quelle rencontre j'ai fait en toi. Merci pour ces moments de sagesse et de gentillesse tous confondus. Merci d'avoir été un sourire permanent et pour tout le soutien que tu as su me donner au cours des épreuves. Et merci de nous avoir partagé tes pâtisseries, ta joie de vivre, et les chocolats de Pâques bien cachés sur le bureau. Ce n'est qu'à travers les pensées des autres que l'on ne part jamais. Tu me l'avais dit une fois, et tu avais encore raison.

Claire, nouvelle membre du bureau (enfin plus trop d'ailleurs), merci pour ces fous rires pendant les moments plus calmes au bureau ou au N4 à chercher un certain géant avec un long torse et des petits pieds. Merci pour les jours d'encouragements et les moments d'écoute scientifique et personnel. Merci de m'avoir boostée et pour ta bonne humeur générale, Coach Claire. J'espère quand même que j'aurai un post-it digne de ce nom pour la fin de cette thèse ! Et bon, pour te surprendre… J'ai faim !

Anthony, petit oisillon, compagnon pendant l'écriture, merci de t'être motivé pour venir à l'IMN de temps en temps. Tu aurais pu rester en chimie, mais bon, il y avait les petits déjeuners du vendredi et des fous rires à avoir (une fois que tu avais décidé qu'en fait tu nous aimais bien !). Tu as bien changé du petit oisillon qui n'osait pas m'interrompre ! Comme il se devait, on a commencé cette thèse en même temps et on la finira à une semaine d'écart. Il fallait bien se soutenir jusqu'au bout, guilli guilli. Bon vent en Suède, je viendrai te voir pour qu'on rigole encore un peu ensemble, Tut tut.

Pour continuer dans les membres du bureau, Morgane, merci pour le soutien pendant l'écriture de cette thèse. Merci de savoir que mon deuxième écran ne marche qu'un jour sur deux (comme mon cerveau à certains moments d'ailleurs). La thèse te réservera pleins de choses, de moments de joie et de frustration, mais ça en vaut la peine ! N'oublie pas « plus ça rate, plus on a de chance que ça marche ». Et j'attends encore la recette du fameux brownie !

Joanna, thanks for reminding me to put things into perspective and encouraging me during this end of PhD. Thanks for being the one that made us astronomical amounts of food for breakfast, for chocolate balls of happiness and for always checking up on me when I was writing, even from Barcelona!

Alexia, je m'abstiendrai d'écrire ton surnom, mais bon tu sais que je n'en pense pas moins. Merci d'avoir été ma voisine d'en face dans le bureau pour une grande partie de cette thèse (Béa et Xav forever), même si tu es insupportable, on le sait tous. Merci pour les pauses, de savoir quand il ne fallait pas parler de choses qui fâchent, tes blagues indécentes et de te moquer de tout (y compris de toi-même). Peut-être qu'un jour on finira par aller boire un verre ensemble, mais rien n'est moins sûr…

Merci à Sophie et Jérémy d'avoir été présents avec moi tout au long de ces épreuves : du stage de M2 jusqu'à la fin de la thèse ! On a réussi le concours ensemble, et on finira cette thèse plus ou moins ensemble (bon Sophie, on t'attend !). Louison, merci de toujours faire des bêtises avec Alexia et de remettre les choses en perspective parce qu'il t'arrive toujours plus de problèmes que les autres pendant une manip !

Nathalie B, Nathaliiie, merci pour les rires pendant nos galères sur le singe quand on apprenait ensemble, les petites danses en histo, ton aide générale et les petites conversations sur la vie.

Giulia, bellissima, merci pour les conversations et les moments complices, pour les soirées Spritz et restaurant italien ! Sinon, on en parle de Grace and Frankie ?

Merci pour tout le soutien que j'ai eu de l'équipe PSP et les autres membres de l'IMN : Mathieu, Julien, Anna, Florent, Vincent, Marie-Hélène, Nathalie D, Sandra, et bien d'autres ! Merci à Thomas Boraud, nouveau directeur de l'IMN, de m'avoir permis de commencer cette thèse. Merci à toute l'administration pour le soutien avec les commandes et les conversations (Faty, toujours dans mes pensées). Enfin, merci à tous les autres membres du laboratoire que je ne cite pas forcément mais qui m'ont soutenue pendant cette thèse.

A ma Mifa : Léa, Emma, Célia, Julien et JB, merci d'avoir été là pour moi ces dix dernières années. Léa, ma pastèque, bon tu sais ce que je pense de toi parce qu'on pense toujours la même chose ! Je ne pensais pas que je trouverai quelqu'un qui me ressemblerait autant et qui pouvait me comprendre dans tous mes aspects (le vert, c'est la vie). Merci pour ces moments partagés de la vie, à Lyon, à Bordeaux, ou n'importe où. Emma, ma Soufette, merci pour ces moments innombrables (elle est bonne ta pizza ?), d'avoir été ma première vraie amie sur Lyon, et d'avoir simplement toujours été fidèle à toi-même sans te soucier de ce que pensent les autres. Vive la Souf'mobile ! Célia, ma Vava, merci d'avoir été une présence calme et sereine dans ma vie, alors que tout le monde sait que je ne suis pas quelqu'un de calme… Ta patience, ta joie et ton sens de l'aventure font de toi une personne exceptionnelle et n'oublions pas ta petite tête d'oie quand tu imites Lab ! Julien, il faut dire Juju que tu n'as pas toujours beaucoup de tact... Et oui, il fallait bien que je parle des hanches pour materner, sinon ça ne serait pas drôle ! Merci de m'avoir supportée, malgré mes moments moins gentils (barbe rousse), de m'avoir fait corriger ton anglais (yeah baby !), et d'avoir été une épaule sur laquelle je pouvais toujours me reposer. JB, jibjib, my blondie, merci pour ces moments de complicité. Merci pour ces soirées sushis et films (avec tes goûts en films qui sont comme les miens !), merci de ne jamais être plus bronzé que moi, et merci d'être un blondie avec moi (et oui, les crevettes ça se cuit !). Merci aux membres plus récents de la mifa : Charlotte, Thomas, Julien et Fanny. Quel bonheur de vous avoir dans la team. Les loulous, je ne vous remercierai jamais assez pour ces moments, c'était « super confort ». Entre les années à Lyon ensemble, les séjours à Biarritz (Jb, t'as pris froid ?), et les weekends retrouvailles, cette aventure ensemble ne fait que commencer !

Marie and Tessia, thank you for being the two best friends I could ask for and my constant pillars in life. We may be completely different people, but thank you for always understanding when I had to put work first, having my back and giving me the support I needed. All three of us know that life takes us to unexpected places, but it's the people that stay with us throughout the challenges and good times that are the most important. Knowing you both for the last (almost) 25 years has been a true blessing and thinking of the next 25 years already puts a smile on my face. Tessia, my pumba, thanks for being nerdy with me, for indian and phish food, and for the unconditional support. Beebs, thank you for all the moments together and apart and for being present for the hardest times in my life. I couldn't be happier for you, you're going to be the most wonderful mom, and I can't wait for us to get manicures with our kids (long time promises are not forgotten).

Clément, merci pour ton soutien énorme et de m'avoir changé les idées quand j'en avais le plus besoin. Merci pour tous les bons moments passés ensemble pendant ces cinq dernières années. J'espère que tu trouveras ton bonheur dans le travail comme à l'extérieur, tu le mérites. Un grand merci aussi à toute ta famille pour leur soutien, de près comme de loin.

Pour finir, je voudrais remercier toute ma grande famille, mais en particulier Maman, Papa, Romain et Thibaud ; Grand-maman, Grand-papa, Claire et François ; Christophe, Fabienne, Laurie et Morgane. Un grand merci aussi à Murielle, Pascal, Alizée, Célia, Jean-Luc, Eline et Maïna. On ne choisit pas sa famille, mais il semblerait que j'ai gagné au loto en vous ayant dans ma vie donc merci à tous.

Grand-maman et grand-papa, merci pour votre soutien permanent tout au long de ma vie, en particulier depuis mon arrivée à Lyon il y a 10 ans. Merci pour les lessives, les après-midis piscine, et les repas des abonnés du dimanche (avec une belle pensée pour Mamie évidemment). Grand-maman, merci pour les histoires de famille qui m'ont fait halluciner (j'attends encore le livre), les mots-croisés, les restaurants quand grand-papa partait aux champignons, les journées portes ouvertes, et d'être la grand-mère la plus connectée. Grand-papa, merci pour les repas fait avec amour (mais sans recettes !), d'être le meilleur apiculteur de la Croix-Rousse, et surtout de m'avoir transmis ton amour pour la médecine.

Claire, merci pour ton sens de la folie, de te rendre toujours disponible, pour les séances bronzage et papotage et pour les petits messages d'encouragement. Merci de me montrer que tant qu'on est jeune dans sa tête, on s'en fiche du reste ! François, merci pour tes blagues,

pour les conversations to the point, et de m'avoir montré que parfois c'est bien quand on ne parle pas. Du coup, could you be quite quick ? Après les aventures en voiture sous la tempête en redescendant d'Avoriaz, il ne reste plus qu'à te dire : monkey on the car !

Les Perriot, il est difficile de mettre en mots tout ce que je ressens pour vous quatre. Christophe et Fabienne, merci pour le soutien, pour les weekends à Pau, les aventures à Biarritz et toutes les conversations depuis ma naissance. Laurie et Morgane, on sait toutes les trois que vous êtes comme mes grandes sœurs : toujours à veiller sur moi, à être prêtes à sortir ensemble malgré la pluie (et oui, on repense à la même soirée !), à se poser sur la plage juste pour partager un moment, et j'en oublie des mieux !

Romain, life's challenges have only made you stronger and I cannot express how proud I am of you. More than a proud sister, I am in awe of your constant strength and perseverance that have reminded me to evaluate what is truly important in life. Thank you for always worrying about me and always taking the time to check on me, no matter what. Thibaud, I know that even the sky is not the limit for you and where you will be going in the future. I've said it once, but I'll say it again, one day we will all see you head into the stars and become the rocket man you were destined to become. Thanks for PhD-complaining with me and general one-hour conversations where one of us needed to vent. Thank you for both being supportive big brothers and protecting me whatever comes our way.

Mom and Dad, few words exist that are able to encompass the love and gratitude I have for you. Thank you for your constant encouragement and for always pushing me to go further, be better, and think harder. Thank you for the blessing that is this life that you have worked so hard to give us, for always reminding us that it is only through hard work that we can get what we want, and for being a true inspiration in all aspects of life. It is only with true encouragement and fortitude that we persevere through all challenges that are thrown at us. Thank you for all the life lessons.

This accomplishment could be only greater without one missing piece, but life does not always give us what we wish for. Through it all, the pride is felt from the stars. Bon, allez, on se serre les coudes et on avance.

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RESUME

Mon projet de thèse s'inscrit dans l'étude des synucléinopathies, une famille de maladies neurodégénératives. Les trois principales synucléinopathies sont la maladie de Parkinson, l'atrophie multisystématisée et la démence à corps de Lewy. Ces maladies sont caractérisées par une perte de neurones dans des régions cérébrales spécifiques et la présence d'inclusions intra-cytoplasmiques positives pour l'α-synucléine dans les neurones (Corps de Lewy) ou dans les oligodendrocytes (Inclusions gliales cytoplasmiques). Les causes d'induction de ces maladies restent encore inconnues et les traitements curatifs sont inexistants. L'objectif de mon travail de thèse visait à étudier les mécanismes neurodégénératifs et de potentielles cibles thérapeutiques dans le contexte des synucléinopathies. Je me suis tout d'abord intéressée aux mécanismes impliqués dans la transmission de l'α-synucléine issue de patients atteints de l'atrophie multisystématisée. Ce travail nous a permis de développer un potentiel nouveau modèle de l'atrophie multisystématisée chez la souris et le primate non-humain, par la transmission de l'α-synucléine dans le cerveau. Dans un deuxième temps, nous nous sommes intéressés à des cibles thérapeutiques éventuelles pour la maladie de Parkinson dans un même modèle animal de la pathologie. Nous avons pu vérifier l'efficacité et la pertinence de trois différentes stratégies ciblant plusieurs mécanismes affectés dans la maladie de Parkinson dans le but d'induire une protection des neurones dopaminergiques de la substance noire des souris. Nous avons pu démontrer une dérégulation des niveaux de zinc au cours de la pathologie qui a suscité l'intérêt de cibler son homéostasie dans le cerveau à travers une molécule chélatrice du zinc. Ensuite, la surexpression d'un facteur de transcription impliqué dans la survie des neurones dopaminergiques ainsi que dans le stress oxydatif et le protéasome a montré son intérêt comme cible thérapeutique de la maladie de Parkinson. Enfin, une molécule anti-agrégative a aussi démontré sa capacité à induire une neuroprotection. En résumé, ces travaux montrent d'abord l'importance de l'α-synucléine dans la mise en place et la progression des synucléinopathies, mais aussi la nécessité de cibler d'autres mécanismes dérégulés dans ces pathologies pour proposer des nouvelles stratégies thérapeutiques.

Mots clés : maladie de Parkinson ; atrophie multisystématisée ; synucléinopathies ; neuropathologie ; thérapeutique

ABSTRACT

My thesis focused on the study of synucleinopathies, a family of neurodegenerative diseases. The three main synucleinopathies are Parkinson's disease, multiple system atrophy, and dementia with Lewy bodies. These diseases are characterized by the loss of neurons in various brain regions and the presence of intracytoplasmic α-synuclein-positive inclusions. These inclusions are located either in neurons (Lewy bodies) or in oligodendrocytes (Glial cytoplasmic inclusions). The trigger and cause for the formation of these inclusions remain unknown, and no curative treatments currently exist. The objective of my thesis was to study the neurodegenerative mechanisms and potential therapeutic strategies of these synucleinopathies. For this, I was first interested in the mechanisms implicated in the transmission of α-synuclein from multiple system atrophy patients. This allowed us to develop a potential new model to study multiple system atrophy in both mice and non-human primates by spreading of α-synuclein within the brain. In the second part, we wanted to investigate potential therapeutic targets in the same model of Parkinson's disease. This study confirmed the efficacy and pertinence of three different strategies that target various mechanisms of Parkinson's disease to induce the protection of dopaminergic neurons of the substantia nigra in a mouse model. By modulating zinc levels, we demonstrated the importance of zinc concentrations in the brain and the therapeutic interest in targeting metal homeostasis via specific chelators. We then used viral vectors to overexpress a transcription factor implicated in dopaminergic neuron survival, oxidative stress and proteasome activity in the substantia nigra of a mouse model of Parkinson's disease. Finally, we used an anti-aggregative molecule to determine its efficacy in protecting neurons in the same mouse model. Altogether, this thesis work showed the implication of α-synuclein in triggering and propagating synucleinopathies, the importance of targeting this protein, and other dysregulated cellular mechanisms to discover potential therapies.

Keywords: Parkinson's Disease; multiple system atrophy; synucleinopathies; neuropathology; therapies

RESUME LONG

Parmi les maladies neurodégénératives, il existe une famille de maladies appelées les synucléinopathies. Les trois principales synucléinopathies sont la maladie de Parkinson (MP), l'atrophie multisystématisée (AMS) et la démence à corps de Lewy. Ces synucléinopathies sont caractérisées par l'accumulation anormale et pathologique de la protéine α-synucléine qui, lorsqu'elle est mal repliée, induit la formation d'agrégats. Au cours du temps, ces agrégats forment des inclusions dans les neurones ou dans les oligodendrocytes, contribuant à la mort de ces cellules. Les causes d'induction de ces maladies restent encore inconnues et les traitements curatifs sont inexistants. Étant donné que ces synucléinopathies touchent surtout les adultes de plus de 50 ans et du fait du vieillissement de la population, leur prévalence augmente, d'où l'intérêt croissant de trouver un traitement curatif.

La MP atteint 1 à 2% de la population des plus de 65 ans et 4 à 5% des plus de 85 ans, avec environ 10% de formes familiales et 90% de formes sporadiques. Cliniquement, la MP est caractérisée par des symptômes moteurs comprenant l'akinésie, les tremblements au repos et la rigidité musculaire. Au niveau anatomo-pathologique, elle est caractérisée à la fois par la présence d'inclusions cytoplasmiques neuronales appelées corps de Lewy (LB, Lewy Bodies) et par la perte progressive des neurones dopaminergiques de la substance noire compacte. L'étiologie de cette maladie est peu comprise, mais plusieurs mutations de gènes ont été montrées comme étant impliquées dans le développement de cas familiaux de la MP, y compris le gène *SNCA*, qui code l'α-synucléine. Malgré l'avancée des connaissances dans le domaine, les seuls options de traitements sont symptomatiques telles que la L-Dopa ou la stimulation profonde du cerveau.

L'AMS est une maladie progressive et sporadique se déclarant à l'âge adulte. Celle-ci est plus rare que la MP, avec 2 à 5 personnes affectées pour 100 000 personnes, mais présente plusieurs formes cliniques : l'AMS-c et l'AMS-p. L'AMS-c touche le système olivo-ponto-cérebelleux et est donc associée à des troubles d'équilibre et de la parole. D'un autre côté, l'AMS-p présente des symptômes moteurs de types parkinsoniens, avec une rigidité musculaire, des tremblements au repos et de l'akinésie. Ces deux AMS présentent des inclusions cytoplasmiques dans les oligodendrocytes, appelées inclusions gliales cytoplasmiques (GCI, Glial Cytoplasmic Inclusions). Ces GCI contribuent à la mort des neurones et des oligodendrocytes, ainsi que la démyélinisation et de l'inflammation. Concernant l'étiologie de l'AMS, celle-ci est encore moins bien comprise que celle de la MP.

Cependant, certains gènes en commun avec la MP, en particulier le gène *SNCA*, ont été montrés comme étant impliqués dans l'AMS également. Les traitements symptomatiques ne sont pas encore établis, par manque de réponse de certains patients à la L-Dopa.

Au vu de l'implication de l'α-synucléine dans ces maladies, il est important de comprendre le rôle physiologique et pathologique de cette protéine. L'α-synucléine est une petite protéine de 14 kDa contenant trois domaines principaux : un domaine N-terminal à hélice α, un domaine central non-amyloïde, et un domaine C-terminal non-structuré. Physiologiquement, l'α-synucléine a démontré une présence au niveau présynaptique, indiquant un rôle potentiel dans la transmission de neurotransmetteurs au niveau de la synapse. Pathologiquement, le monomère d'α-synucléine peut adopter des conformations intermédiaires, commençant par des formes riches en feuillets β, qui peuvent ensuite s'assembler entre elles pour former des oligomères, puis des fibrilles. L'importance de la protéine α-synucléine s'illustre donc par sa capacité à s'agréger, mais également de se propager de cellule en cellule et de transmettre son information pathogénique. A l'aune de ces éléments, cette protéine détient certaines caractéristiques similaires à la protéine prion, d'où sa dénomination de protéine prion-like.

Le rôle central de l'α-synucléine dans ces maladies neurodégénératives a ouvert la possibilité au développement de nouveaux modèles animaux basés sur une surexpression de la forme sauvage ou mutée de la protéine. Ces modèles vont de modèles transgéniques, aux modèles nécessitant des vecteurs viraux, aux modèles basés sur l'injection d'α-synucléine. Néanmoins, aucun modèle animal n'existe actuellement récapitulant tous les aspects des synucléinopathies, en particulier la formation des LB ou des GCI *in vivo*.

Au bout du compte, ces deux synucléinopathies sont très mal comprises à ce jour, et des travaux portant sur la mécanistique de ces maladies ainsi que des potentielles pistes thérapeutiques sont nécessaires. *Dans ce but, mon projet de thèse visait à démêler les mécanismes associés à ces pathologies, ainsi qu'à étudier les effets de certaines molécules comme potentiels traitements de ces maladies*.

L'AMS est très peu étudiée par rapport à d'autres synucléinopathies telles que la MP et les démences à corps de Lewy, du en grande partie à sa prévalence plus faible. De plus, l'apparition d'une accumulation de la protéine α-synucléine dans les oligodendrocytes majoritairement au lieu des neurones reste encore incomprise. Pour comprendre l'importance de ces GCI et leurs mécanismes pathogéniques, un projet visant à injecter ces GCI dans des souris et des primates non-humains (*Papio papio*) a été lancé dans notre laboratoire, en

collaboration avec des laboratoires européens. Ces GCI ont été extraits de cerveaux de patients atteints de l'AMS, purifiés, et injectés dans le cerveau des singes. Après deux ans, la caractérisation des cerveaux de ces babouins a commencé, dans le but de déterminer l'effet de l'injection des GCI sur la survie neuronale. Dans cette étude, nous avons pu montrer que ces extraits issus de patients AMS sont capables d'induire une perte de neurones et de fibres dopaminergiques dans le striatum et la substance noire. De plus, ces singes démontrent également une perte d'oligodendrocytes ainsi qu'une démyélinisation. Enfin, ces singes illustrent une accumulation de l'α-synucléine dans la SN ainsi que le striatum. Tout ceci démontre l'effet pathologique de cette injection intracérébrale de GCI dans les cerveaux de souris et de singes. Ce travail a permis de mieux appréhender l'effet de ces GCI pour la première fois, mais pourrait aussi aider à comprendre la différence de pathologie entre les GCI et les LB, et donc entre l'AMS et la MP. Ce projet s'inscrit dans une compréhension plus large de ces deux synucléinopathies et dans la mise en place de méthodologies de ces deux pathologies distinctes.

Dans une visée plus thérapeutique, nous avons utilisé plusieurs approches en parallèle les unes des autres pour déterminer l'effet de plusieurs potentielles cibles thérapeutiques sur un même modèle de la MP basé sur l'inoculation de LB dans la substance noire de souris. Dans un premier temps, certains métaux ont déjà été impliqués dans la pathogénicité de plusieurs maladies neurodégénératives, dont la MP, en particulier : le fer, le cuivre, le zinc et le manganèse. Toute altération dans les concentrations de ces métaux induit des dysfonctionnements au niveau cellulaire et subcellulaire. En particulier, le zinc, le deuxième métal le plus présent dans le corps après le fer, a été montré comme jouant un rôle particulièrement important dans l'homéostasie du cerveau. Le maintien de l'homéostasie du zinc permet entre autres une protection neuronale des structures cérébrales. Si cet équilibre est perdu, l'activité du cerveau peut être affectée. Malgré son importance dans les mécanismes cellulaires, des concentrations trop fortes en zinc libre dans le cytoplasme sont toxiques. Dans la cellule, ces concentrations sont donc très fortement régulées par une séquestration dans les organelles ou par des protéines se couplant au zinc. Au vu de l'importance de ce métal et devant le manque d'études sur ses effets précis sur la MP, une autre partie de ma thèse visait à étudier l'effet de la modulation du zinc dans la progression de la MP. Nous avons donc utilisé des souris modélisant la MP qui ont ensuite été alimentées avec 2 types de nourritures, l'une supplémentée avec un ionophore du zinc, le clioquinol, et une autre supplémentée avec du zinc. Ces différents régimes ont pour rôle d'augmenter ou de

diminuer la teneur en zinc respectivement, afin de déterminer si ceci affecterait l'initiation et/ou la progression de la MP. Dans ce projet, nous avons observé une protection des neurones dopaminergiques avec le clioquinol, ainsi qu'une diminution de formes agrégées d'α-synucléine. De plus, nous avons voulu voir s'il y avait des changements d'expression dans les transporteurs du zinc, et nous avons pu mettre en avant une diminution du transporteur de zinc dans les vésicules synaptiques. Enfin, étant donné le rôle décrit du clioquinol dans la modulation de l'autophagie, nous avons voulu déterminer si ce mécanisme d'élimination des déchets cellulaires était modifié avec le traitement au clioquinol. Ainsi, nous avons pu détecter une augmentation dans la fonctionnalité des lysosomes avec le traitement au clioquinol, indiquant une meilleure élimination de l'α-synucléine dans ces neurones. En conclusion, nous avons pu démontrer une protection significative des neurones dopaminergiques dans le groupe de souris LB traitées au clioquinol, qui s'expliquerait en partie par une meilleure élimination de l'α-synucléine par les lysosomes et le zinc synaptique.

Dans un deuxième temps, parmi les stratégies pour réduire la toxicité de l'α-synucléine. Dans la MP, l'agrégation de cette protéine est accélérée en présence de membranes riches en lipides, telles que celles des neurones. Cette accélération trouble les vésicules contenant de la dopamine, ainsi que leur relargage. Ceci pourrait conduire à la mort préférentielle des neurones à dopamine. NFE2L1, un facteur de transcription impliqué dans la différenciation et la survie des neurones dopaminergiques, est sous-régulé en conditions pathologiques. Dans ce projet, nous souhaitions restaurer l'expression normale de NFE2L1 afin de diminuer le stress oxydatif ainsi que le dysfonctionnement du système ubiquitine-protéasome dans les neurones dopaminergiques, deux facteurs participant à la mort neuronale. Dans ce cadre, un adénovirus associé contenant NFE2L1 a été produit et a été injecté en parallèle à l'injection des LB dans la substance noire des souris. Nous avons observé une diminution de la perte des neurones dopaminergiques de la substance noire conjointement avec une diminution de l'accumulation de formes agrégées d'α-synucléine avec la surexpression de NFE2L1 dans les souris LB. Ce résultat nous indique un potentiel effet neuroprotecteur de la surexpression du facteur de transcription NFE2L1.

Dans un troisième temps, pour cibler plus particulièrement l'agrégation d'α-synucléine, nous avons utilisé une molécule aux propriétés anti-agrégation déjà décrite comme efficace sur plusieurs protéines dites prion-like, y compris l'α-synucléine. Dans les études précédentes, anle138b a montré son efficacité pour réduire la neurodégénérescence ainsi que l'accumulation de l'α-synucléine dans plusieurs modèles de la MP et de l'AMS, dont des

modèles transgéniques et des modèles de neurotoxines. Au vu de ceci, nous avons voulu déterminer le potentiel de cette molécule à travers deux schémas expérimentaux dans notre modèle basé sur l'injection des LB dans la substance noire des souris. Dans cet objectif, nous avons administré le composé dès l'initiation (« concomitant start ») et deux mois après l'initiation (« delayed start ») de la pathologie. Dans le groupe traité en concomitance, nous avons observé une protection des neurones et des terminaisons dopaminergiques, ainsi qu'une légère diminution de formes agrégées de l'α-synucléine. Dans le groupe traité à mi-parcours, le traitement avec anle138b n'a pas permis d'observer une diminution de la perte de neurones dopaminergiques. De plus, nous avons observé une augmentation d'accumulation de l'α-synucléine dans la substance noire et le striatum. Ces deux protocoles d'administration de la molécule à différents temps ont pu démontrer l'efficacité d'anle138b dans les souris LB lors d'un traitement concomitant alors que ce n'était pas le cas avec un traitement tardif.

Ces deux grands axes ont eu pour but de mieux comprendre les synucléinopathies ainsi que ce qui les distingue entre elles et de trouver des thérapies potentielles visant à diminuer les effets pathologiques délétères de l'α-synucléine.

En prenant tous ces résultats en compte, il est ainsi possible d'apprécier l'importance des modèles animaux dans la compréhension des mécanismes responsables dans le cadre des synucléinopathies. Le développement de ce nouveau modèle basé sur l'injection intracérébrale de GCI a permis de mettre en évidence la capacité pathogénique d'extraits de cerveaux de patients AMS de transmettre la pathologie dans des primates non-humains. Ce modèle est complémentaire avec l'autre modèle utilisé dans ce manuscrit fondé sur l'injection de LB dans la souris. La comparaison de ces deux modèles de synucléinopathie pourrait mettre en avant l'hypothèse de « souches » d'α-synucléine qui induirait la MP ou l'AMS. De plus, aucun modèle utilisé actuellement pour étudier les synucléinopathies ne représente parfaitement la pathologie humaine. Cet état de fait met en avant le besoin de développer des modèles expérimentaux qui représentent plusieurs aspects des maladies selon ce que l'on souhaite étudier.

Concernant les cibles thérapeutiques, nous avons utilisé des approches variées ciblant indirectement l'α-synucléine à travers une modulation du zinc ou d'un facteur de transcription impliqué dans la survie de neurones dopaminergiques, ou directement par une molécule anti-agrégative. De plus, au vu des similarités entre les synucléinopathies, il serait intéressant de prolonger ces projets et de vérifier l'effet d'une même molécule sur les

différentes synucléinopathies et pas juste la MP. Cette approche a déjà débuté sur des molécules telles qu'anle138b, ou dans des cas d'immunothérapies visant à éliminer l'α-synucléine « pathologique ». Néanmoins, vu la complexité de ces synucléinopathies, il parait difficile de croire qu'une seule stratégie sera efficace pour inhiber la progression de la maladie. Une des potentielles solutions serait d'utiliser une approche combinatoire qui ciblerait par exemple l'agrégation de l'α-synucléine et/ou son élimination par une augmentation des différents systèmes de clairance cellulaire, qui freinerait la propagation. Un autre aspect important nécessite de pouvoir réaliser un diagnostic plus précoce des synucléinopathies, par le développement de biomarqueurs fiables pour suivre l'effet bénéfique de composés thérapeutiques. Malgré tout le travail qu'il reste à faire, nous démontrons ici l'intérêt d'explorer les mécanismes impliqués dans les synucléinopathies afin de trouver des stratégies thérapeutiques efficaces.

PUBLICATIONS, POSTERS, PRESENTATIONS

Published articles:

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Posters:

GCI-induced neurodegeneration and synucleinopathy in non-human primates. **M. Teil**, S. Dovero, M. Bourdenx, M.-L. Arotçarena, S. Camus, G. Porras, M.-L. Thiolat, I. Trigo-Damas, C. Perier, C. Estrada, N. Garcia-Carrillo, M. Morari, W. G. Meissner, M. T. Herrero, M. Vila, J. A. Obeso, E. Bezard, B. Dehay. Bordeaux Neurocampus Day, Bordeaux, 2021. → Winner of the best Poster Prize

Zinc chelation reduces α-synuclain-induced neurodegeneration in a mouse model of Parkinson's Disease. **M. Teil**, E. Doudnikoff, M.-L. Thiolat, E. Bezard, B. Dehay. Neurofrance Virtual meeting, 2021.

GCI-induced neurodegeneration and synucleinopathy in non-human primates. **M. Teil**, S. Dovero , M. Bourdenx , M.-L. Arotcarena, S. Camus, G. Porras, M.-L. Thiolat, N. Kruse, B. Mollenhauer, I. Trigo Damas, C. Estrada, N. Garcia-Carrillo, M. T. Herrero, P. Derkinderen, M. Vila, J. A. Obeso, B. Dehay, E. Bezard. AD/PD Virtual meeting, 2021.

GCI-induced neurodegeneration and synucleinopathy in non-human primates. **M. Teil**, S. Dovero , M. Bourdenx , M.-L. Arotcarena, S. Camus, G. Porras, M.-L. Thiolat, N. Kruse, B.

Mollenhauer, I. Trigo Damas, C. Estrada, N. Garcia-Carrillo, M. T. Herrero, P. Derkinderen, M. Vila, J. A. Obeso, B. Dehay, E. Bezard. HOPE, Paris, 2020.

GCI-induced neurodegeneration and synucleinopathy in non-human primates. **M. Teil**, S. Dovero , M. Bourdenx , M.-L. Arotcarena, S. Camus, G. Porras, M.-L. Thiolat, N. Kruse, B. Mollenhauer, I. Trigo Damas, C. Estrada, N. Garcia-Carrillo, M. T. Herrero, P. Derkinderen, M. Vila, J. A. Obeso, B. Dehay, E. Bezard. 50th annual Society for Neuroscience meeting, Chicago, 2020.

Oral Presentations:

2020: HOPE (Physiopathology of Parkinson's Disease) congress in Paris, January 2020. Flashtalk – « GCI-induced neurodegeneration and synucleinopathy in non-human primates. »

2019: Club des Ganglions de la Base (CGB) meeting in Biarritz, April 2019. « Zinc dyshomeostasis and effect of zinc ionophore Clioquinol in models of Parkinson's Disease. »

ABBREVIATIONS

α-syn	α-synuclein
AAV	Adeno-associated virus
AD	Alzheimer's Disease
ALP	Autophagy-lysosomal pathway
ClQ	Clioquinol
CMA	Chaperone-mediated autophagy
CNS	Central nervous system
CSF	Cerebrospinal fluid
DAT	Dopamine transporter
DLB	Dementia with Lewy Bodies
GCase	Glucocerebrosidase
GCI	Glial cytoplasmic inclusions
LB	Lewy Bodies
L-Dopa	L-3,4-dihydroxyphenylalanine
LRRK2	Leucine rich repeat kinase 2
LUHMES	Lund Human Mesencephalic cells
MA	Macroautophagy
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSA	Multiple System Atrophy
MSA-c	MSA-cerebellar
MSA-p	MSA-parkinsonian
NAC	Non-amyloid component
NHP	Non-human primate
PD	Parkinson's Disease
PFFs	Preformed fibrils
PLP	Proteolipid promoter
PTM	Post-translational modifications
SN	Substantia nigra
ROS	Reactive oxygen species
UPS	Ubiquitin-proteasome system
ZIP	Zrt, Irt-like protein
ZnT	Zinc transporter

PREFACE

Around 25% of the French population is over 60 years old, with an estimated 32% in 2050. With this increase in demography and life expectancy, there is an increased risk of developing neurodegenerative diseases. Among these age-related diseases, synucleinopathies are a family of disorders characterized by cytoplasmic inclusions positive for the α-synuclein protein. The most popular synucleinopathies are Parkinson's disease and multiple system atrophy. Parkinson's disease, in particular, is of high socio-economical interest as it affects more and more of the aging population. To this day, little is known about the mechanisms that trigger these diseases. Additionally, symptomatic treatments are the only currently available options for patients as no curative solutions exist. These symptomatic treatments are often temporary and are not recommended in the long term. Faced with these issues, **my thesis project aimed to better understand the mechanisms implicated in these diseases and explore potential therapeutic strategies**.

My interest in biology and medicine led me to complete a master's degree at the Ecole Normale Supérieure of Lyon. During the final internship of my master's degree, I got the opportunity to work in Erwan Bézard's team "Physiopathology of Parkinsonian syndromes" under the supervision of Benjamin Dehay. Through this first experience in the team, I got to work on the underlying mechanisms of Parkinson's disease and then continue on this thesis, where I was able to work on multiple projects in this same thematic.

For this manuscript, I decided to begin by globally introducing synucleinopathies to describe these diseases' clinical and neuropathological aspects and identify the differences between them. I continued by discussing the physiological and pathological characteristics of the α-synuclein protein, a central protein in many of the projects led during my thesis. Following this, I described the *in vitro* and *in vivo* models of synucleinopathies specifically based on α-synuclein expression or gene mutations. In the next part, I discussed the implication of zinc, a predominant heavy metal, in the brain and the development of brain-associated pathologies. I ended the introduction with potential therapeutic approaches to these diseases. This introduction was followed by a description of the results of the four main projects I worked on during this thesis. Finally, I finished with a global discussion on the projects and their place in the field.

INTRODUCTION

A. Synucleinopathies

Synucleinopathies encompass several neurodegenerative diseases, which are characterized by the formation of α-synuclein (α-syn)-positive inclusions in the brain. These synucleinopathies include Parkinson's Disease (PD), Multiple system atrophy (MSA), and Dementia with Lewy bodies (DLB). The discriminating factor between these diseases is the localization of the intracytoplasmic inclusions within the cells. In PD and DLB, these inclusions are localized predominantly in neurons and are termed Lewy bodies (LB). However, in MSA, these inclusions are located in oligodendrocytes and named Glial cytoplasmic inclusions (GCI). Independently of their cellular placement, these inclusions are associated with the loss of neurons in the brain. Nevertheless, each disease has specific clinical and pathological hallmarks that make the disease singular.

1. Parkinson's Disease

History

In 1817, James Parkinson first described PD in "An essay on the shaking palsy". He described that PD, which he termed "shaking palsy", was symptomatically characterized by an involuntary tremor combined with a bent trunk and difficulty to initiate a movement (akinesia) without impact on the senses or the intellect (Parkinson 1817). By 1872, Jean Martin Charcot and his colleagues confirmed and added to Parkinson's description of the disease: resting tremor, postural instability, akinesia, and bradykinesia, the slowing of movements and loss of fine movements. Charcot was the first to change the name of the disease from shaking palsy to Parkinson's disease (Charcot 1877). The neuropathology of PD only started to be described in 1893 by Blocq and Marinesco, when they indicated that a lesion in the substantia nigra could be the basis of the disease (Blocq Marinesco 1893). In 1912, the first description of intra-neuronal inclusions present in the brain of PD patients was described by Fritz Jacob Heinrich Lewis (Lewy 1912). These spherical intracytoplasmic inclusions were later termed Lewy bodies, a persistent hallmark of PD neuropathology. However, the association of lesion in the substantia nigra coupled with the presence of LB in PD patients was first suggested by Constantin Trétiakoff in 1919 (Tretiakoff 1919).

Epidemiology

PD is the second most common neurodegenerative disease after Alzheimer's disease (AD), affecting around 1-2 per 1000 of the total population at any time. With increasing age, PD prevalence increases to around 1-2% of the population over 65 years of age and 4-5% of the population over 85 years of age are affected by the disease (Rocca, 2018). The average onset of PD is usually around 65 to 70 years of age. Importantly, the incidence and prevalence of the disease are 1.5 times higher in men than in women (Moisan *et al.*, 2016). Of all PD cases, genetic PD patients represent around 5-10% of patients, the rest being sporadic cases. In France, in the last 25 years, the number of PD cases has more than doubled, mainly due to the aging population. Projections in 2030 estimate that the prevalence rate in France will continue to increase in both men and women, accompanied by an increase in the number of PD patients (Wanneveich *et al.*, 2018). The projections made in this study estimate 260 000 PD patients in France in 2030, an even higher than previous studies had estimated (Collaborators, 2018). In 2020, the estimated global number of PD patients was 9.4 million. Specifically, countries with the highest prevalence were China (2.7 million), the United States (930 000), Japan (344 000), Germany (266 000), and other western European countries (**Figure 1**) (Rocca, 2018). The distribution of PD cases is highly heterogeneous depending on the country, in large part due to varying risk factors such as industrialization.

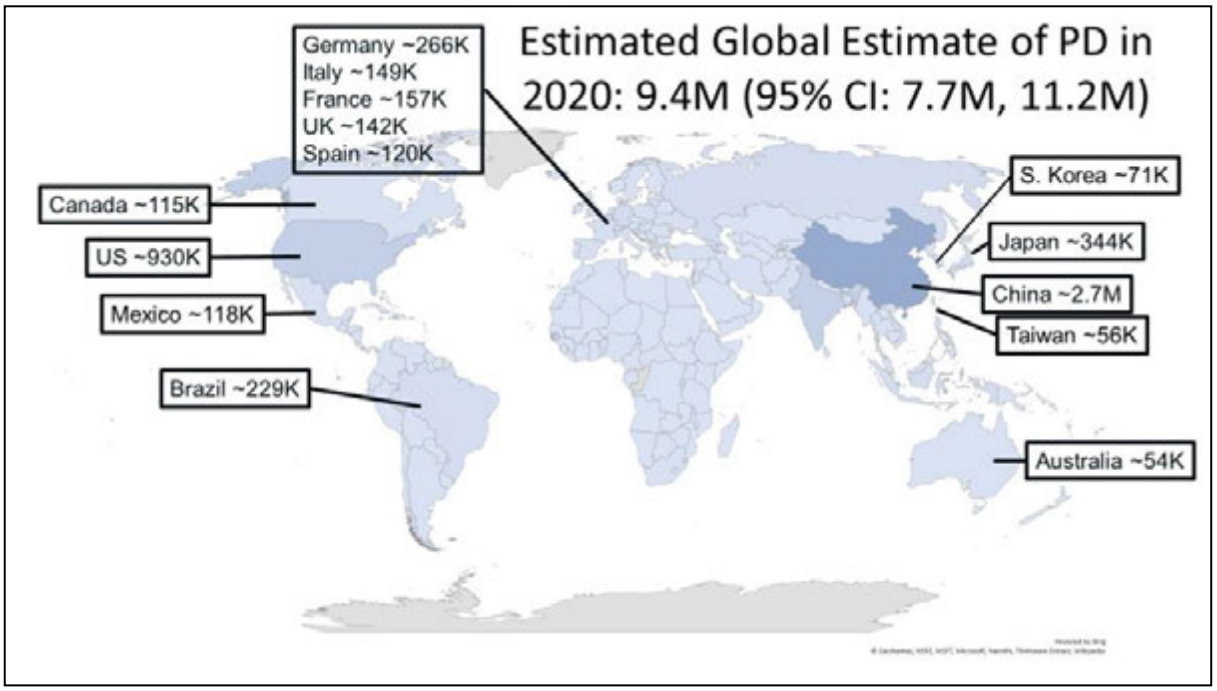

Figure 1: Estimated Global PD cases throughout the world in 2020. *Adapted from Maserejian et al. 2020, MDS Virtual Congress Abstract.*

Clinical assessment and diagnosis

The original description of PD is an association of four major motor symptoms: bradykinesia/akinesia, muscular rigidity, postural instability, and resting tremor (Jankovic, 2008). First, **bradykinesia** and **akinesia** are the slowing and loss of fine movements and the difficulty to initiate a movement, respectively. These two movement disorders are the first to appear and are easily detectable in patients. Combined, bradykinesia and akinesia represent a slowness to initiate and hesitation to execute a movement, described as a delay between the willingness to initiate the movement and its execution. These impairments can be very difficult on the day-to-day life of patients, notably to execute daily tasks, such as eating and getting dressed.

The following symptom, **muscular rigidity**, is due to generalized muscular hypertonia leading to increased movement resistance implicating articulations. Most muscles of the body are affected, but particularly flexing movements. This is also translated by stiffness of limbs, the neck and/or trunk.

In later manifestations of the disease, **postural instability** begins to occur in patients. This represents the development of an imbalance accompanied by a stooped posture, leading the patient to fall more often.

Lastly, the most recognizable symptom of PD, **resting tremor**, represents a slow and constant tremor present usually unilaterally in distal extremities. These tremors occur at a frequency between 4 and 6 Hz. Despite it being one of the most known PD symptoms, resting tremor is absent in around 15 to 30% of PD patients.

In addition to the characteristic motor symptoms, PD patients can also present with a variety of **non-motor symptoms** (**Figure 2**), manifesting earlier than their motor symptoms. Among these non-motor symptoms, PD patients can suffer from generalized dysautonomia manifested through orthostatic hypotension, anosmia, constipation, and urogenital dysfunction. Sleep disorders are also frequently observed with alterations in sleep-wake patterns. Psychiatric disorders can emerge with the progression of the disease with the appearance of depression, dementia, anxiety, and potential hallucinations.

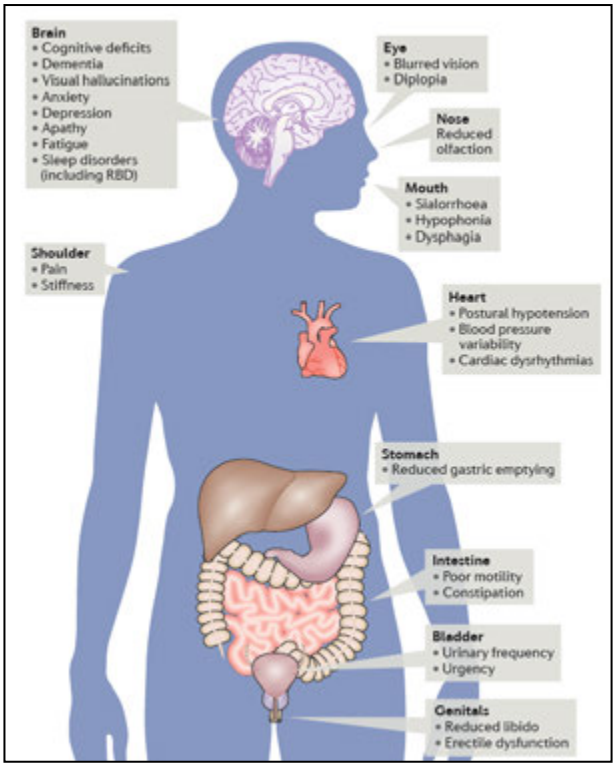

Figure 2: Non-motor symptoms of Parkinson's Disease. RBD: REM sleep behavior disorder. *Adapted from Schapira et al. 2017, Nature Reviews Neuroscience.*

The combination of motor and non-motor symptoms varies between each PD patient. These symptoms can also be impacted by the environment, in particular with stress and emotions. Faced with a multitude of symptoms, motor and non-motor, the clinical diagnosis of the UK Parkinson's Disease Society Brain Bank is based on the observation of bradykinesia/akinesia associated with at least one other motor symptom (rigidity, postural instability, resting tremor). In addition, three other features must be present among the following: unilateral onset, progressive disorder, persistent asymmetry primarily affecting side of onset, excellent response to levodopa, dyskinesia, levodopa response for 5 years or more, and clinical course of 10 years or more (Jankovic, 2008). Diagnosis can also be facilitated by using imaging techniques such as Magnetic Resonance Imagery or Tomography by emission of positrons. Clinical rating scales are also being used to assess the progression, severity and diagnosis of PD, particularly the Unified Parkinson's Disease Rating Scale (UPDRS).

Pathophysiology

Although PD diagnosis is primarily associated with clinical observations, post mortem confirmation of the disease is necessary. PD neuropathology is described by both the loss of dopaminergic neurons in the substantia nigra (SN) and by the accumulation of LB in neurons throughout the brain.

Unlike in AD, PD patients do not present general macroscopic atrophy but with neuronal degeneration, specifically in dopaminergic neurons. The loss of dopaminergic neurons was first characterized by a significant lesion in the midbrain in post mortem PD patients in 1893. This is translated by a visible loss of SN pigmentation due to the natural accumulation of neuromelanin, a brown pigment contained in the cytoplasm of dopaminergic neurons (Graham, 1978) (**Figure 3**). This loss of dopaminergic neurons leads to a significant depletion of the striatum in dopaminergic innervation and thus in dopamine, which clinically explains the motor symptoms observed in patients (Ehringer and Hornykiewicz, 1960). In the early stages of the disease, this dopaminergic neurodegeneration is specific to the ventrolateral SN but spreads throughout the stages of the disease (Parkkinen *et al.*, 2011). Given the vast loss of dopaminergic neurons early in the disease, neuronal loss begins before motor symptoms arise. Furthermore, other monoaminergic systems are altered in PD, including noradrenergic, serotoninergic, and cholinergic systems.

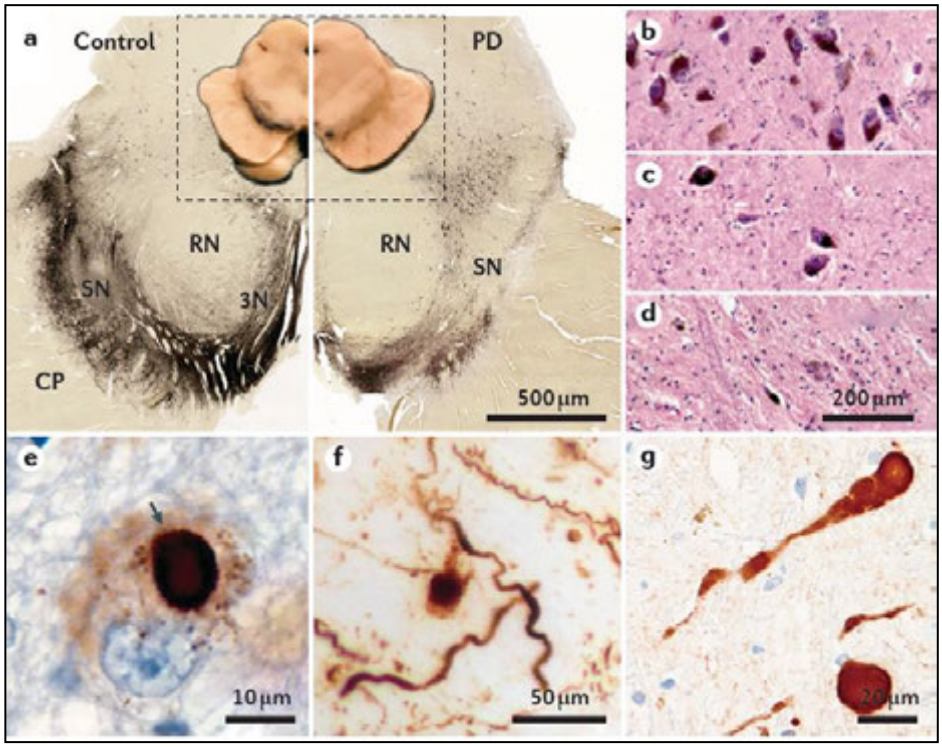

Figure 3: Pathological hallmarks of Parkinson's disease (PD): (**a**) Depigmentation of the substantia nigra (SN) in PD brains (right) compared to control brains (left), associated with loss of dopaminergic neurons. (**b-d**) Haematoxylin-eosin staining in the SN showing a normal distribution of pigmented neurons in control (b) brains or patients diagnosed with moderate (c) or severe (d) PD. (**e-g**) Immunohistochemical staining of α-syn-positive intracytoplasmic Lewy bodies (e), with deposits in neuronal projections (f) and axonal spheroids (g). *Adapted from Poewe et al. 2017, Nature Reviews Disease Primers*.

The second neuropathological marker of PD is the presence of intracytoplasmic inclusions termed LB. The major components of LB are α-syn and ubiquitin, but more than 90 other molecules have been recognized to be present in these inclusions (McCormack *et al.*, 2016; Trinkaus *et al.*, 2021). Certain inclusions can be found in neuronal projections and are thus name Lewy Neurites. LB were first thought to be located only in the dorsal nucleus of the vagal nerve, the brainstem, and the SN. Later on, it was demonstrated that LB localization ranged throughout the brain. In fact, in 2003, Braak and colleagues emitted the hypothesis that synucleinopathies would progress through brain regions coherently and predictively, starting in the dorsal nucleus of the vagal nerve or the olfactory bulb and progressing towards the neocortex in six stages (**Figure 4**) (Braak *et al.*, 2003). Given that this theory rests only on the localization of LB in the brain without relating to the progression of neurodegeneration, it remains controversial to this day.

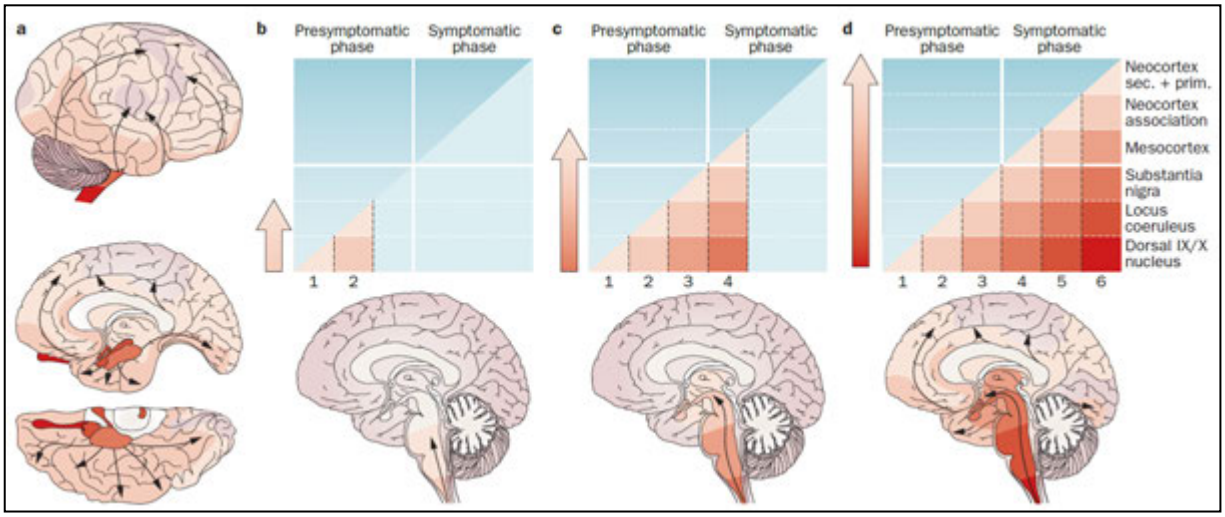

Figure 4: Stages of propagation of PD pathology following the Braak hypothesis. PD can be separated into six stages, each describing an evolution in the dissemination of α-syn aggregates. Stages 1-3 involve a presymptomatic phase where the aggregates progress from the dorsal nucleus of the vagal nerve to the SN. Stages 4-6 involve the symptomatic phase were aggregates spread towards the mesocortex and neocortex. *Adapted from Goedert et al. 2012, Nature Reviews Neurology*

Etiology

The exact cause of PD has yet to be identified, but throughout the last years, several findings have helped to start to decipher some of the potential sources. PD can stem from either a genetic predisposition or can arise sporadically. Given that the median age of onset of PD is 60 years of age, the principal risk factor is age. As with most neurodegenerative diseases, the increase in age is accompanied by natural biological dysfunction such as genomic instability,

mitochondrial and oxidative stress, and autophagy-lysosomal dysfunction. In addition to age, the risk between genetics and environment has been debated through the years (Jankovic and Tan, 2020).

PD counts a majority of sporadic cases, but it is important to note that genetic forms are also present and represent 5-10% of cases. The first familial form was described in 1997 in an Italian family and three Greek families in the *SNCA* gene, located in chromosome 4 (4q21-q23). The *SNCA* gene codes for the α-syn protein and in these families, the protein presented a mutation at Ala53Thr (Polymeropoulos *et al.*, 1997). Since this discovery, seven other missense mutations of α-syn have been identified: Ala30Pro, Ala30Gly, Glu46Lys, His50Gln, Gly51Asp, Ala53Glu, and Ala53Val (Kruger *et al.*, 1998; Zarranz *et al.*, 2004; Appel-Cresswell *et al.*, 2013; Lesage *et al.*, 2013; Proukakis *et al.*, 2013; Pasanen *et al.*, 2014; Yoshino *et al.*, 2017; Liu *et al.*, 2021a). These point mutations are rare but can induce various types of Parkinsonism: some similar to sporadic cases (Ala30Pro mutation) or more rapid and severe forms accompanied by dementia, hallucinations, and autonomic dysfunction. Genetic PD families can also demonstrate duplications or triplications of *SNCA*, demonstrating once more the importance of this gene in PD pathology (Singleton *et al.*, 2003; Chartier-Harlin *et al.*, 2004).

	Phenotypic Features			Genetic Features			
Genetic PD Syndrome	Age at Onset, Motor Features	Cognition, Nonmotor Features	Others	Inheritance	Mutation Spectrum	Locus	Others
Syndromes of dominantly inherited monogenic PD							
PARK-SNCA	Younger AAO, more aggressive course especially in triplication carriers	Prominent nonmotor features, especially in triplication carriers	Family members may have other LB-related diseases, such as dementia	Autosomal dominant, few sporadic cases	Missense mutations, whole gene duplication and triplication more common	PARK1 and PARK4 4q21	Low penetrance (duplications); common variants also contribute to risk for idiopathic PD
PARK-LRRK2	Typical PD, tremor-dominant at onset	Nonmotor features are less common	Very common among North African Arabs	Autosomal dominant or sporadic	Missense mutations	PARK8 12q12	Common variants confer risk for idiopathic PD
PARK-VPS35	Typical PD	Probably similar to typical PD	Good levodopa response	Autosomal dominant, sporadic	Missense mutations	PARK17 16q12	Reduced penetrance
PARK-DNAJC13	Late-onset PD	Probably similar to typical PD	Brainstem LB pathology and taupathy	Autosomal dominant, few sporadic	Missense mutations	no PARK locus 3q22.1	Mutations in *DNAJC6* and *SYNJ1* linked to atypical parkinsonism
PARK-GBA	Typical PD	Probably similar to typical PD	LB pathology	Autosomal dominant with reduced penetrance	Missense mutations	no PARK locus 1q21	Homozygous mutations in GBA cause Gaucher disease
Syndromes of recessily inherited early-onset PD							
PARK-PARKIN	Early-onset PD, dystonia is a common early sign	Dementia almost always absent	Absence of brain LB pathology	Autosomal recessive	Mutations, exonic duplication and deletion more common	PARK2 6q25.2-q27	Mutations have been described in other neurologic disorders
PARK-PINK1	Early-onset PD	Higher rate of psychiatric symptoms vs *PARK-PARKIN*	May mimic sporadic typical PD	Autosomal recessive, some sporadic	Nonsense, frameshift, missense mutations more common	PARK6 1p36	Heterozygous mutations may predispose to PD
PARK-DJ1	Early-onset PD	Similar to other recessive PD syndromes	Significantly less common than *PARK-PARKIN* or *PARK-PINK1*	Autosomal recessive	Missense mutations and exonic rearrangements	PARK7 1p36.23	Heterozygous mutations are frequently identified, unkown if pathogenic

Table 1: PARK-designated genes involved in genetic cases of PD. *Adapted from Domingo and Klein 2018, Handbook of Clinical Neurology.*

Besides *SNCA*, other gene mutations have been implicated in the development of genetic PD, hence the terminology "PARK" genes (**Table 1**). Other loci besides *SNCA* have been confirmed to be implicated in PD: *PARK-Parkin* encodes for the Parkin protein, *PARK-PINK1* encodes for PTEN-induced putative kinase 1- PINK1 protein, *PARK-DJ1* encodes for the DJ1 protein, *PARK-LRRK2* encodes for the Leucine Rich Repeat Kinase 2 (LRRK2) protein, *PARK-ATP13A2* encodes for the ATP13A2 protein. Of these mutations, *PARK-LRRK2* mutations are associated with an autosomal dominant transmission and the other cited *PARK* mutations are of autosomal recessive transmission. Among these six loci, the roles of certain proteins that are known have indicated to play a role in cellular functions associated with protein quality control. PINK1, DJ1, and Parkin have all three been implicated in mitochondrial function and, in particular, in mitophagy, allowing the degradation of deficient mitochondria. ATP13A2 is a transmembrane lysosomal P5-type ATPase that transports ions, including manganese and zinc, to maintain lysosomal homeostasis. Mutations in the *PARK-ATP13A2* gene provoke several lysosomal alterations, including lysosomal membrane permeabilization (Dehay *et al.*, 2012). Lysosomal dysfunction has also been associated with PD through the *PARK-LRRK2* gene and glucocerebrosidase (GCase) mutations.

Outside of genetic risk factors, environmental factors have shown high importance in the last years. In particular, pesticide exposure and heavy metal exposure have been correlated with a higher risk of developing PD in various studies (Jankovic and Tan, 2020). On the other hand, certain lifestyles have been shown to reduce the risk of PD, including cigarette smoking and caffeine consumption, despite being controversial in the field (**Figure 5**).

Given the various risk factors described here, the exact etiology of PD remains to be fully elucidated, but certain genetic and environmental factors show an undeniable correlation with PD development.

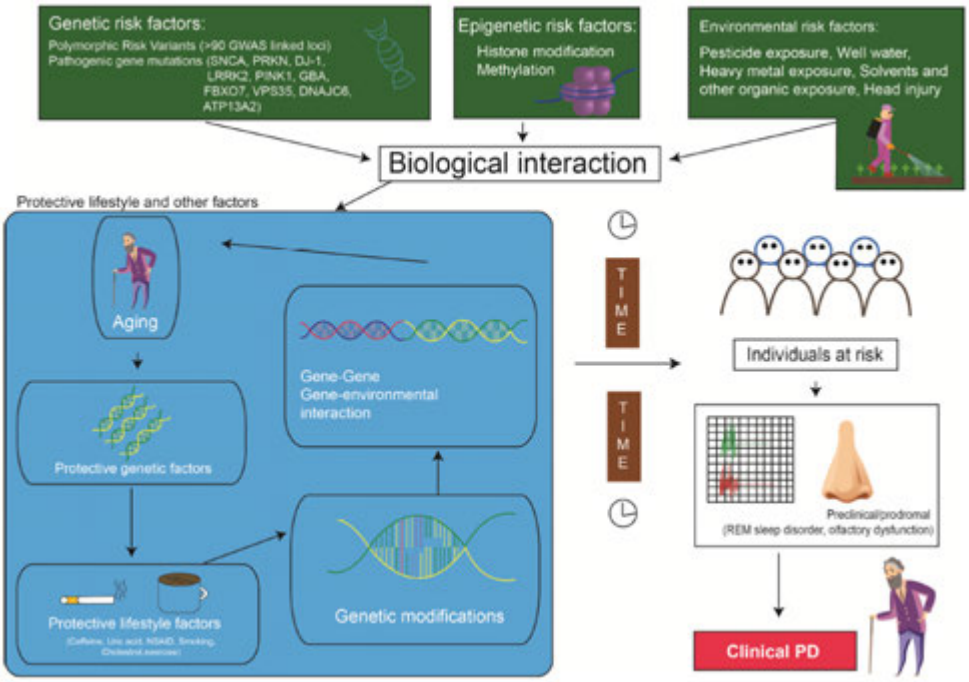

Figure 5: Etiologies of PD pathology. These involve the interaction between genetic predispositions, epigenetic factors and environmental factors, combined with lifestyle. *Adapted from Jankovic et al 2020, Neurol Neurosurg Psychiatry.*

Treatments

Currently, no treatments exist that are capable of halting the progression of PD pathology. The only treatments currently used are to manage the motor and non-motor symptoms associated with the disease (Meissner *et al.*, 2011; Elkouzi *et al.*, 2019; Armstrong and Okun, 2020). There exist two types of treatment approaches: pharmacological or surgical. Pharmacological treatments rely on the synthesis of dopamine to compensate for the loss of dopaminergic neurons and restore the correct function of the brain. For this, the gold standard treatment, discovered in the 1960s, is L-Dopa (L-3,4-dihydroxyphenylalanine), a direct dopamine precursor. Nonetheless, despite its efficiency, L-Dopa can have long-term side effects with the development of dyskinesias in 40-50% of cases. Other pharmaceutical approaches include dopamine agonists and monoamine oxidase B inhibitors, which have their respective side effects (Jankovic and Tan, 2020). Concerning surgical approaches, the most used approach is Deep Brain Stimulation, which consists of implanting stimulation electrodes in the subthalamic nucleus or the internal globus pallidus. This approach, however, is not suitable for all patients and requires specific criteria. Altogether, these treatments are purely used to reduce the motor and non-motor symptoms of PD, but a real need remains for a treatment that will be able to decrease the progression of the disease.

2. Multiple System Atrophy

History

MSA was first described in the early 1900s by Dejerine and Thomas in Paris with patients presenting adult-onset sporadic ataxia that developed extra-pyramidal symptoms and died a few years after (Dejerine, 1900). These patients were then confirmed to show severe olivopontocerebellar atrophy (Quinn, 1989). In 1925, Bardbury and Egglestone described cases of "postural hypotension" associated with symptoms of autonomic nervous system dysfunction such as orthostatic hypotension, anhidrosis and impotence. In 1960, Shy and Drager also described patients with similar autonomic failure symptoms associated with severe parkinsonism and ataxia, thus termed Shy-Drager Syndrome (Wenning and Quinn, 1997). Post-mortem analyses later indicated that patients presented with striatonigral degeneration, with gliosis in the striatum and cerebellar, olivary, and pontine contribution (Adams, Van Bogaert, Van Der Eecken, 1961). The patients presented with varying levels of olivo-ponto-cerebellar degeneration, but all had striatonigral loss accompanied by a parkinsonian syndrome. In 1969, to avoid multiple names for a common syndrome presenting neuronal atrophy in various brain regions, they found the broader term of multiple system atrophy (Graham and Oppenheimer, 1969).

Given the variability of MSA symptoms, MSA neuropathology was confirmed only in 1989 by Papp and colleagues with the first description of GCI in patients presenting with various forms of MSA (Shy-Drager syndrome, olivo-ponto-cerebellar atrophy, striatonigral degeneration) (Papp and Lantos, 1994).

Epidemiology

The prevalence of MSA is 2-4 cases for 100 000 people, with an incidence of 0.1 to 0.8 new cases per year per 100 000 people (Vanacore *et al.*, 2001; Winter *et al.*, 2010). New cases are predominantly revealed in individuals around 55 to 60 years old, with a life expectancy of 6 to 10 years after diagnosis (Winter *et al.*, 2010). Similarly to PD, men are slightly more affected than women, with a ratio of 1.3:1 (Wenning and Quinn, 1997). When compared to PD, MSA is less common in terms of prevalence but with a life expectancy that is very short after diagnosis.

Depending on their symptoms, MSA patients are divided into two main groups after diagnosis: MSA-parkinsonian (MSA-p) and MSA-cerebellar (MSA-c). Patients presenting

parkinsonian symptoms are classified as MSA-p, whereas patients presenting a dysfunction in the olivo-ponto-cerebellar system are classified as MSA-c. Nonetheless, the separation into the two classes of MSA-p and MSA-c is difficult with certain MSA-c patients presenting parkinsonian symptoms and MSA-p patients presenting cerebellar symptoms as well. MSA-p patients are predominantly present in Europe and North America, whereas MSA-c patients are more frequent in Asia (Kollensperger *et al.*, 2010).

Clinical assessment and diagnosis

Patients with MSA present heterogeneous symptoms with a combination of autonomic failure, parkinsonian symptoms, cerebellar ataxia, pyramidal signs and urogenital dysfunction (**Figure 6**). As previously stated, MSA patients are separated into two main groups: MSA-MSA-p and MSA-c (Kollensperger *et al.*, 2010; Fanciulli and Wenning, 2015; Jellinger, 2018).

The majority of MSA patients (87%) present a **parkinsonian syndrome**. MSA-p patients display the same four major motor symptoms as PD: **bradykinesia/akinesia**, **muscular rigidity**, **postural instability,** and **resting tremor**.

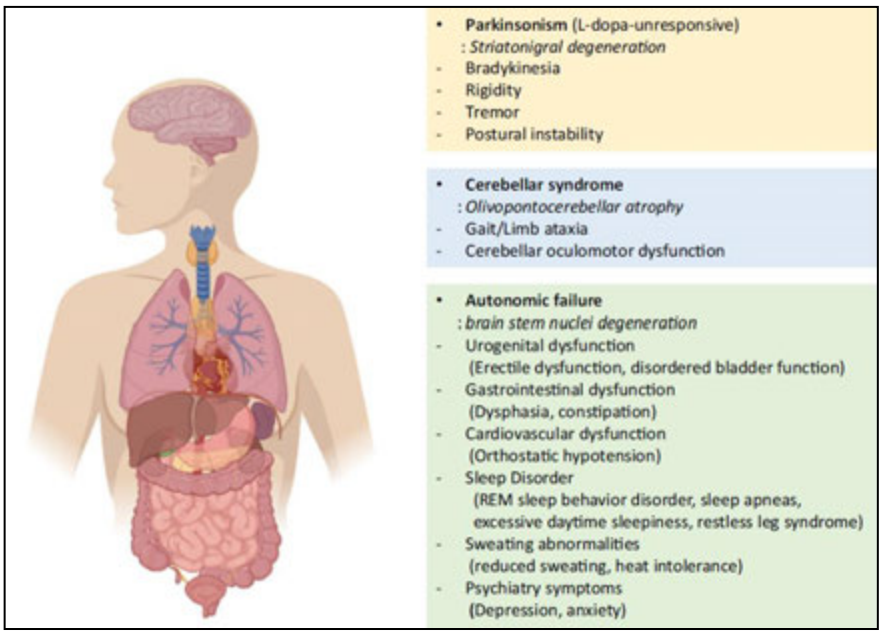

Figure 6: Clinical characteristics of MSA. Each subtype of MSA has particular characteristics, with Parkinsonism symptoms for MSA-p and cerebellar syndrome for MSA-c. Both subtypes share common autonomic failure. *Adapted from Lee and al. 2019, Experimental & Molecul Med.*

On the other hand, **cerebellar syndromes** represent 64% of MSA patients. MSA-c patients display **cerebellar ataxia**, a loss of coordination in movements. Cerebellar ataxia can be accompanied by **gait/limb ataxia**, a modified walk pattern with spread legs and difficulty to execute fluid movements in the extremities, **oculomotor dysfunction** with eye movement abnormalities, and **ataxic dysarthria** with slurred speech and poor coordination.

In addition to these syndromes, around 30-50% of MSA patients present a **pyramidal syndrome**, translated by a generalized hyper-reflexia and/or the Babinski sign, an abnormal plantar reflex.

Similar to PD, MSA patients present many non-motor symptoms, which can often be the first symptoms to appear. The most significant non-motor symptoms in MSA are regrouped as **dysautonomia**, a dysfunction of the autonomous system, which can be seen in 99% of patients. Dysfunction of the autonomous system can be manifested through urogenital dysfunction (urge incontinence, incomplete bladder emptying, and erectile dysfunction), orthostatic dysregulation (blood pressure of at least 20 mmHg systolic, orthostatic syncope), respiratory dysfunction, and gastro-intestinal disruption (constipation). Other non-motor symptoms are mainly **sleep disturbances** and **neuropsychiatric features**, such as depression, hallucinations, dementia, and anxiety. Of all non-motor symptoms, respiratory disorders are the leading cause of death in patients with MSA.

Given the wide variety of MSA symptoms, accurate diagnosis of MSA is a difficult feat. This diagnosis takes place through three degrees of certainty: definite MSA, probable MSA, and possible MSA (**Figure 7**).

- **Definite MSA** is characterized through post-mortem diagnosis with the confirmation of the presence of GCI throughout the brain. Depending on the predominant localization of the GCI, patients are then separated into MSA-p or MSA-c.
- **Probable MSA** is characterized through clinical analyses by the combination of autonomic failure via demonstration of urinary incontinence and severe orthostatic hypotension
- **Possible MSA** is characterized through clinical analyses through the combination of Parkinsonism or cerebellar syndrome with at least one feature suggesting autonomic failure and one additional feature (**Figure 7**).

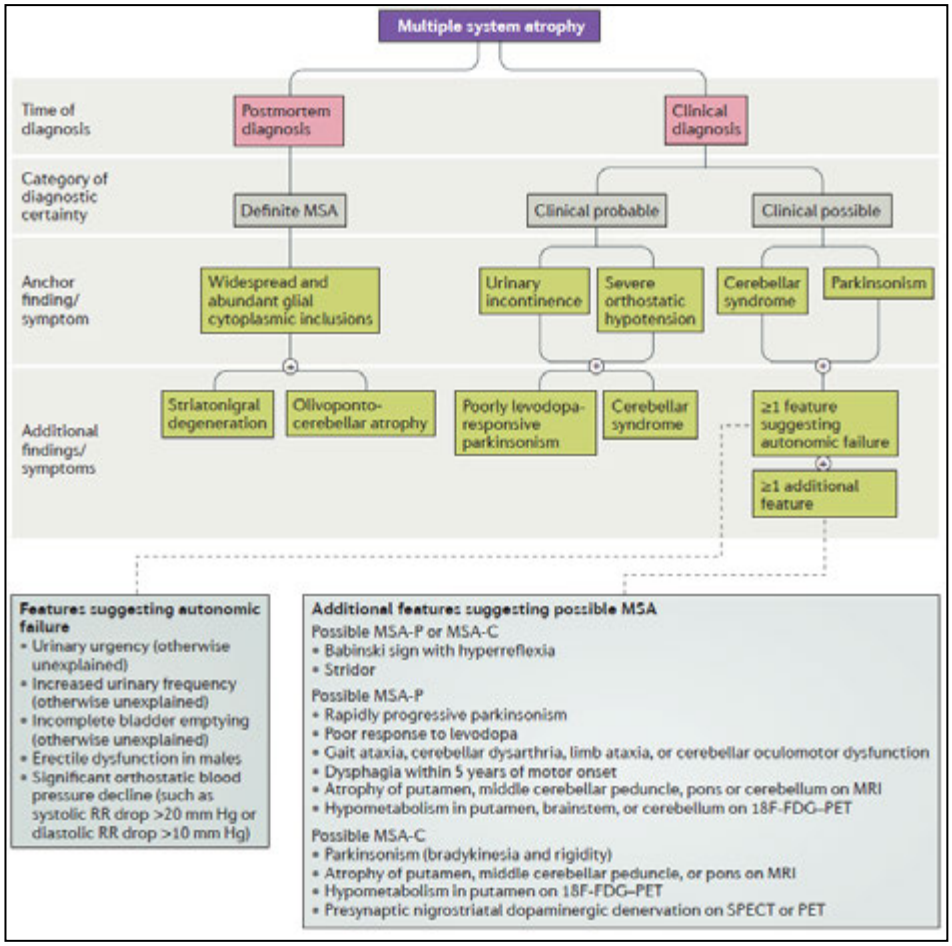

Figure 7: Scheme of MSA diagnosis. Definite MSA can only be characterized through post-mortem confirmation. Clinical diagnosis reveals either probable or possible cases of MSA. *Adapted from Krismer and al. 2017, Nature Rev Neurol.*

Pathophysiology

Similarly to PD, MSA patients present with neurodegeneration that is dependent on the clinical phenotype. Several brain regions are affected by MSA and this is visually observed by the atrophy affecting white and/or grey matter in multiple areas. MSA-parkinsonian patients present neurodegeneration predominantly located in the nigrostriatal pathway, similar to PD patients. MSA-cerebellar patients demonstrate neurodegeneration in the olivo-ponto-cerebellar system. Autonomic brainstem nuclei can also show degeneration, which are at the origin of the characteristic autonomic failure seen in MSA patients.

The core feature in MSA pathology is the presence of intracytoplasmic inclusions present in oligodendrocytes, GCI. The observation of these GCI is required for a confirmed MSA diagnosis but, as previously stated, this can only be done post-mortem (**Figure 7**). GCI density and localization correlate with neurodegeneration and disease progression. In 2005,

Jellinger proposed a pathological grading system to quantify GCI distribution and density, associated with neurodegeneration following three stages of propagation (Jellinger *et al.*, 2005). Given the general similarities between PD and MSA, the answer as to why inclusions form in oligodendrocytes instead of neurons is of great interest within the field.

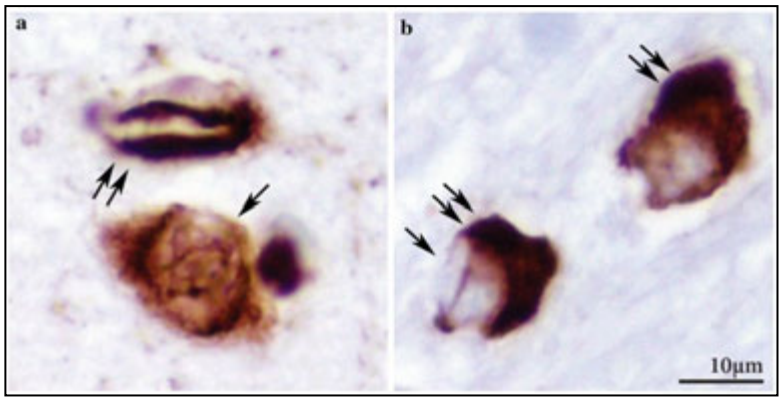

Figure 7: Glial cytoplasmic inclusions from the putamen of MSA-patient brains. Immunohistochemical staining of α-syn-positive inclusions, GCI, found in the putamen of MSA brains. *Adapted from Halliday et al. 2011, Acta Neuropatho*.

Oligodendrocytes are capable of ensheathing axons via their projections, allowing the production of myelin, which protects axonal projections. The myelin sheath has both a protective role and an insulation role to allow for better diffusion of neuronal stimulations. In MSA patients, neuropathology shows a loss of myelination in the region affected by neurodegeneration, accompanied by the relocalization of certain myelin-associated proteins. P25α, also known as tubulin polymerization promoting protein, is an oligodendrocyte-specific protein associated with myelination. In MSA, p25α is relocated from the myelin sheath to the cytosol of oligodendrocytes preceding GCI formation (Song *et al.*, 2007; Ota *et al.*, 2014; Mavroeidi *et al.*, 2019).

In addition to neurodegeneration, GCI formation and demyelination, inflammation is also observed in MSA neuropathology. Microglia are the resident immune cells of the brain and are capable of phagocytosis. Astrocytes are, on the other hand, implicated in maintaining homeostasis and protecting the blood-brain barrier. When alterations are made in the brain, both of these cell types react to the environmental change and are able to activate to protect the central nervous system. In MSA, both of these cell types are found to be activated in regions affected by neurodegeneration.

Etiology

By contrast with PD, few families have reported a history of MSA and, in the families that do, no one specific mutation has been found responsible for the disease (Wullner *et al.*, 2004; Hara *et al.*, 2007; Itoh *et al.*, 2014). Concomitantly to what can be observed in PD, mutations, duplications and triplications of the *SNCA* genes can cause PD with similar traits to MSA in some instances. Specifically, the G51D *SNCA* mutation has demonstrated juvenile Parkinsonism with neuropathological findings associated with both PD and MSA (Kiely *et al.*, 2013). In addition, certain mutations in the *COQ2* gene, encoding coenzyme Q10, have been reported in Japanese families and sporadic MSA cases, which are to date the only potential genetic association with MSA development specifically (Fanciulli *et al.*, 2019). However, no *COQ2* mutations have been detected in occidental countries yet.

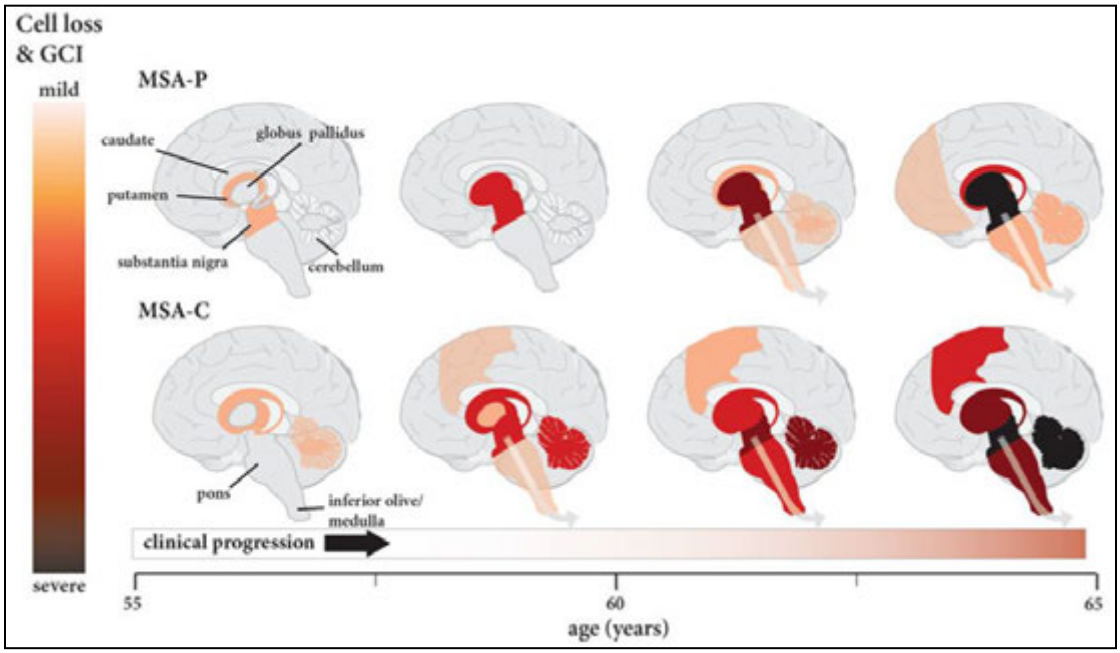

Figure 8: Progression of both subtypes of MSA pathology, MSA-p and MSA-c over time. *Adapted from Halliday et al. 2011, Acta Neuropatho*.

Treatments

Concerning treatments for MSA, similarly to PD, no disease-modifying or neuroprotective treatments exist to decrease the progression of the disease. In MSA-p patients, some of the motor symptoms can be reduced by the use of L-Dopa. However, unlike in PD, L-Dopa is only efficient in 30% of MSA cases and only temporarily for a duration ranging from 1-3 years. Other symptomatic treatments can be used for specific symptoms of MSA, particularly

for autonomic failure, but none cover the entirety of MSA symptoms. Given that, diagnosis only occurs when patients have motor deficits, neurodegeneration is already in advanced stages and this limits the effect of therapeutic strategies.

3. Dementia with Lewy Bodies

DLB is the second most common degenerative dementia in older people, after AD. Historically, the discovery of LB by Lewy was in 1912 and was confirmed by Tretiakoff in 1919, but DLB had yet to be discussed at that time. In 1976, Kenji Kosaka and colleagues described a first post mortem case of dementia accompanied by Lewy body pathology, with the presence of both senile plaques and LB (Kosaka *et al.*, 1976). This was the first description of DLB as we understand it today. In 1984, Kosaka identified additional cases and introduced the disease as "diffuse Lewy body disease" (Kosaka *et al.*, 1984). It wasn't until 1996 that McKeith and colleagues described specific criteria to identify and diagnose DLB (McKeith *et al.*, 1992; McKeith *et al.*, 1996). Currently, it is believed that DLB accounts for 4-7% of dementia cases seen in secondary care, but this disease remains still largely under-diagnosed (Vann Jones and O'Brien, 2014).

Clinically, diagnosis of DLB is a challenge, as half of all patients are misdiagnosed with another type of dementia. Between PD and DLB, most patients are easy to differentiate due to the predominance of extrapyramidal motor features versus dementia. However, this is not always the case, and in some patients, dementia and extrapyramidal signs occur concomitantly, making diagnosis difficult. The core clinical features are the fluctuation in cognition and attention, recurrent visual hallucination, rapid eye movement, sleep behavior disorder, and the later development of parkinsonian motor deficits.

The neuropathological signs of DLB are similar to those of PD, an accumulation of intracytoplasmic LB throughout the brain. This posits the possibility that PD and DLB could stem from similar pathological processes. Nonetheless, in DLB, this is accompanied by the presence of amyloid-β and tau plaques, explaining the dementia symptoms of the disease. It is also important to note that the distribution of these plaques and LB differ in PD and DLB. In PD, they are predominantly located in the SN before progressing to more cortical regions, whereas in DLB, they are found largely in the cortex.

The etiology of DLB remains unclear but, given its similarities with PD, certain gene mutations have been found. Typically, *GBA* mutations in the lysosomal enzyme GCase have

been identified as a risk factor. In addition to this, mutations in *PARK-Parkin*, *APP, SQSTM1*, *MAPT,* and others have been associated with the development of DLB (Kun-Rodrigues *et al.*, 2019; Orme *et al.*, 2020). Genetic heritability for DLB was estimated at 31%, but recent studies have estimated that it might reach up to 60%, making heritability a more critical factor than previously believed (Guerreiro *et al.*, 2016; Guerreiro *et al.*, 2019).

Similar to PD and MSA, no disease-modifying treatments are currently available for DLB. To help with symptoms, a combination of dementia treatments (antipsychotics, cognitive symptoms, depression, and visual hallucinations) as well as dopamine agents can be used. Unfortunately, dopamine agents tend to increase psychotic symptoms, limiting their use as potential treatments for patients with DLB.

B. α-Synuclein

Synucleinopathies hold their name from the α-syn protein present in all three diseases in the intracytoplasmic inclusions found in patient brains. These intracytoplasmic inclusions, either termed LB for PD and DLB, or GCI in MSA, are hallmarks of these neurodegenerative diseases. The exact role of α-syn in the development of these synucleinopathies remains unclear, but understanding α-syn seems to be one of the keys to better understanding these diseases.

1. Physiological structure and function

The first synuclein protein was identified in different mammals in parallel, including in the *Torpedo californica* for its association with synaptic vesicles, in rat and bovine brains, or as a protein implicated in song-learning in birds (Maroteaux *et al.*, 1988; Nakajo *et al.*, 1990; George *et al.*, 1995). For its association to synaptic vesicles and its concomitant nuclear localization, this protein was termed “syn” (synaptic vesicle) and “nuclein” (nucleus) (Maroteaux *et al.*, 1988). A fragment of α-syn sequence, corresponding to the central domain was also a component in amyloid plaques in AD (Ueda *et al.*, 1993). α-Syn was shown to belong to the family of synuclein proteins which contain three homologs: α-syn, β-synuclein, and γ-synuclein.

α-Syn is encoded by the *SNCA* gene and is a small natively unfolded protein of 14kDa, around 140 amino acids long. α-Syn is composed of three principal domains: an N-terminal α-helix domain (residues 1-60), a central non-amyloid component (NAC) domain (residues

61-95), and an unstructured C-terminal domain (residues 96-140) (**Figure 9**). Its N-terminal domain contains 11 amino acid imperfect repeats, which are highly conserved throughout vertebrates and other synuclein proteins. These repeats are thought to form an amphiphilic α-helix, similar to those in apolipoproteins and could confer synuclein proteins the ability for lipid-binding. Importantly, all known mutations of α-syn associated with neuropathology are found in this N-terminal domain, emphasizing its importance in α-syn aggregation. The central NAC domain has a high content in hydrophobic amino acids, which gives it the propensity to aggregate. The C-terminus of α-syn contains a high proportion of charged residues, giving it negative polarity, and is an intrinsically disordered domain. This C-terminus can be phosphorylated at multiple sites, notably the Ser129 phosphorylation. In contrast to the N-terminus, the C-terminal region is less conserved among species and synuclein isoforms (Landeck *et al.*, 2020).

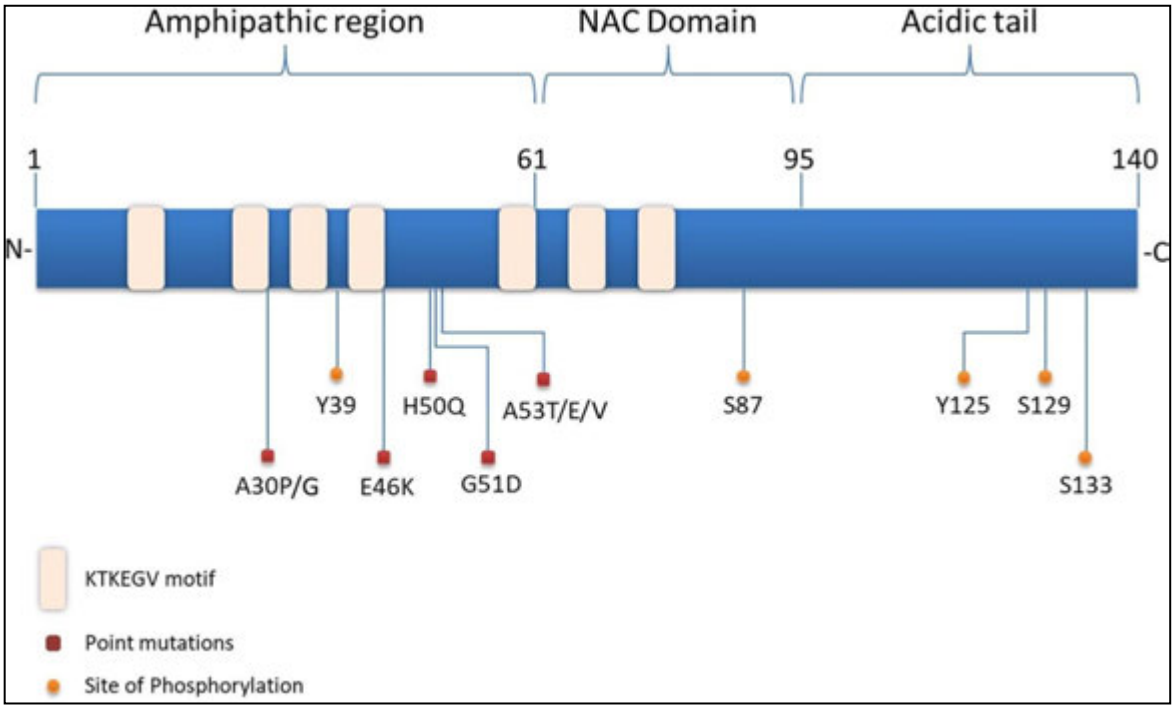

Figure 9: Primary protein structure of α-Synuclein. The N-terminal region is characterized by the presence of a repeated lipid-binding motif and all point mutations associated with PD. The NAC domain favors the aggregation of the protein. The C-terminal acidic tail carries most of the potential phosphorylation sites. *Adapted from Fouka et al. 2020, Front Cell Dev.*

The structure of α-syn remains contested as it appears to be natively unfolded in solution but could adopt a helical conformation in the presence of membranes and bind to membrane lipids. This α-helix conformation, when binding to the membrane, could reduce misfolding as a β-sheet. This hypothesis is strengthened by the observation that all PD-related mutations are

found in the N-terminal region. α-Syn mutations could impair the balance between unfolded and membrane-bound conformations, leading to an increased formation of monomeric, oligomeric, or fibrillary structures (Burre *et al.*, 2015).

α-Syn is primarily found in the central nervous system, in particular in the neocortex, striatum, hippocampus and cerebellum, but can also be found outside of the nervous system. α-Syn has also been detected in cerebrospinal fluid (CSF), muscles, liver, heart, lungs, blood plasma, and red blood cells. This protein is primarily found at axon terminals, where it can associate with synaptic vesicles, but also in cellular compartments such as the nucleus, the mitochondria, and the endoplasmic reticulum.

The specific roles of α-syn in the brain remain unclear, but certain mechanisms have been shown to involve α-syn and give indications of its function (**Figure 10**). The pre-synaptic localization of α-syn indicates its potential role in the synaptic trafficking of neurotransmitters. This presence at synaptic terminals could, in large part, be explained by its small membrane-binding potential. α-Syn has been shown to participate in the control of neurotransmitter release through its interaction with proteins of the SNARE (soluble N-ethylmaleimide-sensitive factor (NSF) Attachment protein Receptor) family, synaptobrevin-2 and VAMP2. The chaperone-like interaction of α-syn with these SNARE proteins allows for the promotion of stable SNARE complex assembly, allowing the presynaptic release of neurotransmitters (Burré *et al.*, 2010). A triple knockout of the three isoforms of synuclein induced a decreased SNARE complex assembly and premature death of these mice (Burré *et al.*, 2010), however loss of just α-syn has little effect on neurotransmitter release. The C-terminal region of α-syn has also been shown to interact with VAMP2 and inhibition of this interaction combined with α-syn overexpression context has been shown to inhibit synaptic vesicle exocytosis (Sun *et al.*, 2019).

In addition to this, α-syn has been shown to have the ability to sense membrane curvature and remodel membranes (Varkey *et al.*, 2010). This has been specifically seen in the case of mitochondria, where α-syn has been shown to mediate mitochondrial fission (Nakamura *et al.*, 2011). This propensity to sense membrane curvature has also implicated α-syn in endocytosis, particularly in the synaptic vesicle cycle once again. In fact, triple synuclein knockouts have been shown to have impaired vesicle recycling when submitted to repeated stimulations (Vargas *et al.*, 2014). α-Syn could also have a specific function on the fusion between synaptic vesicles and the plasma membrane by increasing the fusion pore dilation

and vesicle collapse after fusion to the plasma membrane (Logan *et al.*, 2017). This modulation of endocytosis by α-syn has also been demonstrated by the ability of α-syn to modulate the localization of Dopamine Transporter (DAT) and NMDA (N-methyl-D-aspartate) receptors through the induction of clathrin-mediated endocytosis (Cheng *et al.*, 2011; Kisos *et al.*, 2014).

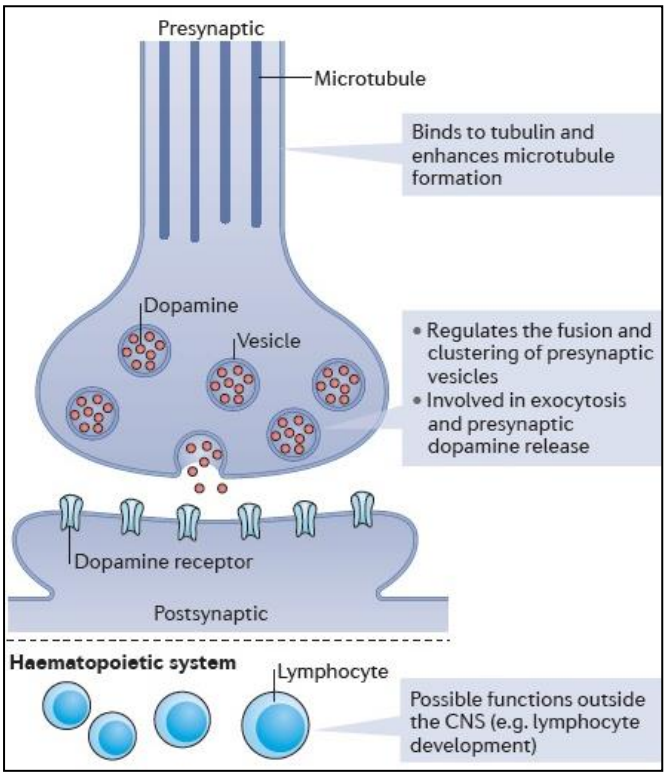

Figure 10: Physiological functions of α-Synuclein. α-Syn is involved in multiple functions at the synapse including in exocytosis and dopamine release at the presynaptic terminal. *Adapted from Koprich et al. 2017, Nature Rev.*

α-Syn can also undergo post-translational modifications (PTMs), which can alter its function within the cell. The most studied and renown of these PTMs is the phosphorylation on Ser129, which has been observed to be present in LB (Fujiwara *et al.*, 2002). However, the pathological implication of this phosphorylation remains debated given the plethora of contradictory data reported in the field. Other PTMs implicated in PD pathology include the phosphorylation on Ser87, Tyr39, Tyr125 or nitration on Tyr39, Tyr125, or Tyr133. The exact roles of these modifications have yet to be completely elucidated as they have been shown to be both neuroprotective and neurotoxic (Negro *et al.*, 2002; Tenreiro *et al.*, 2014; Zhao *et al.*, 2020). Importantly, the implication of a truncation in the C-terminus of the α-syn protein has also been implicated in pathology, with an increased propensity of α-syn to form fibrils *in vitro* and *in vivo*. Though the list of characterized PTMs is long, their exact implication in PD pathology remains unclear (**Figure 11**). This remains largely due to the varying results obtained by different studies and the observation of PTMs in α-syn

overexpressing contexts, which probably influences the manner in which α-syn is altered within the cell.

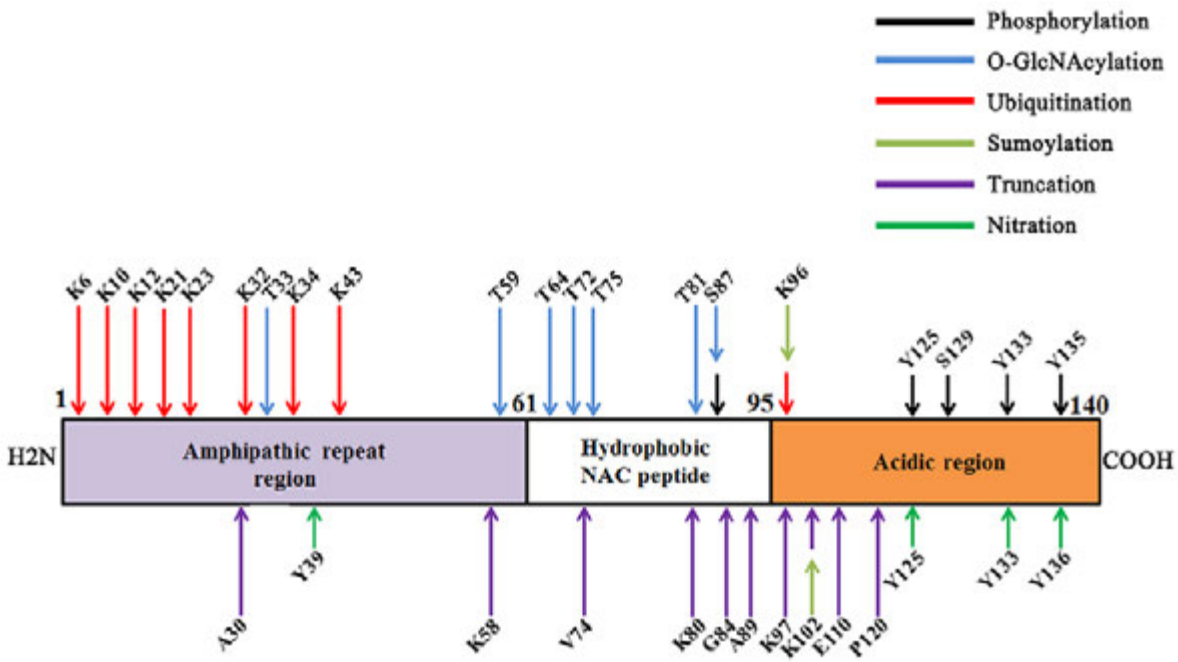

Figure 11: Major post-translational modifications found on the different amino acids of the α-synuclein protein. *Adapted from Zhang et al. 2019 Frontiers in Neuroscience.*

Although the precise physiological functions of α-syn remain unclear, it has been shown that the loss of α-syn does not induce a particular pathology. Nevertheless, the implication of overexpression and aggregation of α-syn in the progression of synucleinopathies remains undeniable.

2. Pathological α-syn

As previously stated, α-syn-positive intracytoplasmic inclusions are one of the hallmarks of synucleinopathies. Depending on the synucleinopathy, these inclusions can be found predominantly in neurons, in the case of PD and DLB, or in oligodendrocytes, in the case of MSA. In this part, we will discuss all aspects of pathological α-syn: its aggregation, spreading, seeding, and to conclude its prion-like characteristics.

α-Syn aggregation

α-Syn in its monomeric form has a natural ability to aggregate through its central NAC domain. This monomeric disordered structure can adopt different intermediary structures

starting with a β-sheet rich pathogenic structure which can then assemble between each other to form dimers, tetramers, and oligomers, also called the nucleation step. When assembled, these oligomers can form protofibrils and fibrils; this is called the polymerization step (**Figure 12**). The aggregation of endogenous α-syn can occur either through a self-seeding mechanism, in which the NAC domain is involved (Giasson *et al.*, 2001) or through the participation of other proteins to initiate α-syn aggregation, such as tau or amyloid-β (Badiola *et al.*, 2011; Bassil *et al.*, 2020).

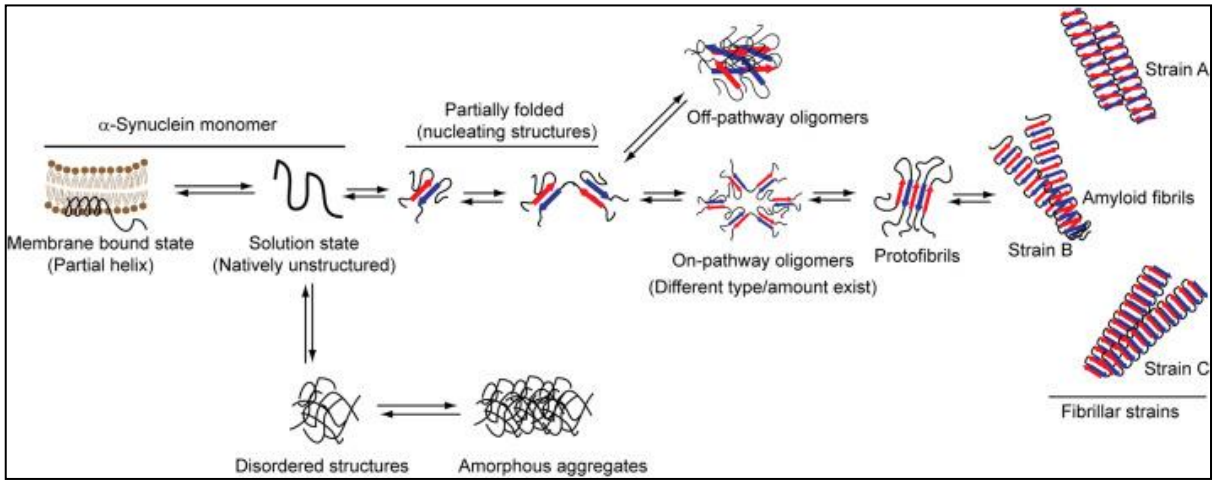

Figure 12: α-Synuclein aggregation pathway. α-Syn exists in two monomeric states: either membrane-bound or natively unstructured. The unstructured monomer can convert to oligomers or other disordered structures. From oligomers, there can be the formation of protofibrils, to then induce the potential formation of multiple strains of amyloid fibrils. *Adapted from Mehra et al 2019, Biochim Biophys Acta Proteins Proteom.*

The identification of which species is the toxic species remains a big unanswered question in the field. Two potential hypotheses have emerged concerning the toxic species: in the first, the toxic species represent the large mature amyloid aggregates, in the second, the toxic species would be smaller intermediate species such as oligomers (Winner *et al.*, 2011; Dehay *et al.*, 2015). From recent studies, it appears that the second hypothesis is more probable while mature amyloid aggregates are somehow composed of trapped toxic species. In fact, a considerable effort has been made to characterize these potentially different oligomers, which could represent different α-syn conformations, or strains, responsible for the variations in α-syn associated pathologies (Bousset *et al.*, 2013; Peng *et al.*, 2018). Certain studies have demonstrated that strain-specific differences can be observed and produce pathologically different diseases (Peng *et al.*, 2018; Lau *et al.*, 2020). These strains could also target specific cellular populations, explaining differences seen between PD and MSA (Lau *et al.*, 2020).

The mechanisms behind the induction of aggregation and the formation of distinct α-syn strains remain largely unknown. However, these different α-syn conformations could be impacted by PTMs, cellular populations, cellular milieu or other unknown mechanisms (Oliveira *et al.*, 2021). This notion will be further addressed in the final discussion of this thesis.

α-*Syn spreading*

In 2003, Braak and colleagues proposed the classification of six neuropathological stages of PD that were classified based on the spreading of α-syn deposits throughout interconnected regions of the brain (Braak *et al.*, 2003). The model suggests that environmental factors or predispositions could trigger the formation of LB pathology. Following this hypothesis, a spreading would begin in the enteric nervous system, olfactory bulb or in the brainstem, extending into the brain and expanding spatiotemporally through neurons until reaching cortical regions. It has been suggested that this could occur through the vagus nerve, but other studies have found no α-syn pathology in the vagus nerve (Braak *et al.*, 2006; Arotcarena *et al.*, 2020). Through observations of many PD cases, it has been hypothesized that there could be two different subtypes of patients: brain-first or body-first. Brain-first patients would be qualified by an appearance of PD in the brain, which then spreads throughout the body, whereas body-first patients would have a disease beginning in the enteric or peripheral autonomic nervous system before spreading to the brain (Borghammer and Van Den Berge, 2019; Horsager *et al.*, 2020). In any case, the spreading of α-syn could occur in susceptible vulnerable cells or through preferential routes, leading to a unique spatiotemporal distribution of the pathology (Walsh and Selkoe, 2016; Surmeier *et al.*, 2017). This progressive propagation of α-syn inclusions was also observed in MSA by characterization of stages of the disease correlated with GCI inclusion spread (Jellinger *et al.*, 2005). In 2008, this hypothesis was further validated by the discovery of Lewy pathology in fetal neurons grafted in PD patient brains more than 10 years before, underlining a potential propagation of LB and α-syn from cell to cell (Kordower *et al.*, 2008; Li *et al.*, 2008; Mendez *et al.*, 2008). This α-syn spreading was further demonstrated *in vitro* using co-cultures of donor and receptor neuroblastoma cells, and *in vivo* with the transfer of α-syn to grafts in mice (Desplats *et al.*, 2009). This host-to-graft transfer was further demonstrated by the transfer of α-syn in 6-hydroxydopamine rats to their grafted embryonic cells (Kordower *et al.*, 2011).

In an experimental context, synthetic preformed fibrils (PFFs) have shown their potential to induce α-syn propagation to interconnected regions, after injection in the striatum of wild-type mice, leading to nigral neurodegeneration and motor impairments (Luk *et al.*, 2012a). This spread of α-syn species was also demonstrated by the injection of LB fractions extracted and purified from PD patient brains in both mice and NHP, where α-syn species were present throughout interconnected brain regions and induced dopaminergic cell loss in the SN (Recasens *et al.*, 2014). This potential to spread using patient-derived extracts was more recently confirmed in a larger size study on NHP (Arotcarena *et al.*, 2020; Bourdenx *et al.*, 2020). These experimental models based on α-syn spreading will be elaborated in greater detail in subsection D discussing models of synucleinopathy of this introduction.

The exact mechanism underlying the spread and transfer of α-syn from a donor to a recipient cell remains unclear (**Figure 13**). Transmission of α-syn can occur between neurons, between neurons and astrocytes, between oligodendrocytes, and between oligodendrocytes and astrocytes. Given that α-syn does not go through classic endoplasmic reticulum/Golgi-dependent exocytosis, its mechanism of secretion remains unclear. However, certain non-classical secretory pathways have been proposed to explain this cell-to-cell propagation, including endoplasmic reticulum/Golgi-independent exocytosis, misfolding-associated protein secretion, exosomal release, tunneling nanotubes or passive secretion through the plasma membrane (Bras and Outeiro, 2021).

Altogether, both clinical observations and experimental studies have proven the possibility of α-syn spreading from cell to cell, but the mechanisms involved in this transmission remain uncertain. Further studies should focus on the manner in which α-syn spreading occurs, which could be a potential therapeutic strategy to limit the expansion of LB.

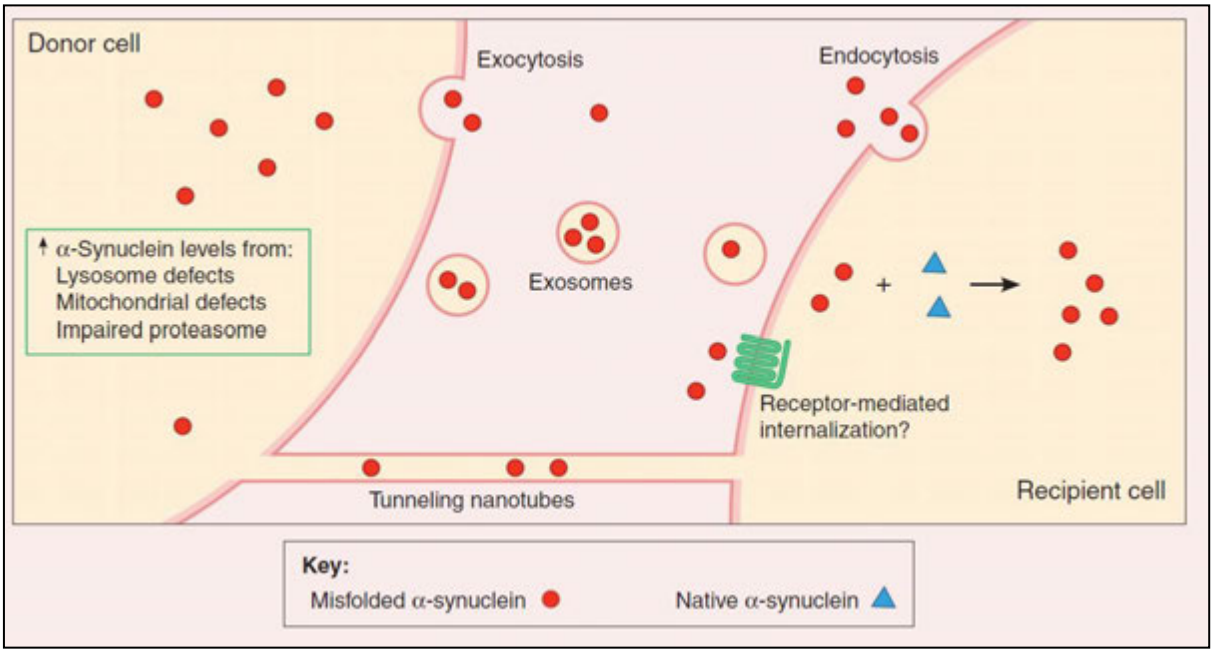

Figure 13: Potential pathways of cell-to-cell transfer of α-synuclein. The possible routes include exocytosis from a donor to the recipient cell, which could possibly be mediated by a receptor on recipient neurons. Exosome-mediated transport and tunneling nanotubes are other routes that could be used to transfer from donor to recipient cell. *Adapted from Hansen et al 2012, Trends Mol Med, Cell Press.*

α-*Syn seeding*

After its ability to spread, α-syn is believed to have the capacity to transmit its pathological information to endogenous and naïve α-syn proteins of infected cells. First, the exposure of cells to exogenous α-syn fibrils has been shown to induce the accumulation of endogenous α-syn in multiple independent studies (Volpicelli-Daley *et al.*, 2011; Bousset *et al.*, 2013). Adding human exogenous PFFs to primary cultures of mouse hippocampal neurons leads to the formation of inclusions positive for both human and murine α-syn. This double-positivity suggests recruitment of endogenous murine α-syn by human α-syn fibrils once in the cell (Hansen *et al.*, 2011; Volpicelli-Daley *et al.*, 2011). This was then confirmed by studies *in vivo* with the injection of these same human α-syn fibrils leading to the formation of inclusions positive for murine α-syn (Luk *et al.*, 2012b). These seeding properties were also assessed using patient-derived inclusions in mice, which once more confirmed the seeding potential of α-syn (Recasens *et al.*, 2014). They showed that human α-syn was not detectable in mice brains after just 24 hours and that endogenous murine α-syn accumulates after just 4 months. Most importantly, they determined that these phenomena were not seen in α-syn knockout mice or α-syn immunodepleted fractions.

By demonstrating that exogenous aggregates of α-syn are able to recruit endogenous α-syn monomers and serve as templates in their aggregation, these studies have shed light on the capacity of α-syn to expand the progression of pathology. This self-seeding ability, along with its aggregation and propagation, give α-syn similar aspects to prion proteins.

α-Syn: a prion-like protein?

Prion diseases are infectious neurodegenerative diseases that hold their infectious potential from the prion protein (Prusiner, 1982). With a change in conformation to a β-sheet rich structure, this prion protein becomes pathological and induces the formation of amyloid fibrils. These pathological prion proteins have the ability to propagate and seed the formation of additional fibrils. This prion protein can also be transmitted between individuals, making it an infectious disease, leading to spongiform encephalopathies such as Creutzfeldt-Jakob disease.

In the last years, it has been described that other proteins could hold this same prion-like potential inducing their associated diseases. Among these proteins, there is Aβ and Tau for AD, TDP-43, and SOD1 for amyotrophic lateral sclerosis, huntingtin for Huntington's disease, and α-syn for synucleinopathies.

As seen in this section, α-syn holds many of the same characteristics as the prion protein including:

- The ability to change from its native conformation into a β-sheet rich pathogenic structure
- The ability to spread from cell-to-cell
- The potential to seed the formation of new pathogenic species in newly infected cells

However, synucleinopathies do not seem to exhibit the potential of being transmitted between individuals of the same species (Asher *et al.*, 2020). This lack of infectious capacity excludes α-syn from being a proper prion protein and confers it the status of a "**prion-like**" protein.

3. Cellular mechanisms impaired in synucleinopathies

α-Syn-induced toxicity seems to act on multiple levels within the cell, through multiple independent and complementary mechanisms. The accumulation of intracellular aggregated α-syn has pleiotropic pathological effects on the cell, including synaptic vesicle impairments, mitochondrial dysfunction, oxidative and endoplasmic reticulum stress, or dysfunction of

clearance pathways including the autophagy-lysosomal pathway (ALP) and the ubiquitin-proteasome system (UPS) (**Figure 14**) (Serratos *et al.*, 2021).

The physiological interaction of α-syn with vesicles and its role in vesicle trafficking may explain the disruption of synaptic motility by α-syn oligomers associated with dopamine release (Choi *et al.*, 2013; Burre *et al.*, 2015). In addition, misfolded α-syn aggregates have also been shown to associate with the endoplasmic-reticulum membrane. This association causes morphologic dysfunction, perturbations in chaperone levels, an increased level of reactive oxygen species (ROS) and/or calcium leakage in the cytosol (Smith *et al.*, 2005). α-Syn aggregates can also modulate the transport of proteins from the Golgi apparatus to the endoplasmic reticulum, leading to impairment in protein production and quality control (Cooper *et al.*, 2006). Concerning the mitochondria, α-syn oligomers induce mitochondrial swelling and depolarization combined with an accelerated release of cytochrome c. α-Syn aggregates could also lead to high oxidative stress by inhibiting the mitochondrial complex I subunit and increasing the production of ROS (Choi *et al.*, 2012; Luth *et al.*, 2014).

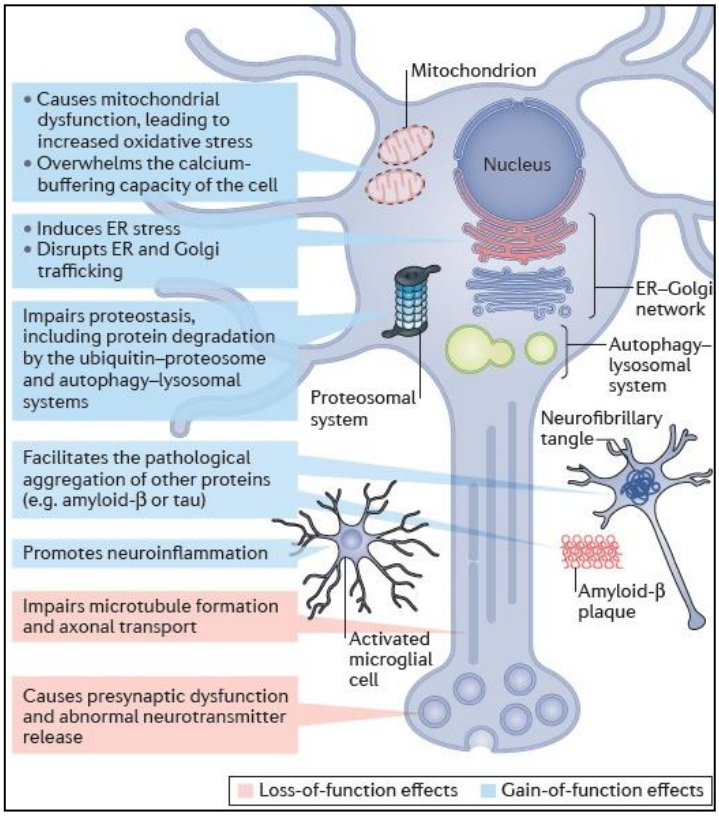

Figure 14: Pathological effects of α-synuclein on cellular mechanisms. These include gain-of-function effects which can induce mitochondrial dysfunction, endoplasmic reticulum (ER) stress, protein degradation impairment, and neuroinflammation. There can also be loss-of-function effects including the impairment of microtubule formation and abnormal neurotransmitter release. *Adapted from Koprich et al. 2017, Nature Rev.*

Outside of the neuron, other cell non-autonomous impairments are caused by α-syn accumulation. The implication of glia has been increasingly shown in the last years, partly due to the aggregation of α-syn in oligodendrocytes in MSA pathology. However, this accumulation of α-syn could be transferred from neurons to glia, which remains to be confirmed. Neuroinflammation has also been shown to contribute to pathogenesis, both from astrocytes and microglia. Astrocytes have been shown to uptake α-syn via endocytosis, which leads to an inflammatory response (Lee *et al.*, 2010). Microglia, on the other hand, have been shown to be activated via α-syn binding to Toll-like Receptors, leading to a pro-inflammatory response (Kim *et al.*, 2013; Kim *et al.*, 2015; Kim *et al.*, 2018).

In parallel to these cellular impairments, a dysfunction in α-syn clearance can lead to the abnormal accumulation of the protein, which can, in turn, disrupt autophagy-lysosomal and ubiquitin-proteasomal functions, entering in a vicious circle. Despite the importance of dysfunctions of the cell, including endoplasmic reticulum stress, impaired proteostasis, or the promotion of neuroinflammation (**Figure 14**), I will focus particularly on the impairment of clearance mechanisms by α-syn in synucleinopathies.

Autophagy-Lysosomal pathway

ALP is a complex process in charge of long-lived and aggregated protein degradation, as well as clearance of damaged organelles through multiple pathways, including the non-selective macroautophagy (MA) and the selective chaperone-mediated autophagy (CMA) (**Figure 15**). MA non-selectively degrades cellular waste through the fusion of the autophagosomes carrying the material into the lysosomes containing the enzymatic material. On the other hand, CMA degrades proteins after the specific recognition of a pentapeptide KFERQ-like motif by the cytosolic chaperone heat-shock cognate 70kDa and delivery of the targeted protein to the lysosomes through the Lysosomal-Associated Membrane Protein 2A (LAMP2). Alterations in both ALP pathways involved in α-syn clearance have been implicated in PD pathology by both genetic and post-mortem studies (Arotcarena *et al.*, 2019).

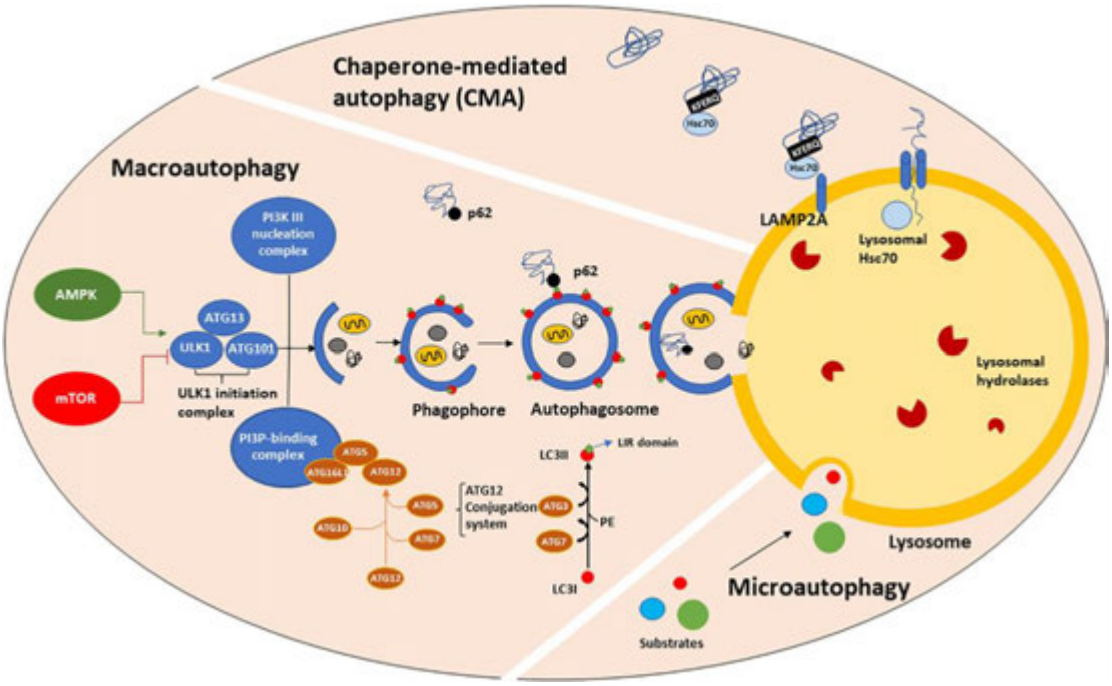

Figure 15: Illustration of the Autophagy-Lysosomal Pathway. This pathway includes non-selective macroautophagy, which relies on an activation and initiation complex to induce the formation of the phagophore and autophagosome. The Chaperone-mediated autophagy relies on the recognition of the KFERQ motif, which is then uptaken by Hsc70 towards the lysosome. Microautophagy also allows for direct degradation to the lysosome. *Adapted from Albanese et al. 2019, Frontiers in Neurosci.*

ALP is essential for maintaining neuronal health since neurons are particularly susceptible to the accumulation of defective organelles, but paradoxically autophagy becomes impaired in normal aging. In synucleinopathy patients, post-mortem analyses have demonstrated dysregulation of this clearance mechanism. In 1997, Anglade and collaborators observed a decrease in autophagic vacuoles in the SN of PD patients (Anglade *et al.*, 1997). Increased post-mortem observations of ALP in synucleinopathies were further confirmed by the detection of the autophagosomal marker LC3 in LB from PD patients (Chu *et al.*, 2009; Alvarez-Erviti *et al.*, 2010; Dehay *et al.*, 2010), in the hippocampus and cortex of DLB patients (Crews *et al.*, 2010), and in GCI located in the pons of MSA patients (Schwarz *et al.*, 2012; Tanji *et al.*, 2013). These observations demonstrate a possible accumulation of autophagosomes in all three synucleinopathies.

The relationship between α-syn accumulation and autophagy-lysosomal impairment is a dual loop: autophagy impairment induces an increase in α-syn accumulation, and conversely, α-syn impairs correct autophagy function. With this vicious circle, it is difficult to understand which comes first, autophagy impairment or α-syn accumulation (**Figure 16**).

Given that α-syn is degraded either by the ALP or the UPS, any deficits in this machinery will directly impact its accumulation. In fact, inhibitions of the ALP by knockout of the *Atg7* gene or the *PARK9* gene have both shown an accumulation of α-syn. With the induction of MA, using the mTOR inhibitor rapamycin, in PC12 cells overexpressing α-syn, there was an increased clearance of both wild-type and mutant forms of α-syn. Conversely, increased intracellular α-syn was observed when autophagosomal formation and fusion with the lysosome were inhibited (Webb *et al.*, 2003). Similarly to MA implication in α-syn degradation, CMA has also been shown to be involved in α-syn clearance. Cuervo and colleagues demonstrated that when inhibiting lysosomal proteolysis, the half-life of α-syn increased however, inhibition of MA did not inhibit α-syn degradation (Cuervo *et al.*, 2004). In this same study, mutations of the CMA-recognition motif in the α-syn sequence decreased its translocation to the lysosome and thus implicated CMA in α-syn degradation. Knockdown of *LAMP2A* also induced autophagic defects and increased α-syn accumulation (Vogiatzi *et al.*, 2008a; Xilouri *et al.*, 2013). These data thus suggest that α-syn is degraded by the ALP, either by MA or CMA, and dysfunctions in this pathway participate in α-syn accumulation and aggregation.

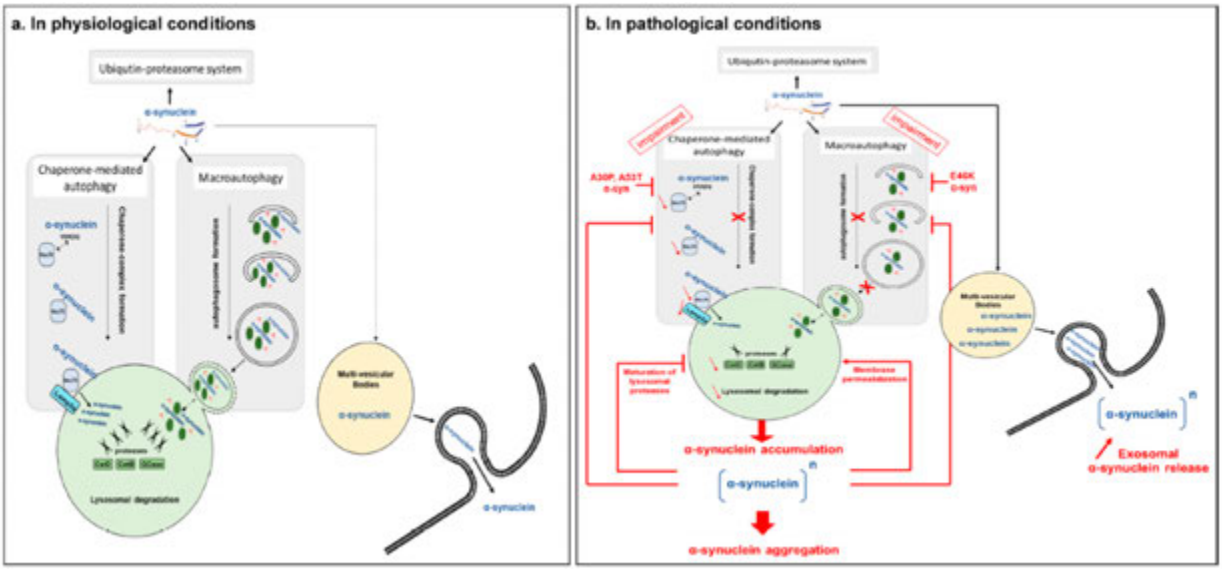

Figure 16: Dual loop between autophagy lysosomal pathway and α-synuclein. (a) In physiological conditions, α-syn is degraded through the ubiquitin-proteasome system, chaperone-mediated autophagy or macroautrophagy. (b) In pathological conditions, the impairment of the two types of autophagy and α-syn inhibition of these pathways induce an increased α-syn accumulation. This increases once more the inhibition of degradative pathway, turning into a vicious cycle. *Adapted from Teil et al 2019, Cells.*

On the other hand, the presence of α-syn inclusions has also been shown to affect correct autophagy function, particularly the fusion of autophagosomes with lysosomes, resulting in a decreased protein clearance. This was first observed by an overexpression of α-syn *in vitro,* which led to an accumulation of vesicular structures associated with lysosomal impairment (Stefanis *et al.*, 2001). This could be due to the decrease in Rab1 activity induced by α-syn, which impairs the correct localization of the ATG9 protein involved in autophagosome formation (Winslow *et al.*, 2010). Similarly, the interaction between autophagic trigger HMGB1 and α-syn has also been shown to decrease Beclin-1 protein levels, which in turn inhibit autophagic degradation (Song *et al.*, 2014). α-Syn has also been demonstrated to impact CMA function by binding to LAMP2, forming oligomeric species, and inhibiting its correct translocation to the lysosomal lumen (Martinez-Vicente *et al.*, 2008). Lysosomal function can also be impacted by α-syn accumulation, in particular lysosomal hydrolases, such as Cathepsin B, GCase, and β-galactosidase used for acidification and degradation of the cargo (Mazzulli *et al.*, 2011; Mazzulli *et al.*, 2016). Altogether, these studies and others have demonstrated the role of α-syn in perturbing the ALP function.

Given that ALP plays a role in α-syn accumulation, its impact on neurodegeneration must also be assessed. In fact, autophagic genes *Atg5* and *Atg7* have both been implicated in neurodegeneration, with knockout mice for these genes inducing the formation of ubiquitin-positive inclusions and neuronal loss (Hara *et al.*, 2006; Komatsu *et al.*, 2006; Komatsu *et al.*, 2007). In synucleinopathies, autophagy dysfunction has been observed through different mechanisms within the cell from the lysosomal stress, to mitophagy defects (a mitochondrial-centered autophagy), and finally in cellular trafficking disturbances that lead to neuronal loss (**Figure 17**) (Arotcarena *et al.*, 2019).

Altogether, ALP clearance plays a vital role in neurodegeneration and α-syn accumulation, with α-syn also impacting correct ALP function. The fine and specific regulation of autophagy could thus become an important aspect in initiating a better clearance of α-syn and halting neurodegeneration.

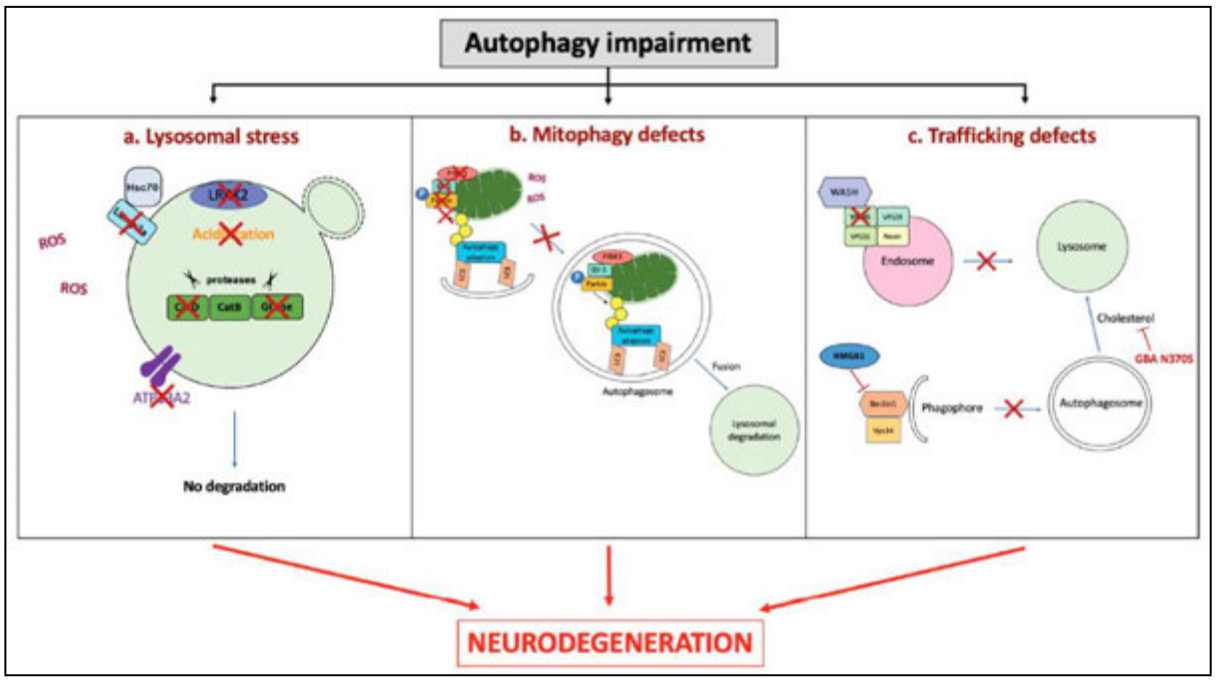

Figure 17: Autophagy impairment induces neurodegeneration at multiple levels within the cell. (a) Lysosomal stress can induce the loss of acidification or defective lysosomal proteases, making degradation impossible. (b) Mitophagy defects are caused by alterations in proteins implicated in priming defective mitochondria. (c) Trafficking defects impair autophagosome formation, and thus macroautophagy as a whole. *Adapted from Teil et al 2019, Cells.*

Ubiquitin-proteasome system

UPS is involved in short-lived, damaged and misfolded protein degradation through a step of ubiquitination followed by proteolysis, involving the action of multiple proteases. The proteasome is a large cylindrical protease complex made up of a proteolytic core known as the 20S proteasome and a 19S regulatory cap, which together form the active 26S proteasome. Proteins destined for proteasomal degradation undergo poly-ubiquitination, a procedure that is tightly controlled by three enzymes: E1 activates and transfers ubiquitin to the E2 ubiquitin-conjugating enzyme, a carrier enzyme that conjugates ubiquitin to the Lysine of the target protein through a specific E3 ubiquitin ligase. There are hundreds of E3 ligases with affinities for distinct substrates, conferring selectivity to the process. This cycle between E1, E2 and E3 enzymes occurs multiple times to confer the poly-ubiquitination to the protein, which acts as a signal to target the protein to the proteasome. Once recognized by the 19S regulatory cap, the protein is brought towards the lumen of the proteasome, where it is degraded into small peptides which can be recycled by the cell (**Figure 18**).

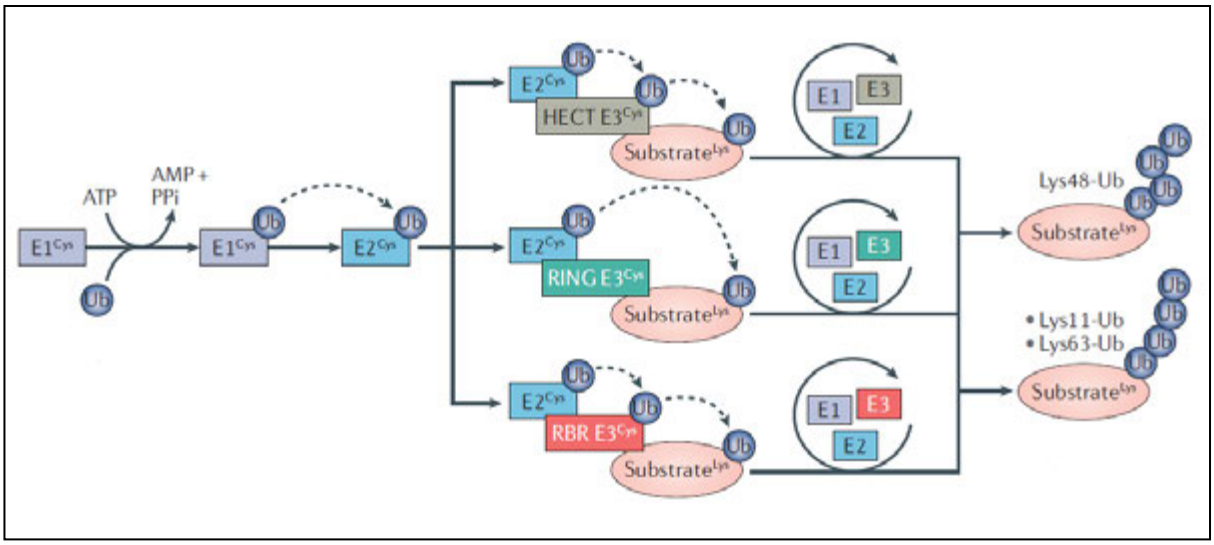

Figure 18: The ubiquitin-proteasome system. With the help of E1, E2 and E3 enzymes, substrate proteins are poly-ubiquitinated to then be delivered to the proteasome for degradation. *Adapted from Meyer-Schwesinger, 2019 Nature Reviews Nephrology.*

UPS dysfunction has often been believed to participate in the pathology of synucleinopathies. This was first due to the observation of ubiquitin in LB (Lowe *et al.*, 1988; Iwatsubo *et al.*, 1996). These LB were then seen to contain UPS machinery, including proteasome subunits and enzymes (Lowe *et al.*, 1990; Ii *et al.*, 1997). The importance of the UPS was further confirmed by post-mortem analyses of the SN of PD patients that demonstrated a decrease in proteasomal subunit α and a decreased protease activity (McNaught *et al.*, 2002; McNaught *et al.*, 2003). Additionally, a truncated form of α-syn was shown to be ubiquitinated in LB (Tofaris *et al.*, 2003). However, certain studies did not observe accumulation of α-syn after inhibition of the proteasome *in vitro* (Vogiatzi *et al.*, 2008b) and α-syn has not shown to be a consistent proteasomal substrate depending on the cell line (Ancolio *et al.*, 2000). Nonetheless, oligomeric forms of α-syn can impair proteasomal function and, conversely, the inhibition of UPS activity induces the formation of oligomeric α-syn structures (Emmanouilidou *et al.*, 2010).

Mutant forms of α-syn (A53T and A30P) have been shown to inhibit the UPS more than wild-type α-syn (Stefanis *et al.*, 2001; Smith *et al.*, 2005). In mouse primary cultures, TH-positive neurons were found to be selectively vulnerable to the overexpression of mutant α-syn and proteasome inhibition (Petrucelli *et al.*, 2002). The baseline activity of the UPS was also reduced in the cortex of α-syn transgenic mice (Ebrahimi-Fakhari *et al.*, 2011). In addition, in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD, the toxin was showed to decrease the proteasome levels in both mice and marmosets (Fornai *et al.*,

2005; Zeng *et al.*, 2006). Oxidative stress has also been shown to influence the UPS activity, in particular, an excess in oxidative stress and ROS production can overload the proteasome and lead to its inhibition (Okada *et al.*, 1999). More recently, the various strains of α-syn have been shown to influence the degradation systems in charge of clearance. A PFF-treated neuroblastoma cell line has suggested that the uptake of larger fibrils of α-syn is done by the ALP whereas smaller oligomers are degraded, at least partially, by the UPS (Pantazopoulou *et al.*, 2021). Collectively, these results suggest that the proteasome could be inhibited as a consequence of α-syn accumulation, even though the UPS is probably not the major degradation system for α-syn.

Together, both ALP and UPS have been shown to be responsible for the degradation of α-syn within the cell. In the case of synucleinopathies, these degradative systems are altered and become largely dysfunctional. This dysfunction leads to an even larger increase in α-syn accumulation, increasing the neurodegenerative effects of α-syn.

C. Zinc and its implication in neurodegeneration

Certain metal ions such as iron, copper, zinc, and manganese are components of thousands of proteins and are indispensable for certain functions throughout the cell. These metals are essential for many reactions occurring in the body, but of particular importance in the brain. These metals are responsible for many cellular functions, including synaptic transmission, energy production, myelination, and regulation of oxidative stress. Alterations of these metals have been commonly reported in multiple brain diseases, including PD. These dysregulations in metal concentrations can be detrimental to neurons and other brain cells. Here, in my thesis, I was particularly interested in zinc and its role in neurodegeneration that I will specifically detail below.

1. Function and localization of zinc

Zinc function

Among heavy metals, zinc is the second most predominant metal in the body after iron. Zinc is a transition metal with an atomic number of 30 and an atomic mass of 65.39. Zinc is relatively stable when coupled with nitrate, oxygen, and sulfur. According to nutritionists, it is an essential micronutrient of which the daily intake should be around 7 to 15 mg depending

on the individual. To meet these needs, the zinc contained in our food is the principal source of zinc intake.

It is estimated that 10-15% of genes in the human genome encode proteins that use zinc as a cofactor. These genes include transcription factors, enzymes, and structural and signaling proteins. Zinc plays a significant role in multiple cellular functions such as: regulation of the cell cycle, DNA replication, cell differentiation, apoptosis, and lipid metabolism (Marger *et al.*, 2014). Two other essential functions of zinc are its antioxidant and anti-inflammatory roles. The antioxidant properties of zinc are mediated by its ability to interact with amino acid residues that present oxidation, its structural roles in enzymatic antioxidants such as Cu-Zn-superoxide dismutase, and metallothionein synthesis. The anti-inflammatory effect of zinc has been shown to rely on the inhibition of NF-κB and pro-inflammatory cytokines. Zinc has also shown its importance in inflammation by regulating the proliferation and maturation of immune cells such as lymphocytes and dendritic cells. Zinc insufficiency can thus be translated by a diminished immune response, difficulties with healing, and a certain amount of neurological disorders.

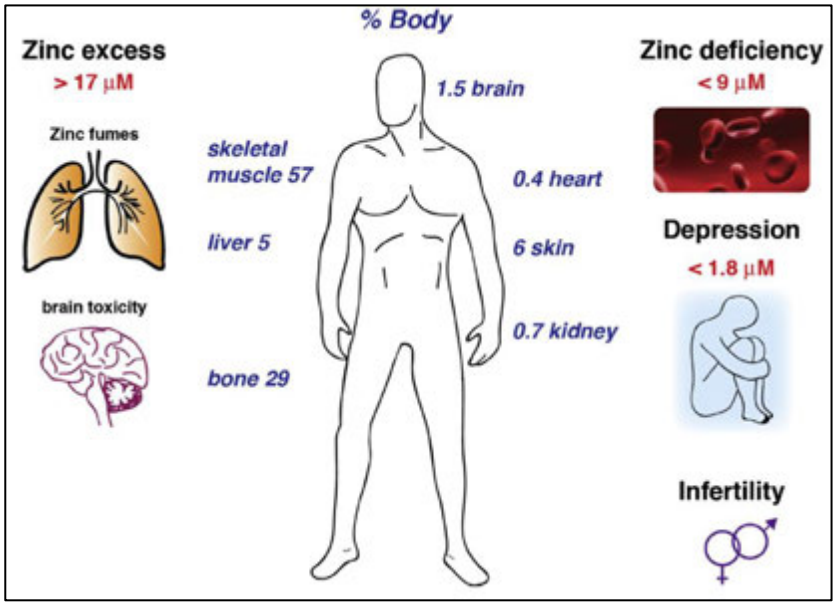

Figure 19: Distribution of zinc and the effects of zinc dysregulation in the human body. *Adapted from Marger et al 2014 Biochem Pharm.*

Zinc homeostasis

In the human body, most of the zinc is stored within the skeletal muscle (57%), in bones (30%), in the liver and skin (5%), and the brain (1.5%). Given its importance, concentrations

of zinc must be tightly regulated due to its toxicity in high concentrations (**Figure 19**). For correct zinc-binding protein function, labile zinc concentration must be in the picomolar range within the cytoplasm. Over these concentrations, zinc shows toxicity for proteins and can lead to their misfolding. Cellular zinc is mainly distributed in the cytoplasm (50%), nucleus (30-40%), and at the plasma membrane (10%). To maintain zinc homeostasis, the cell relies on multiple zinc transporters present at the plasma membrane and at the membrane of organelles. In case of excess labile zinc in the cytosol, transcription of transporters coding for the elimination of zinc from the cytosol is activated. When there is a deficiency in zinc, transporters importing zinc in the cytosol are transcriptionally activated. Mammalian zinc transporters come from two major families: Zrt-Irt-Proteins (ZIP / SLC39) family and the Zinc-transporters (ZnTs / SLC30) family (Kambe *et al.*, 2015). Both of these transporter families and zinc-binding proteins are the main regulators of intracellular zinc concentrations.

The ZIP transporter family is responsible for the import of zinc. The ZIP family consists of 14 isoforms, from ZIP1 to ZIP14, which allow the passage of zinc from the extracellular space, intracellular vesicles, or the lumen of organelles towards the cytoplasm. The majority of ZIP transporters are located at the plasma membrane and their expression increases under zinc-deficient conditions. In case of excess zinc, these transporters are rapidly internalized and degraded. All ZIP transporters are relatively similar forming homodimers composed of eight transmembrane domains. Despite these similarities, ZIP proteins are not expressed in the same area of the cell (plasma membrane or specific organelle) or in the same cell types (**Figure 20**).

The ZnT transporter family is responsible for the export of zinc from the cytoplasm. The ZnT family consists of 10 isoforms, from ZnT1 to ZnT10, which remove zinc from the cytoplasm towards the extracellular space or its sequestration in organelles. Among these ZnTs, only ZnT1 is expressed at the plasma membrane, whereas most other ZnT transporters are located at the membrane of organelles for zinc sequestration or storage. Similarly to ZIP transporters, ZnTs have a similar structure and are composed of six transmembrane domains and their localization is mostly tissue-specific (**Figure 20**).

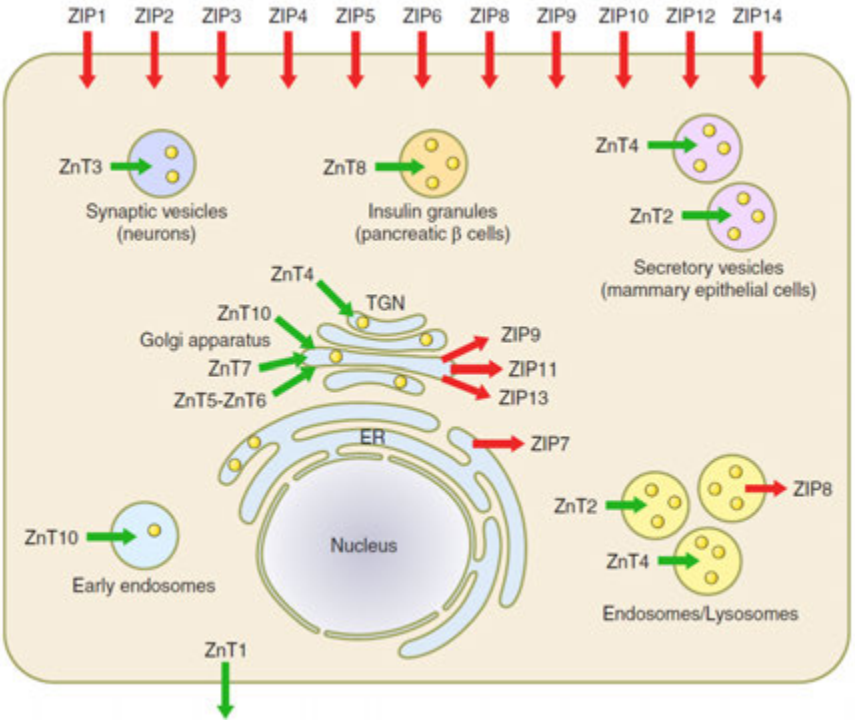

Figure 20: Localization and direction of transport of zinc transporters within the cell. Zinc transporters (ZnT) export zinc outside of the cytoplasm and Zrt-Irt-Proteins (ZIP) import zinc toward the cytoplasm. *Adapted from Kambe et al. 2015, Physiol Rev.*

In addition to transporters, certain zinc-binding proteins are capable of providing a rapid response to excess and low zinc in the cytosol: metallothioneins. Metallothioneins are small, cysteine-rich metal-binding proteins that are highly involved in zinc homeostasis. With variations in cytosolic zinc, metallothioneins are transcribed through the activation of the metal regulatory transcription factor, increasing metallothionein synthesis and adjusting zinc concentrations. This metal regulatory transcription factor has been shown to regulate the transcription of metallothioneins but also of ZnT1.

Brain zinc

The brain represents the organ with one of the highest zinc concentrations in the body, with around 150 µM of labile zinc present. There are three pools of zinc in the brain: free zinc, protein-bound zinc, and synaptic zinc. Zinc is capable of being released at the synapse via synaptic vesicles, where it can physiologically regulate many synaptic processes. In the synaptic cleft, extracellular zinc levels increase to modulate neurotransmission. In general, zinc seems to have an important role of second messenger in the cell, giving it the name of "the calcium of the twenty-first century" (Frederickson *et al.*, 2005).

Zinc concentrations in the brain increase during development until adulthood and then remain stable. Zinc is present throughout the brain but particularly in the hippocampus, cerebral cortex, and amygdala. Glutamatergic neurons contain more zinc than other neurons and are located predominantly in the cerebellum, olfactory bulb, and hippocampus. At the synaptic cleft, the zinc released modulates the post-synaptic response particularly by binding with NMDA, GABAergic, AMPA and other receptors. In the case of NMDA and GABA receptors, zinc seems to inhibit their activity. Through its binding and modulation of synaptic receptors, zinc has been shown to play a role in sensory processing and synaptic plasticity.

Maintaining zinc homeostasis in the brain is excessively important as zinc dyshomeostasis has been associated with many neurological disorders. To avoid its neurotoxicity, brain zinc is tightly regulated by its ZnT and ZIP transporters. Synaptic vesicle zinc is estimated to represent 5-10% of neuronal zinc. The discovery of ZnT3 as the transporter responsible for sequestering zinc into synaptic vesicles helped understand a certain aspect of zinc homeostasis in the brain. Indeed, ZnT3 knockout mice demonstrated a 20% decrease in brain zinc, with a total elimination of synaptic zinc. Despite this, there seemed to be no behavioral changes in learning, memory, and sensorimotor function in young ZnT3 knockout mice (Cole *et al.*, 2001). Later, other studies using older ZnT3 knockout mice demonstrated that vesicular zinc is required for memory function in adulthood, particularly in maintaining synaptic health during aging (Adlard *et al.*, 2010; Takeda *et al.*, 2017).

Altogether, zinc homeostasis is of great importance in the brain, and more generally, in the entire human body, as it mediates many cellular activities indispensable for correct function.

2. Cellular mechanisms altered by zinc

Dyshomeostasis of zinc can have many effects at the level of the cell, which then translate to physiological alterations, including a myriad of zinc-associated neurological disorders. These effects of zinc alterations are in large part due to the multifaceted role of zinc in cellular mechanisms, including mitochondria, autophagy-lysosomal pathway, and apoptosis and necrosis (**Figure 21**).

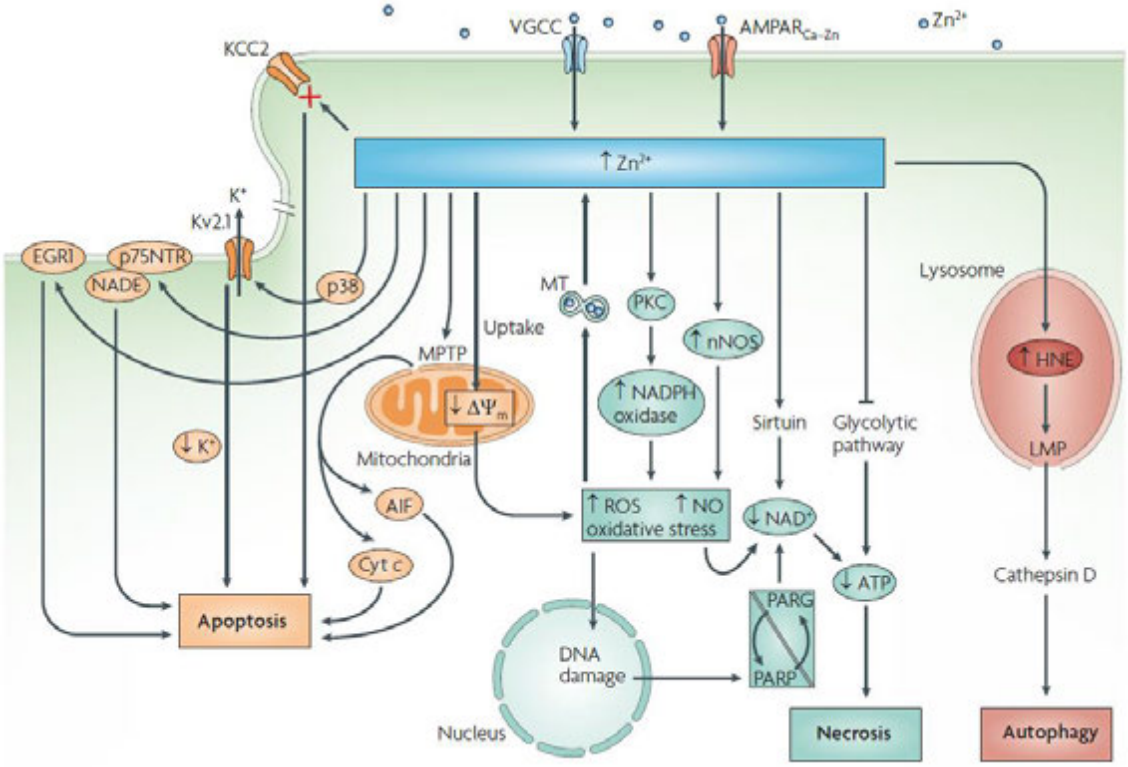

Figure 21: Neuronal pathways activated by zinc. Zinc activates multiple pathways leading to the death of the cell. EGR1: early growth response factors 1, p75NTR: neurotrophin receptor p75, MPTP: mitochondrial permeability transition pore, cyt c: cytochrome c, AIF: apoptosis-inducing factor, PKC: protein kinase C, VGCC: voltage-gates calcium channel, ROS: reactive oxygen species, NO: nitric oxide, nNOS: meuronal nitric oxide synthase. *Adapted from Sensi et al 2009, Nature Reviews.*

Mitochondrial dysfunction

Mitochondria are a primary target for intracellular zinc as it is able to accumulate in this organelle. Given that deregulation of neuronal zinc has been previously linked to mitochondrial dysfunction and oxidative stress, this metal could be at the source of pathological pathways involved in the aging brain.

At physiological levels, zinc regulates certain mitochondrial processes, including glycolysis, the tricarboxylic acid cycle, the electron transport chain, and mitochondrial permeability transition. Mitochondrial zinc uptake occurs through the mitochondrial calcium uniporter or via the ZnT2 transporter and can provide clearance of the accumulated cytosolic zinc. However, the uptake of zinc through the activation of the mitochondrial calcium uniporter can participate in the initiation of neuronal death (Ji *et al.*, 2019; Ji *et al.*, 2020). An increase in intra-mitochondrial zinc can lead to a loss of mitochondrial membrane potential, which eventually prompts to the production of ROS and cell death, similarly to calcium accumulation. Within mitochondria, zinc also acts by inhibiting the activity of the complex

III of the electron transport chain or by altering the Krebs cycle, also initiating ROS production. Zinc can also trigger mitochondrial membrane permeabilization by opening the mitochondrial permeability transition pore, a key promotor of cell death, generating the production of pro-apoptotic factors (cytochrome c, apoptosis-inducing factor).

Finally, zinc is also capable of impairing mitochondrial dynamics. Zinc is capable of impairing mitochondrial fusion-fission dynamics through the interaction of zinc with DRP1. This zinc-induced fission leads to morphological changes such as mitochondrial fragmentation. This cation can also modify mitochondrial motility, thus impairing mitochondrial trafficking and ATP production, leading to axonal degeneration and inhibiting synaptic growth (Liu *et al.*, 2021b).

Autophagy- Lysosomal pathway

Given the importance of autophagy in cellular homeostasis, it is conceivable that such a process could be regulated by the availability of zinc in the cell. First, oxidative stress has an impact on zinc accumulation within the lysosome. An acute increase in zinc eventually induces the permeabilization of the lysosomal membrane, releasing both zinc and lysosomal enzyme cathepsin D into the cytoplasm. The toxicity of both of these molecules in high quantities in the cytoplasm leads to neuronal death (Hwang *et al.*, 2008). This lysosomal membrane permeabilization also occurs with the increase of zinc in the lysosome due to mutations in the ATP13A2 protein, a cation transporter (Dehay *et al.*, 2012; Tsunemi *et al.*, 2014; Tsunemi and Krainc, 2014).

The exact effect of zinc on autophagy signaling has yet to be completely elucidated. Several studies have demonstrated that zinc could activate the ERK1/2 pathway, in turn initiating the activation of autophagy. In addition, zinc-binding metallothioneins have shown their role in regulating autophagy through the rapid release of zinc by pro-oxidants leading to the activation of autophagy. Importantly, chemical and genetic inhibition of autophagy has been consistently associated with decreased cellular zinc levels (Liuzzi and Pazos, 2020) (**Figure 22**).

Among other organelles such as the endoplasmic reticulum and mitochondria, lysosomes are important zinc storage organelles. To mediate the import and export of zinc, lysosomes have specific transporters such as Znt2, ZnT4 that allow the import of zinc towards the lumen of lysosomes, and ZIP8 that allows the export of zinc towards the cytoplasm. Additional zinc

non-specific transporters exist at the lysosomal membrane, including ATP13A2 and TRPML1-3. Mutations in genes coding for ATP13A2 and TRPML1 have previously been shown to impact lysosomal function, particularly lysosomal pH in the case of TRPML1.

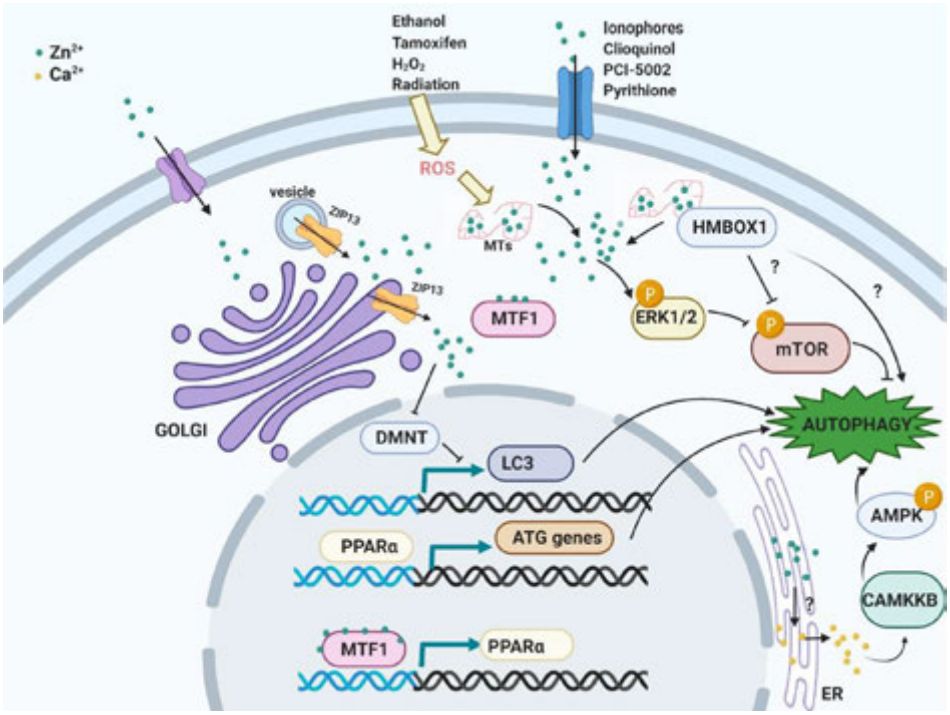

Figure 22: Zinc and its effect on autophagy. With the entry of certain molecules, this induces the formation of reactive oxygen species (ROS), in turn releasing zinc from metallothioneins (MTs). Ionophores such as Clioquinol can also allow the entry of zinc into the cell. With an increase of zinc in the cytosol, there is an activation of ERK1/2, in turn inhibiting mTOR and thus activating autophagy. The increase in zinc allows for its binding to the Metal transcription factor 1 (MTF1), leading to an activation in the LC3 and ATG genes, both activating autophagy. *Adapted from Liuzzi et al. 2020, Journal of Trace Elements in Med and Biol.*

The induction of autophagy by excess zinc is most likely a protective response of the cell. However, a continuous surplus of zinc triggers uncontrolled autophagy, leading to cell death. Altogether, zinc and autophagy go hand in hand as they influence one another. Autophagy promotes zinc recycling and mobilization and, conversely, zinc activates basal and induced autophagy.

Apoptosis/Necrosis

In addition to its roles in mitochondrial function/dysfunction and the ALP, zinc also affects many cytosolic pathways that, in turn, activate cellular death pathways. An increased zinc concentration in the cytosol prompts the formation of ROS, alters the dynamic of the cytosqueleton and modulates the expression of transcription factors. In the central nervous

system (CNS), zinc promotes oxidate stress by activating NADPH oxidase through activation of protein kinase C, and similarly activates the neuronal isoform of nitric oxide synthase and promoting the production of nitric oxide (Sensi *et al.*, 2009). Zinc is also capable of promoting the activation of PARP, AMPK, and cyclin-dependent kinase 5, which are all implicated in cell death pathways (**Figure 21**).

Taken together, zinc seems to be at the center of multiple cellular processes that regulate both the health and the viability of cells. This role in cellular homeostasis but also in neuronal excitability and cell death mechanisms make it of critical importance to the general equilibrium of the brain. Any changes in brain homeostasis have been shown to be implicated in neurological disorders.

3. Zinc in CNS disorders

As previously stated, zinc dyshomeostasis is implicated in various CNS disorders ranging from neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, to neurological disorders, such as autism and depression.

Alzheimer's disease

AD is the most common neurodegenerative disease worldwide and is characterized by the extracellular accumulation of amyloid-β and the intracellular accumulation of tau. Zinc and amyloid-β have been shown to colocalize at the synapse of glutamatergic neurons. This interaction seems to occur because of the metal-binding domain present in amyloid-β, which leads to the oligomerization and aggregation of the protein (Cherny *et al.*, 2001; Istrate *et al.*, 2016; Khatua *et al.*, 2019). Inhibition of this metal-binding domain has shown beneficial effects in AD pathology *in vivo*. As seen previously, any changes in zinc homeostasis can be detrimental. Surprisingly, zinc at specific concentrations seems to also have a positive effect on amyloid plaque formation as zinc can reduce the interaction of amyloid-β proteins among each other.

In addition to zinc, the expression of zinc transporters is altered in this disease in post-mortem analysis of AD patient brains. These studies showed modifications in mRNA levels of ZnT1, ZnT4, ZnT6, and ZIP1. *In vivo* rodent studies have also indicated the potential for other transporters to be implicated in this disease, in particular ZnT3. Aging ZnT3 knockout mice demonstrate a phenotype that resembles AD, with difficulties in memory and decreased

synaptic health (Adlard *et al.*, 2010). In human patients, there seem to be various findings , with CSF ZnT3 being associated with cognitive decline and cerebral ZnT3 being associated with a reduction in the formation of tau tangles.

Altogether, despite inconsistent findings, it is clear that zinc interacts with both amyloid-β and tau, implicating it in the progression of AD and its neuropathology.

Parkinson's disease

The etiology of PD, as seen previously, is complex and many factors have been implicated in the development of the disease. Among these factors, heavy metals have recently been associated with environmental factors that could influence the development of PD, and could play a role in neuronal vulnerability. Throughout various studies, the implication of zinc in PD patients has been a source of controversy, some demonstrating no changes in zinc concentrations and others measuring variations in zinc (Du *et al.*, 2017; Genoud *et al.*, 2017). Nevertheless, the association of heavy metals with PD pathology has mostly focused on metals such as copper and iron, which have been shown to interact directly with α-syn (Gonzalez *et al.*, 2019).

With these contradictory studies, understanding the link between the dysregulation of zinc and PD has taken a more important place in the field. Exposure of rats to zinc overtime induces neurodegeneration in the SN, associated with impairment of the UPS and accumulation of α-syn. This was one of the first indications that zinc could induce a spontaneous PD-like pathology *in vivo* (Kumar *et al.*, 2018). Increases in zinc were also found in α-syn-rich regions such as the olfactory bulb, with associations of high labile zinc in LB and in mitochondria (Gardner *et al.*, 2017). In addition, loss of ATP13A2 transporter protein led to zinc dyshomeostasis in the lysosomes, in turn inducing lysosomal disruptions and α-syn accumulation (Tsunemi and Krainc, 2014).

In addition to inducing α-syn accumulation, zinc also plays a role in dopaminergic neuron viability. Similarly to pesticides like paraquat, increases in zinc have deleterious effects on dopaminergic neurons and lead to oxidative stress with the release of cytochrome c and, ultimately, cell death (Mittra *et al.*, 2020). Zinc could also be an important player in the toxicity of 6-hydroxydopamine in rats, with 6-hydroxydopamine leading to an extracellular increase in zinc specifically in the SN, which in turn induces dopaminergic neurodegeneration and the 6-hydroxydopamine-based PD model (Tamano *et al.*, 2019).

Moreover, zinc contributes to the behavioral deficits seen in 6-hydroxydopamine mice since ZnT3-deficient mice, which have no synaptic zinc release, were more resistant to 6-hydroxydopamine motor deficits (Sikora *et al.*, 2020).

Taken together, zinc seems to not only have an effect on α-syn and its accumulation, but also on the health of dopaminergic neurons. This implicates this metal in the potential α-syn aggregation, but also on the selective vulnerability of dopaminergic neurons, two important aspects of PD pathology.

Other neurological disorders

Zinc dyshomeostasis has been implicated in a multitude of neurodegenerative diseases as well as neurological disorders, including autism, depression, and epilepsy (Skalny *et al.*, 2021). As previously discussed, the role of zinc in neurodegenerative diseases has yet to be fully elucidated, but zinc seems to be implicated in more than Alzheimer's and Parkinson's disease and in amyotrophic lateral sclerosis. This neurodegenerative disease affects motoneurons of the spinal cord and familial cases of the disease have implicated mutations in the superoxide dismutase 1 (SOD1) protein. SOD1 is an enzyme that is implicated in oxidative stress and binds both zinc and copper. Variations in zinc have been shown to modulate this enzyme and its activity, accelerating the progression of amyotrophic lateral sclerosis (Sannigrahi *et al.*, 2021).

Zinc dyshomeostasis can affect synaptic plasticity, glutamatergic signaling, GABA receptors and many other cellular functions, which can modify behavior and cause certain neurological disorders. In the case of epilepsy, zinc has been demonstrated to increase convulsions, possibly through both the increase in mossy fiber and the modulation of GABA receptors (Sensi *et al.*, 2009). Together, mossy fibers lead to zinc liberation, which binds to GABA receptors and inhibits their activity, in turn facilitating the propensity for epileptic episodes.

However, in depression, increased zinc seems to be beneficial to the neurological disorder. This could occur through multiple mechanisms such as the zinc inhibition of NMDA receptors or increasing brain-derived neurotrophic factor (BDNF) activity, either directly or indirectly, by increasing CREB activity or AMPA receptor activity (Petrilli *et al.*, 2017). In autism spectrum disorders, zinc has also demonstrated positive effects by improving glutamatergic signaling, synaptic plasticity and density, which has been shown to reduce

repetitive behavior and decrease anxiety (Kirsten *et al.*, 2015). This, in turn, relieves autism patients and reduces the severity of their autism.

As seen through these various neurological disorders, zinc is able to act at different levels in the brain that then translates in behavioral changes. In the case where zinc is detrimental and worsens the disease, it could be interesting to target zinc accumulation to relieve certain pathological effects.

D. Models of synucleinopathy

To better understand the mechanisms behind synucleinopathies and potential therapeutic targets, using models that represent certain aspects of the pathologies are necessary. Experimental models have been developed *in vitro*, through the use of cell-culture models and yeast, and *in vivo*, through the use of flies, worms, rodents and non-human primates (NHP). Each type of model presents its advantages and drawbacks, which will be briefly discussed in this section. Here, we will focus our discussion on α-syn-based models, despite the high interest of other mutations involved in synucleinopathies (**Figure 23**).

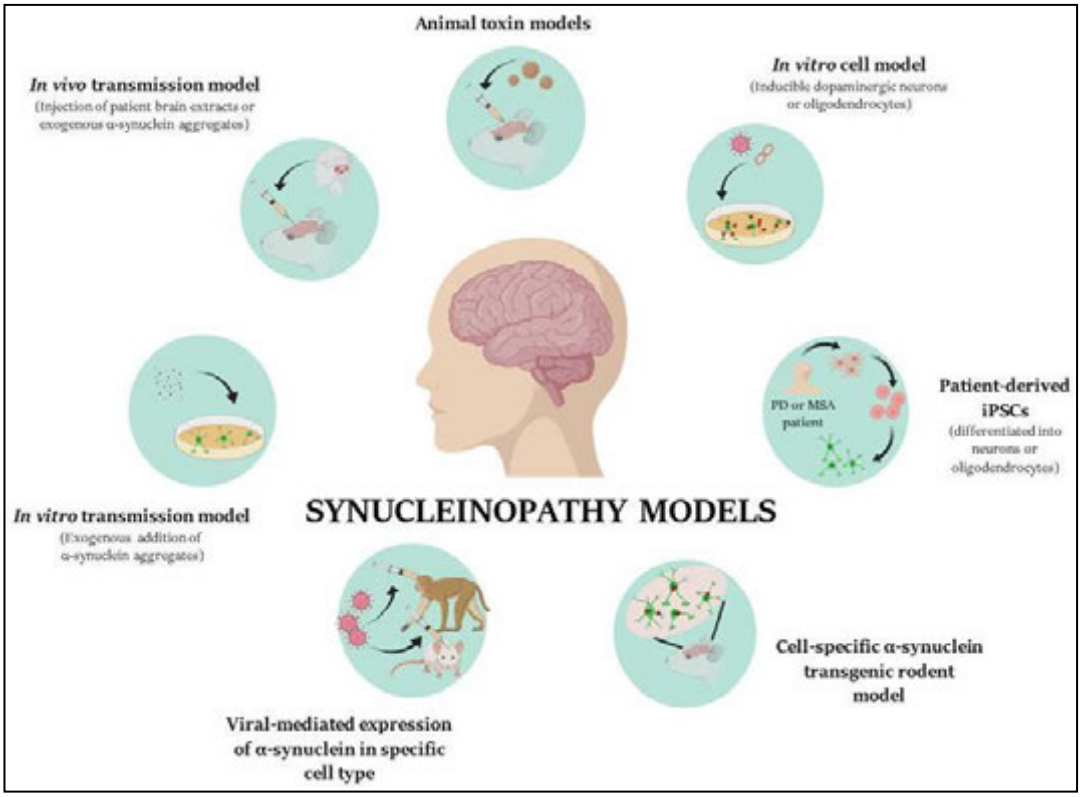

Figure 23: Models of synucleinopathy. Various *in vitro* and *in vivo* models have been used to induce models of synucleinopathies. These include the use of transgenic, viral-injected, toxin-injected models or transmission models. *Adapted from Lee et al. 2019, Exp and Mol Medicine.*

1. *In vitro* models

Given the complexities of studying the physiological and pathological functions of α-syn, the simplification of *in vitro* models allows for a better understanding of the protein. *In vitro* studies permit the exclusion of complexities of the cell with its interaction with other cell types and the tissue as a whole. Since synucleinopathies implicate numerous cellular functions as well as the interaction with other cell types, the simplification of models makes interpretation of results easier, but does not recapitulate the non-cell-autonomous features seen in animal models. Nevertheless, these cellular models give the ability to easily modify target genes and environmental factors, and avoid the complex ethical associations with animal models.

Simplified models such as yeast (*S. cerevisiae*) allowed for an early understanding of the implication of α-syn in synucleinopathies, as well as the cellular mechanisms implicated in its toxicity (Tenreiro and Outeiro, 2010). Increased α-syn expression induced a toxicity associated with increased oxidative stress, altered lipid metabolism, and vesicular trafficking (Outeiro and Lindquist, 2003). With further inquiries in the role of α-syn in yeast, conserved pathways altered by α-syn in patients were discovered in yeast, including endoplasmic reticulum trafficking and lipid metabolism alterations (Zabrocki *et al.*, 2008). Despite all expectations, yeast studies have brought much insight into many genes implicated in PD, including α-syn, and into potential molecules able to target α-syn. However, yeast are not enough to understand the full scope of cellular alterations induced by α-syn and cellular models closer to human neurons were necessary.

Primary neurons

Primary neurons, prepared from embryonic or post-natal mouse or rat pups, simulate a neuronal environment with a variety of cell types. Due to this environment, they can produce results that are more similar to what can be seen *in vivo*. Cell types of interest can also be isolated and enriched to develop a more "pure" culture. Synucleinopathies, and in particular PD, are characterized by the selective vulnerability of dopaminergic neurons, with a lower targeting of cholinergic neurons. The primary culture of dopaminergic neurons from the ventral midbrain of mice has thus been extensively studied. To induce a model similar to what can be observed in PD, the use of transgenic mice overexpressing α-syn to prepare primary neurons or transfection of already cultured midbrain neurons has been used. These

cultured primary neurons can present with a decrease in viability with overexpression of α-syn, as well as an accumulation of α-syn.

Another technique to induce the aggregation of α-syn has been the generation of PFFs. PFFs are *in vitro* generated recombinant α-syn monomers in which aggregation is induced, usually by shaking, leading to the formation of fibrils (Volpicelli-Daley *et al.*, 2014). When primary cell cultures are put in contact with these fibrils, the PFFs induce the formation of insoluble, filamentous, hyper-phosphorylated aggregates through the recruitment of endogenous α-syn (Volpicelli-Daley *et al.*, 2014). In addition, live-cell imaging has allowed the demonstration of the transfer of fluorescent α-syn PFFs to other neurons and the visualization of the internalization of α-syn. Microfluidic chambers have also allowed the study of this internalization of α-syn through intracellular trafficking. These primary neurons exposed to PFFs have also been used in more mechanistic approaches to better understand the cellular pathways affected by PFFs, their mode of entry in the cell, and their potential to propagate towards other cells.

In an attempt to study MSA pathology, oligodendroglial cultures have been developed to better understand the mechanisms behind this disease. First, a combination of a glioblastoma astrocytoma cell line and primary oligodendrocytes from mixed rat glial cultures have been used. When exposed to high levels of α-syn and TNFα, a pro-inflammatory cytokine, cytotoxic changes were observed and suggested that a toxic environment and α-syn overexpression represented a high risk in the development of MSA. The OLN-93 cells, primary oligodendroglial cells derived from rat brain cultures, have also been used to study MSA. When co-expressing p25α and α-syn in OLN-93 cells, these cells demonstrated an inhibition of NF-κB signaling, which blocked cytoprotective effects, triggering the activation of apoptosis via caspase-3 signaling (Kragh *et al.*, 2009; Kragh *et al.*, 2014). This sequestration of NF-κB is also seen in MSA patients.

Despite the benefits of coming from an *in vivo* model and having a variety of cell types within the culture, primary cultures also present disadvantages. One of their first disadvantages is the presence of only 5-10% of dopaminergic neurons on average, mostly taking into account the importance of these neurons in PD pathology. In addition, primary cultures are difficult to maintain and prepare, but their composition is also variable between two cultures. This variability makes it difficult to compare results from different cultures and thus difficult to conclude on certain results.

Differentiated immortalized cells

In order to try to be more faithful to PD, it was important to develop cell models that would be more physiologically similar to dopaminergic neurons found *in vivo*. For this, immortalized cells with the ability to differentiate into dopaminergic neurons have been used, such as the SH-SY5Y and PC12 lines. These cell lines, when differentiated, bear striking similarities to PD dopaminergic neurons and are widely used to study cellular mechanisms implicated in the disease. The differentiation of these cells using substances such as BDNF, retinoic acid, glial cell-derived neurotrophic factor (GDNF), or neuronal growth factors leads to changes in morphology and function. In addition to their similarities to mesencephalon-derived primary dopaminergic neurons, they have the distinct advantage of retaining their ability to divide and are less difficult to culture.

Similarly to primary neurons, these cells can be induced to overexpress wild-type or mutant α-syn through transfections which can be maintained over time, the use of PFFs, or other treatment methods such as patient brain lysates. PC12 cells, which are derived from rat adrenal medulla, once differentiated become electrically excitable and respond to neurotransmitters. They are particularly sensitive to neurotoxins, but can stably overexpress α-syn. PC12 cells overexpressing A30P mutant α-syn have demonstrated an impaired release of dopamine, proteasome dysfunction, and mitochondrial abnormalities (Larsen *et al.*, 2006). SH-SY5Y cells, human neuroblastoma cells, have demonstrated an increased vulnerability to toxic environments when overexpressing A53T-mutant α-syn.

However, compared to primary neurons from rodents and PC12 cells, SH-SY5Y cells are derived from human neurons and express several human-specific proteins, making them closer to PD patient dopaminergic neurons. Using SH-SY5Y cells, the transmission of α-syn to neighboring cells via endocytosis and the formation of LB-like aggregates was demonstrated (Desplats *et al.*, 2009). Other cells similar to SH-SY5Y cells include the BE(2)-M17 cells, which are also neuroblastoma cells that are able to be differentiated into dopaminergic neurons (Filograna *et al.*, 2015).

Alternatively, Lund human mesencephalic cells (LUHMES), a clone of the MESC2.10 line derived from ventral mesencephalic tissue of a human fetus, is another well-used model of immortalized cells that can be differentiated into human dopaminergic neurons (Lotharius *et al.*, 2002). The differentiation process involves a combination of dibutyryl cyclic adenosine monophosphate and GDNF, which induce changes in the proteome of these cells (Tushaus *et*

al., 2021). These LUHMES cells have electrical properties and can produce wild-type human α-syn, similarly to authentic human dopaminergic neurons. They have been used to study synucleinopathy through their exposure to mutant α-syn, after which they showed transcriptional deregulation and DNA damage (Paiva *et al.*, 2017). LUHMES cells have also been used for high-throughput screening of drugs to identify compounds that could have neuroprotective effects (Hollerhage *et al.*, 2017). Recently, more and more studies have aimed at using LUHMES cells and their similar characteristics to human dopaminergic neurons to test the efficacy and toxicity of novel molecules (Paiva *et al.*, 2017; Rosado-Ramos *et al.*, 2021). Toxicity could be of particular interest since differentiated LUHMES cells have shown higher toxicity than other immortalized cell lines, making them relevant in the study of potential therapeutic compounds (Tong *et al.*, 2017). Nonetheless, despite the benefits that come with using LUHMES cells, they present limitations in their use to study PD. First, they are challenging to culture, and differentiation is most efficient in early passages of the cells, making them more complicated to maintain. More importantly, these cells are difficult to transfect. This makes the overexpression of α-syn a challenge and requires the use of lentiviral infections to have a correct amplification of α-syn. In the next years, the ideal would be to develop LUHMES cells that overexpress the different mutant forms of α-syn could be a way to compare the effects of each mutation in dopaminergic neurons.

Patient-derived cell lines

In the last years, the discovery of induced pluripotent stem cells has revolutionized how we study diseases *in vitro*. Indeed, one of the barriers in the study of synucleinopathies is the lack of accessibility to live brain tissue to understand cellular mechanisms and test potential therapies (Wray *et al.*, 2012). By being able to use skin fibroblasts that are transformed into pluripotent stem cells to then reprogram them into cells of interest, the study of synucleinopathies and the mechanisms implicated have taken a new turn. These induced pluripotent stem cells have multiple differentiation potentials, are capable of self-renewal, and have the patient's complete genomic background. These cells also offer the possibility of high-throughput screenings and allow the combination of CRISPR-Cas9 genome-editing technologies to better understand the role of specific mutations. With 10% of PD cases being of genetic origin, the study of genetic mutations in these patients and their implications in cellular mechanisms has been invaluable. Patients with mutations and triplications in the

SNCA gene have been mainly studied, given the implication of this gene in synucleinopathies.

In dopaminergic neurons differentiated from pluripotent stem cells of PD patients with *SNCA* triplication mutation, the amount of the α-syn protein expressed is twice the levels observed in dopaminergic neurons from controls (Byers *et al.*, 2011). Importantly, these transformed patient cells demonstrate alterations in cellular mechanisms, and in particular an increase in oxidative stress (Byers *et al.*, 2011; Deas *et al.*, 2016). Neurons with this *SNCA* triplication also show changes in growth, viability, neuronal activity, and stress resistance, which can be reversed by a knockout of endogenous α-syn (Flierl *et al.*, 2014; Oliveira *et al.*, 2015). Mitochondrial toxicity has also been demonstrated in neurons with this triplication, with α-syn aggregates leading to the premature opening of the mitochondrial permeability transition pore, leaving neurons more vulnerable to cell death (Ludtmann *et al.*, 2018). In addition, lysosomal dysfunction is also present in the *SNCA* triplication-derived neurons through the impairment of protein trafficking, leading to α-syn accumulation (Mazzulli *et al.*, 2016). Finally, when differentiated into microglia, *SNCA* triplication-derived cells demonstrated impaired phagocytosis, linking α-syn accumulation to a lack of microglial clearance (Haenseler *et al.*, 2017).

The p.A53T mutation, probably one of the most famous mutations of the *SNCA* gene, has also been investigated using induced pluripotent stem cells. These A53T-mutant derived neurons demonstrated higher levels of nitrated α-syn species, associated with nitrosative stress and endoplasmic reticulum stress, associated with increased cell death (Chung *et al.*, 2013; Ryan *et al.*, 2013). This mutation also induced the generation of higher levels of nitrous oxide after exposure to environmental toxins, resulting in disruption of mitochondrial transport and subsequent neuronal loss (Stykel *et al.*, 2018). Mitochondrial morphology was also altered in A53T-mutant neurons, with reductions in membrane potential, which was associated with bioenergetic dysfunction (Zambon *et al.*, 2019).

In MSA patient-derived cells, establishing a stable expression of α-syn in mature oligodendrocytes has been a challenge. Indeed, oligodendrocyte precursor cells were able to stably express α-syn, but this expression was progressively reduced as the cells differentiated into mature oligodendrocytes (Djelloul *et al.*, 2015). This difficulty in inducing mature oligodendrocytes expressing α-syn was not seen when differentiating MSA-patients fibroblasts into dopaminergic neurons, which demonstrated mitochondrial dysfunction and

autophagy impairment (Monzio Compagnoni *et al.*, 2018). The *COQ2* mutation has previously been implicated in MSA pathology, thus making it of interest in the generation of induced pluripotent stem cells. In these *COQ2* mutants, deficiencies in mitochondrial respiration and anti-oxidative system were observed in neural differentiated cells (Nakamoto *et al.*, 2018).

These various studies show that induced pluripotent stem cells are the most relevant models to study PD *in vitro*. For now, many studies have focused on the understanding of PD genetic mutations and their implications in the disease, but next steps could involve understanding the changes seen in sporadic PD patients. Knowing that 90% of PD cases are sporadic, the use of patient-derived cells could allow the identification of novel biomarkers or genetic implications. Nonetheless, these induced pluripotent stem cells present limitations, including the difference in epigenetic profiles between cultures cells and *in vivo* neurons. These cells also lack a persistent accumulation of α-syn and are immature compared to aged neurons. This neuronal immaturity requires either a long culture or the use of compounds to stimulate aging, which has yet to be extensively studied in synucleinopathies. In the case of MSA, the lack of studies successfully attaining α-syn-expressing mature oligodendrocytes limits their use in the study of this disease.

Altogether, *in vitro* models represent an invaluable tool to study synucleinopathies. They allow for a better understanding of cellular mechanisms and genetic mutations associated with diseases but also allow the testing of potential therapeutic targets and their exact impact on neuronal functions. Despite their usefulness, it is also of crucial importance to combine the use of cell-based models with *in vivo* models that recapitulate the non-cell-autonomous features of synucleinopathies.

2. *In vivo* models

Many *in vivo* models have been developed in the last 40 to 50 years, all of which have tried to recapitulate most of the pathological aspects observed in patients with PD or MSA (Koprich *et al.*, 2017). Of these many *in vivo* models developed, the most challenging aspect has been observing the presence of LB or GCI as is seen in humans, dense bodies with a peripheral halo. Another recurrent issue is the lack of neuromelanin expression and the difference in α-syn sequence in rodents, making the susceptibility and general pathology quite different in rodents compared to humans. Finally, as previously seen, the most important risk factor that exists for the development of synucleinopathies is age. *In vivo*, it remains a challenge to

recapitulate all of these vulnerabilities that allow for the development of synucleinopathies in humans.

The first developed models for PD relied on the use of toxins, particularly the injection of MPTP and 6-hydroxydopamine. These toxins induce the selective loss of dopaminergic neurons, reflect the modulation of neurotransmitters, and induce motor deficits observed in PD patients. MPTP was used in a multitude of animal species, including rodents and NHP (Porras *et al.*, 2012; Teil *et al.*, 2021). MPTP allowed the induction of oxidative stress and lysosomal dysfunction, two characteristics of parkinsonian neurodegeneration (Dauer and Przedborski, 2003). MPTP models are still used to study PD pathology, in particular with the current analysis of α-syn pathology and the assessment of the efficacy of L-Dopa in models of the disease. This was the interest of one of the projects that I collaborated on (Deffains *et al.*, 2020). 6-Hydroxydopamine has principally been used in rats to induce hemi-parkinsonism and test the efficacy of transplantations and gene therapies. However, these toxin-models present multiple caveats, including the acute induction of PD-like pathology, the unpredictable presence of α-syn aggregates, and cellular mechanisms modulated only by the induction of the toxin. Given these difficulties, it was important to find other models of synucleinopathies to better understand other aspects of the disease.

Transgenic models

With the discovery of essential genes implicated in familial forms of synucleinopathies, and PD in particular, the development of transgenic models has become more important to study cellular mechanisms and a progressive installment of the disease. Here, we will discuss transgenic models based on the overexpression of α-syn in its various forms.

The majority of transgenic PD models have relied on the modulation of human α-syn in rodents, whether it is wild-type, mutant, or truncated α-syn. The results from these different models are variable and, at times, contradictory as certain show signs of neurodegeneration whereas others do not (Jiang and Dickson, 2018). Neurodegeneration has been shown to be age-dependent with around 30% in mice expressing wild-type α-syn (Janezic *et al.*, 2013), 40% in mice expression A53T-mutant α-syn (Lin *et al.*, 2012), and up to 50% in mice expressing either truncated α-syn or a combination of A53T and A30P α-syn mutations (Thiruchelvam *et al.*, 2004; Wakamatsu *et al.*, 2008). Most transgenic models drive pan-neuronal α-syn expression through the use of different potential promoters, including the human platelet-derived growth factors subunit B (PDGFB), the mouse thymus cell antigen 1

(Thy1), or the prion protein (Prnp) promoters. For higher efficiency, certain models used the artificial bacterial chromosome in combination with α-syn to induce transgenic rats (Nuber *et al.*, 2013). This combination proved to be efficient in inducing the accumulation of α-syn, neurodegeneration and motor deficits. These α-syn-based transgenic models are capable of inducing the formation of α-syn aggregates and motor behavioral changes, but very few are able to recapitulate substantial loss of dopaminergic neurons of the SN.

In the case of MSA pathology, the expression of human α-syn under oligodendrocyte promoters has been thoroughly used to create multiple MSA mice models. The proteolipid promoter (PLP)-driven α-syn transgenic mice exhibit accumulation of phosphorylated α-syn as well as α-syn-positive aggregates similar to GCI (Kahle *et al.*, 2002). These mice also demonstrate a loss of dopaminergic neurons in the SN and neurons in the pons and medulla oblongata, as well as gliosis. Progressive motor deficits are observed starting at 6 months and progress until 18 months. Mice overexpressing human α-syn under the cyclic nucleotide 3'-phosphodiesterase (CNP) promoter present an accumulation of α-syn in oligodendrocytes, loss and demyelination of oligodendrocytes, and gliosis in both the brain and the spinal cord (Yazawa *et al.*, 2005). The motor phenotype of this model begins from 7 to 9 months but demonstrates atypical MSA symptoms. Finally, myelin basic protein (MBP)-driven human α-syn transgenic mice display pathological features that are different from the two others transgenic MSA mice. These mice demonstrated widespread neurodegeneration and behavioral modifications, as well as demyelination and axonal degeneration throughout several brain regions (basal ganglia, brain stem, neocortex, and cerebellum) (Shults *et al.*, 2005). Altogether, these three transgenic mice models of MSA represent an accumulation of α-syn in oligodendrocytes, combined with motor deficits but no consistent neurodegeneration. Despite all overexpressing human α-syn in oligodendrocytes, these transgenic mice demonstrate variable pathological patterns that are not complete replicates of MSA pathology.

Taken together, these transgenic models have shown both interesting and potential limits in their use to better understand PD and MSA pathology. It is important to note that transgenic mice models of MSA seem to resemble more aspects of neuropathology than transgenic PD mice models.

Viral-based models

Over the past two decades, models based on the viral over-expression of α-syn have provided effective means to model synucleinopathies. Viral vectors have the potential to induce a long-term expression of the target gene with little toxicity, low inflammatory response, and high titers. For efficient action of viral vectors, it is important to choose both a good vector system, including in the case of adeno-associated vectors a corresponding serotype, as well as an efficient promoter.

The injection of viral vectors for PD models has induced an increased expression of α-syn, accompanied by accumulation of α-syn species, and loss of dopaminergic neurons in the midbrain of several species, including mice rats and NHP. In 2002, models using recombinant adeno-associated viruses (AAV) or lentiviruses overexpressing either wild-type or mutant (A53T or A30P) α-syn in rats were developed (Kirik *et al.*, 2002; Klein *et al.*, 2002; Lo Bianco *et al.*, 2002). In each independent study, these rats demonstrated a progressive accumulation of α-syn and a variable but visible loss of dopaminergic neurons in the SN. Given the more pronounced effects of AAV compared to lentiviruses, most future studies opted for the use of AAV. Building on their first study in rats, AAV were first injected in NHP to assess their potential use in a higher-order species (Kirik *et al.*, 2003). These first studies paved the way for many studies conducted in both rodents and NHP in the twenty years to come. During these twenty years, multiple studies have changed the serotype of AAV, the promoter, the type of α-syn to be overexpressed (mutant or wild-type), and the time course after injection. These studies have shown variability in the species of α-syn accumulated, the presence of ubiquitination, and the manifestation of a motor phenotype but, despite this, they have all demonstrated both the loss of dopaminergic neurons (ranging from 20 to 80%) and an accumulation of α-syn (**Table 2**).

α-Synuclein	Vector system	Promoter	Species	Time course	DN loss in SN	α-syn aggregation	p-α-syn	Motor phenotype	Reference
1st generation									
WT, A53T	rAAV2/2	CBA	Rat	8-16 weeks	30-80%	Yes	-	Mild	Kirik 2002
A30P	rAAV2/2	CBA	Rat	1 year	53%	Yes	-	-	Klein 2002
WT, A30P, A53T	LV	PGK	Rat	20 weeks	24-35%	Yes	-	-	Lo Bianco 2002
WT, A30P	LV	CMV	Mice	40-52 weeks	10-25%	Yes	-	-	Lauwers 2003
WT, A53T	rAAV2/2	CBA	NHP	16 weeks	30-60%	Yes	-	Mild	Kirik 2003
WT	rAAV2/2	ND	Rat	13 weeks	50%	Yes	Yes	No/mild	Yamada 2004
A53T	rAAV2/2	CBA	Rat	14 weeks	~40%	-	-	-	Maingray 2006
A30P	LV	CMV	Rat	46 weeks	20-52%	Yes	-	Mild	Lauwers 2007
WT	rAAV2/2	CBA	Mice	24 weeks	25%	No	-	-	St Martin 2007 Theodore 2008
A53T	rAAV2/2	Syn-1	Rat	17 weeks	35%	-	-	-	Chung 2007
2nd generation									
WT, A53T	rAAV2/5	CBA	NHP	1 year	40% A53T	Yes	Yes	Moderate	Eslamboli 2007
WT, S129D/A	rAAV2/5	CBA	Rat	26 weeks 4 weeks	WT 60% S129A 70%	Yes	-	-	Gorbatyuk 2008
WT, A30P, S129D/A	rAAV2/6	CMV	Rat	8 weeks	WT, A30P 20% S129A 70%	Yes	Yes	-	Azeredo da Silveira 2009
WT, S129D/A	rAAV2/8	CBA	Rat	6 weeks	26%	Yes	-	-	McFarland 2009
A53T	rAAV1/2	CBA	Rat	6 weeks	28%	Yes	-	Mild	Koprich 2011
WT, A53T, A30P	LV	CMV	Rat	3 weeks	17-40%	-	-	-	Winner 2011
WT	rAAV2/5	CAG	Rats	4-12 weeks	35-60%	Yes	-	Yes	Gombash 2013
WT, S129D/A	rAAV2/5	CAG	Rats	4-15 weeks	40-56%	Yes	Yes	Yes	Febbraro 2013
A53T	rAAV1/2	CAG	Mice	8-12 weeks	34-50%	Yes	Yes	Mild	Song 2015
A53T	LV	UBC	NHP	8 weeks	66%	Yes	-	-	Yang 2015
A53T	rAAV1/2	CBA/CMV	NHP	16 weeks	50%	Yes	-	-	Koprich 2016
A53T	rAAV1/2	CAG	Mice	10 weeks	33%	Yes	Yes	Yes	Ip 2017
WT	rAAV1/2	MBP	Rat	12-24 weeks	41-66%	Yes	-	Mild	Bassil 2017
3rd generation									
WT	rAAV2/6	Syn-1	Rat	6-16 weeks	80%	Yes	Yes	Yes	Decressac 2012
A53T, WT	rAAV2/7	CMV/Syn-1	Mice	8 weeks	82%	Yes	Yes	-	Oliveras-Salva 2013
A53T	rAAV2/7	CMV/Syn-1	Rat	4 weeks	80-90%	Yes	-	Yes	Van der Perren 2014
WT	rAAV2/6	Syn-1	Rat	3-8 weeks	43%	-	-	Mild	Caudal 2015
A53T	rAAV2/7	CMV/Syn-1	Rat	2-48 weeks	80%	Yes	-	Yes	Van der Perren 2015
A53T	rAAV2/9	CMV/Syn-1	Mice	20 weeks	30%	Yes	Yes	-	Bourdenx 2015
A53T	rAAV2/9	CMV/Syn-1	Rats	16 weeks	80%	Yes	Yes	Yes	Bourdenx 2015
A53T	rAAV2/9	CMV/Syn-1	NHP	11 weeks	20%	Yes	Yes	-	Bourdenx 2015
WT	rAAV2/6	Syn-1	Rat	8 weeks	50%	-	-	Mild	Tozzi 2016
WT	rAAV2/6	Syn-1	Rat	3-24 weeks	20-50%	Yes	Yes	-	Thakur 2017
WT	rAAV2/9	Syn-1	Rat	1-4 weeks	57%	-	-	Mild	Rodriguez-Perez 2018
A53T	rAAV2/9	CAG	Rat	10 weeks	55%	Yes	-	Yes	Yang 2020
WT	Olig001		Rat	20 weeks	21-36%	Yes	Yes	-	Marmion 2021
WT	Olig001		NHP	24 weeks	12%	Yes	Yes	No	Marmion 2021

Table 2: Models inducing the viral overexpression of α-synuclein in rodents and non-human primates. rAAV: recombinant adeno-associated virus, LV: lentivirus, CBA: chicken β-actin, PGK: phosphoglycerate kinase, CMV: cytomegalovirus, Syn-1: synapsin, CAG: CMV, β-Actin and β-Globin promoter, UBC: Ubiquitin C, MBP: myelin basic protein, NHP: non-human primates.

For MSA models, viral-vector approaches have been less used than for PD models. However, Bassil and colleagues used AAV to overexpress α-syn using the MBP promoter in both rats and NHP. Rats displayed overexpression of α-syn in oligodendrocytes accompanied by loss of dopaminergic neurons and motor deficits that were L-Dopa unresponsive (Bassil *et al.*, 2017). The AAV-injected NHP demonstrated a majority of α-syn overexpression in oligodendrocytes, with a residual neuronal expression. More recently, two consecutive studies in macaques using AAV-Olig001-α-syn demonstrated dopaminergic

neurodegeneration, accumulation of α-syn, demyelination, and microglial activation (Mandel *et al.*, 2017; Marmion *et al.*, 2020). Compared to PD, these viral vectors have been rarely used for MSA pathology.

Injection of viral vectors demonstrated for the first time that, by using a cell-specific promoter, it was possible to create models for different synucleinopathies. In fact, the change in α-syn overexpression between a neuronal and an oligodendroglial promoter manifested the different neuropathological hallmarks of either PD or MSA, proving their interest in the field. Viral vectors have the additional advantage of being easily applicable to different species and can be administered at any time in the life of the animal, adding to its interest in modeling age-related diseases. However, these viral-based vector models are based on the non-physiological overexpression of α-syn, which could lead to a questioning of the validity of this model. These models have yet to recapitulate all aspects of synucleinopathies but retain their interest in testing potential therapeutic options for these diseases.

Transmission models

After testing in cell-based models, indications of the potential for spreading of the α-syn protein have developed the idea that a model based on the way that α-syn could propagate throughout the brain could be of interest in studying synucleinopathies. The basis of using α-syn and its prion-like properties stem from its proven potential to propagate from a host to grafted neurons (Desplats *et al.*, 2009; Hansen *et al.*, 2011; Kordower *et al.*, 2011). These studies fueled the intracerebral injections of various α-syn forms, including synthesized α-syn PFFs or brain-derived α-syn extracts, in multiple species to assess the propagating effect these extracts throughout brain areas.

Injection of PFFs has demonstrated their interest in cell-based models where they can induce the transmission of α-syn and the formation of *de novo* fibrils (Luk *et al.*, 2009). After assessing their potential *in vitro*, these PFFs were injected into mice brains to determine their effect (Luk *et al.*, 2012a). These PFFs induced the formation of α-syn aggregates, accompanied by a loss of dopaminergic neurons and behavioral changes. Surprisingly, these PFFs had no effect in α-syn knockout mice, confirming the importance of endogenous α-syn to initiate and propagate the pathology (Luk *et al.*, 2012b). A large number of studies followed this with the injection of PFFs either in the striatum, the SN, or the cortex of mice (Volpicelli-Daley *et al.*, 2011; Luk *et al.*, 2012a; Masuda-Suzukake *et al.*, 2013), rats (Paumier *et al.*, 2015; Harms *et al.*, 2017) or NHP (Shimozawa *et al.*, 2017; Chu *et al.*, 2019).

The intracerebral injection of PFFs generally leads to the formation of phosphorylated α-syn puncta, in addition to α-syn aggregates and dopaminergic neuron loss, but some exceptions have also been observed.

The use of PFFs has been questioned due to their synthetic nature and the unknown existence of these types of fibrils within PD-patient brains. Indeed, the pathogenic properties of human α-syn aggregates have not been fully identified and the use of artificial aggregates to initiate pathology could be debated. To have a better understanding of aggregates present in patient brains, certain studies have aimed at using patient-derived extracts to induce pathological aspects of PD and MSA. A first study used LB extracts from PD patients that were purified by a sucrose gradient. The inoculation of these LB fractions in wild-type mice and NHP induced the accumulation of α-syn and its progression through inter-connected regions as well as a dopaminergic cell loss (Recasens *et al.*, 2014). The lack of pathology induced in α-syn depleted LB or in α-syn knockout mice strengthens the importance of the bond between endogenous and exogenous α-syn to induce pathology. This study was followed by multiple studies in mice using the intracerebral injection of LB fractions that induced a loss of around 30% of dopaminergic neurons after 4 months of inoculation, accompanied by accumulation of proteinase K-resistant α-syn (Bengoa-Vergniory *et al.*, 2020; Bourdenx *et al.*, 2020; Soria *et al.*, 2020). In NHP, a large study was conducted to better understand the pathogenicity of these LB fractions and noLB fractions, corresponding to small aggregates (Arotcarena *et al.*, 2020; Bourdenx *et al.*, 2020). Using an artificial neural network commonly used in machine-learning, LB and noLB fractions showed similar pathogenicity after intra-striatal injections in NHP, indicating similar effects of large fibrils compared to small aggregates. This study also provided evidence for the multifactorial nature of PD, with a unique signature for pathological induction of large and small aggregates (Bourdenx *et al.*, 2020). The gut-to-brain propagation of α-syn was explored in monkeys having received intra-striatal or enteric LB injections. These monkeys showed lesions in the SN and the enteric system regardless of the injection received, with a pathology that did not spread through the vagal nerve (Arotcarena *et al.*, 2020).

Concerning MSA models using patient-derived extracts, transgenic M83 heterozygous mice have received extracts from MSA-patient brains. These mice presented with dopaminergic cell loss and neuronal accumulation of α-syn 4 months post-injection (Watts *et al.*, 2013; Prusiner *et al.*, 2015; Woerman *et al.*, 2018). Transmission of α-syn was confirmed by later inoculation of extracts from mice brains having received these MSA-patient extracts into

other mice. Given that these secondary injections were able to induce pathology, the potential of α-syn to be propagated was demonstrated. However, these mice did not demonstrate MSA-like pathology, nor did these extracts induce pathology in wild-type mice. The use of patient-derived MSA extracts *in vivo* has yet to be thoroughly investigated, hence the interest taken in **Project 1 of this thesis** to explore this approach and the possible effects of these extracts to induce an MSA-like pathology.

Taken together, these studies have indicated the prion-like potential of α-syn and its ability to induce pathogenicity in animal models. However, the limitations of such transmission models rely on the importance of propagation of α-syn, which is still debated given the possibility that the propagation could be explained by the selective vulnerability of neurons, inducing oxidative stress and neuroinflammation. The use of these models is also debated due to the induction of supra-physiological quantities of α-syn injected in the brain, which could be at the source of the pathogenic effects. Despite this, the use of these models of spread could be invaluable in therapeutic strategies that target the propagation of α-syn within the brain and remain important for future steps in studying synucleinopathies.

E. Therapeutic approaches

As seen in this introduction, it seems that finding therapeutic strategies to decrease the progression of these pathologies is primordial for the future. With only symptomatic treatments currently available and that are not efficient over long periods of time, understanding a way to inhibit neurodegeneration and synucleinopathy could be the only solution to helping with patients.

One of the central issues associated with halting neurodegeneration is diagnosing these pathologies early enough to have an efficient treatment. Indeed, as previously stated, before motor symptoms arise, neurodegeneration has already significantly progressed in patients, enough to be severely detrimental. A potential option for an earlier diagnosis is the identification of biomarkers in PD patients. These biomarkers could also give the ability to stratify patients according to the progression of the disease. Previous studies have looked into the possibility of α-syn as a biomarker for PD, but for now, a consensus has yet to be found. Analyses of CSF levels of total α-syn have been measured in multiple studies and in all synucleinopathies (Mollenhauer *et al.*, 2008; Mollenhauer *et al.*, 2011; Mollenhauer *et al.*, 2013; Fairfoul *et al.*, 2016). These studies have shown variable results, with certain studies

demonstrating a decrease of α-syn in the CSF and others showing no differences. On the other hand, the potential for using oligomeric α-syn or post-translationally modified α-syn as a biomarker has also been evaluated, with once again no conclusive results. The idea of finding an appropriate biomarker could be hugely important in the field and remains of crucial necessity, but complications in finding correct cohorts, having uncontaminated samples, and the general variability observed between patients could skew the results.

With that in mind, finding potential therapeutic approaches is also of high importance. To do this, multiple strategies could be used: either targeting α-syn directly or indirect targeting of neurodegeneration and synucleinopathy. Here, we will discuss both potential options in the second section of the results, in which both direct and indirect targeting were investigated.

1. α-Syn targeting strategies

All the above-mentioned findings show the central implication of α-syn in synucleinopathies, leading to the disruptions of physiological processes and giving rise to neurodegeneration. For this, several options are available and could be combined to have a more substantial effect on α-syn accumulation (Teil *et al.*, 2020) (**Figure 24**):

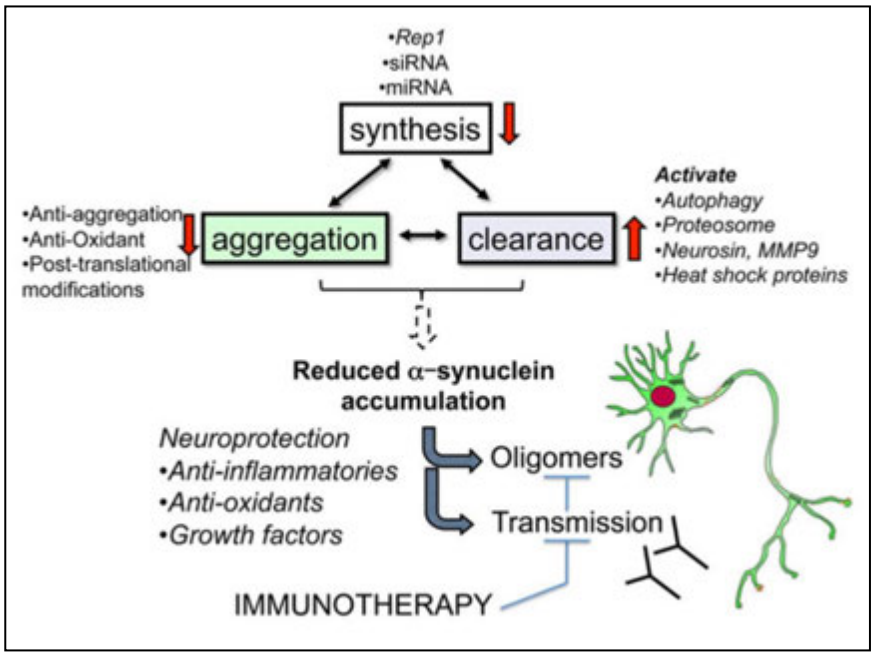

Figure 24: Targeting α-synuclein to reduce its accumulation and neurotoxic effects. α-Syn can be targeted either by a decrease in synthesis, a decrease in aggregation or an increase in clearance. Neuroprotection can also be conferred by molecules with protective effects, and decreasing α-syn transmission through immunotherapy. *Adapted from Lashuel et al. 2013, Nature Reviews.*

(1) To decrease α-syn expression or synthesis via the use of siRNA/miRNA or other inhibiting techniques;

(2) To inhibit the aggregation of the protein either through the use of anti-aggregative molecules, antioxidants, or by targeting PTMs that could induce aggregation;

(3) To increase α-syn clearance via autophagy activation, proteasome activation, and activation of proteins and molecules associated with clearance mechanisms;

(4) To decrease circulating α-syn through the use of antibodies targeting α-syn in its different conformations in the extracellular space (i.e., immunotherapies).

In **Project 3 and 4 of my thesis**, I will discuss the targeting of α-syn through both anti-aggregative small molecule Anle138b and the proteasome activation Nfe2L1.

Through the multiple studies undergone, several of these targeting therapies have made it into clinical trials (**Table 3**). With these efforts, finding helpful treatments for synucleinopathies in the near future could be a possibility. Nevertheless, to ensure the potential of these treatments, it is necessary to have suitable animal models to have the required preclinical testing.

	Molecule	Mechanism	Clinical Trial Phase	Year	Location of Trial	Reference
Immunotherapy	PRX002	Monoclonal antibody targeting C-terminal sequence of α-syn (amino acids 118–126)	1 (healthy volunteers)	2016	United States	NCT02095171 [190]
			1 (healthy volunteers and PD patients)	2017	United States	NCT02157714 [191]
			2 (PD patients)	Active	United States	NCT03100149
	MEDI1341	Monoclonal antibody targeting C-terminal sequence of α-syn	1 (healthy volunteers)	Recruiting	United States, United Kingdom	NCT03272165
	BIIB054	Monoclonal antibody targeting N-terminal aggregated forms of α-syn	1 (healthy volunteers and PD patients)	2018	United States	NCT02459886 [198]
			2 (PD patients)	Recruiting	United States, Japan	NCT03318523 NCT03716570
	BAN0805	Antibody targeting protofibrils of α-syn	1 (healthy volunteers)	Recruiting	United States	NCT04127695
	PD01/PD03 Affitopes	Vaccines targeting the C-terminal sequence of α-syn via small peptides	1 and 2 (healthy volunteers and PD patients)	2018	Austria	NCT01568099 NCT02216188
Clearance	Nilotinib	Tyrosine kinase Abelson (cAbl) inhibitor	1 (PD, PDD and DLB patients)	2016	United States	NCT02281474 [329]
			2 (PD patients)	2019	United States	NCT02954978 [330]
	Ambroxol	Pharmacological chaperone of β-glucocerebrosidase	2 (PDD patients)	Recruiting	Canada	NCT02914366 [348]
			2 (PD patients)	2020	United Kingdom	NCT02941822 [349]
Small molecules	ANLE138B	Small molecule targeting oligomeric forms of α-syn	1 (healthy volunteers)	Recruiting	United Kingdom	NCT04208152
	NPT200-11	Small molecule targeting the C-terminal region of α-syn	1 (healthy volunteers)	2016	United States	NCT02606682
Anti-oxidants	CoQ10 + Vitamin E	Antioxidant activity	3 (early PD patients)	2013	United States	NCT00740714
Metals	Deferiprone	Chelation of iron	1 (PD patients)	2012	France	NCT00943748 [434]
			2 (PD patients)	2019, Recruiting	France, Canada, Austria	NCT02728843 NCT02655315

Abbreviations: α-syn, α-synuclein; PD, Parkinson's Disease; DLB, Dementia with Lewy Bodies; PDD, Parkinson's Disease Dementia.

Table 3: Ongoing clinical trials targeting α-synuclein directly or indirectly. These include molecules using immunotherapy, increased clearance, small anti-aggregative molecules, anti-oxidants or metal targeting. *Adapted from Teil et al 2020, Biomolecules.*

2. Heavy metal therapeutic strategies

As seen throughout multiple studies, the relevance of metals in the progression of synucleinopathies is undeniable. Multiple studies have used targeting of metals with a certain degree of success, in particular the targeting of iron in PD. In fact, clinical trials are in place to establish the efficacy of an iron chelator, deferiprone, in PD patients.

On the other hand, zinc has proved itself to be an interesting target, not only because of its multiple implications in neurodegenerative disorders but also its potential to be targeted in the brain. Zinc targeting can be done through multiple mechanisms, including modulation of the total free zinc concentrations via chelators or zinc-binding proteins or redistributing zinc across biological membranes. These options rely on pharmaceutical targeting of zinc either by chelators or by ionophores. Chelation of metals relies on the inactivation of a single (or several) metal, whereas ionophores rely on the transfer of metals across membranes and the redistribution of metals within the cell. Both options are potentially interesting to target zinc, but chelation relies on a more drastic approach, leading to zinc depletion. Certain chelators and ionophores have already been evaluated for their interest in neurodegenerative diseases such as PD, AD, and Huntington's disease, but also diseases such as cancer (Ding and Lind, 2009; Adlard *et al.*, 2011; Devos *et al.*, 2014; Aguirre *et al.*, 2017; Das *et al.*, 2017). Here, I will discuss one particular ionophore which has been found to be of interest in all the previously stated diseases.

Clioquinol (5-chloro-7-iodo-8-hydroxyquinoline) was first prepared in the first part of the 1900s and was widely used as an antibiotic (Ding and Lind, 2009). In the 1970s, its use was banned in several countries due to its association with subacute myelo-optic neuropathy in Japanese patients. It was later determined that this antibiotic was not at the source of the secondary effects but due to deficiencies in vitamin B (Bareggi and Cornelli, 2012). Clioquinol (ClQ) was thought to be an iron and copper chelator, but also showed properties as a zinc ionophore (Ding and Lind, 2009). Its use was of interest for its hydrophobicity and its ability to cross the blood-brain barrier. This ability to cross the blood-brain barrier made it of interest as a therapeutic agent, particularly when targeting neuronal metals. Its first introduction to the field of neurodegenerative diseases was in 2001 by Cherny and colleagues who used ClQ as a treatment to reduce amyloid-β accumulation in an AD mouse model, an efficiency that was later confirmed in another mouse of the disease (Cherny *et al.*, 2001; Adlard *et al.*, 2008). ClQ was then tested in a mouse model of Huntington's disease and

proved once again to be efficient in down-regulating the accumulation of huntingtin and in alleviating the progression of the disease (Nguyen *et al.*, 2005). The efficacy and bioavailability in humans were assessed in clinical trials in 2003 in AD patients, which estimated that ClQ presented no toxicity, was efficient on certain patients, and remained present in the blood throughout the trial (Ritchie *et al.*, 2003).

ClQ efficiency was then assessed in PD models, in which multiple studies demonstrated its efficiency to improve PD pathology. In A53T transgenic mice, ClQ demonstrated its ability to improve motor function and cognition, but more importantly to decrease loss of dopaminergic neurons and α-syn accumulation (Finkelstein *et al.*, 2016). This positive effect of ClQ was once again demonstrated in another PD model where motor deficits and dopaminergic loss were once again reduced with ClQ treatment (Lei *et al.*, 2015). Despite all these beneficial effects in multiple neurodegenerative diseases, the exact mechanisms modified by ClQ have yet to be discovered and could be of interest to better understand how ClQ prevents neurodegeneration.

Recent studies have demonstrated the potential effect of ClQ as an anti-cancer molecule (Ding *et al.*, 2005). Its role as an anti-cancer molecule comes from its ability to induce apoptosis and inhibit proteasomal activity (Chen *et al.*, 2007). This ClQ-induced apoptosis did not implicate either Beclin-1 or PI3K, but was through mTOR downregulation. This mTOR inhibition by ClQ activates autophagy leading to cell death, which was inactivated by autophagy inhibitor 3-methyladenine (Cao *et al.*, 2014). However, other studies have demonstrated that in neuroblastoma cells submitted to oxidative stress, ClQ activated PI3K, which played a protective role on the cell by inhibiting p53-mediated cell death (Filiz *et al.*, 2008). The ability of ClQ to activate autophagy was determined in astrocytes and neurons in neurodegenerative conditions leading to neuroprotective effects (Park *et al.*, 2011). ClQ has also proved to be efficient in an MPTP-induced NHP model with the decrease of motor and non-motor deficits as well as an improvement in neuropathology. This effect of ClQ was demonstrated to occur through the activation of the Akt/mTOR survival pathway by inhibiting p53-dependent cell death (Shi *et al.*, 2020). This effect on activation of the Akt pathway could be largely due to the regulating properties that ClQ has on the transcription factor FOXO1 (Cameron *et al.*, 2012).

Several studies have also speculated that ClQ could be implicated in the lysosome (Yu *et al.*, 2009). Given the role of ClQ as a ionophore, it has the capacity of redistributing zinc levels

within the cell, potentially to the lysosome. By increasing the intracellular or lysosomal zinc levels, ClQ could be responsible for restoring lysosomal pH, thus restoring correct autophagic activity. This made it of interest for the **Project 2 of this PhD**, in which the effect of zinc modulation and its mechanisms were assessed.

Despite these positive effects of ClQ in neurodegeneration and cellular mechanisms impaired in diseases, metal targeting presents with certain caveats. Since metal chelators and ionophores cannot be completely controlled, it is important to know when, where and how much zinc redistribution must take place to avoid the side effects of excess zinc chelation. Indeed, treatment with ClQ can negatively affect short and long-term memory by inhibiting BDNF and synaptic plasticity (Frazzini *et al.*, 2018). Determining these optimal parameters require many more experimental conditions to better understand the potential primary and secondary effects of ionophores such as ClQ.

OBJECTIVES

Synucleinopathies represent an important family of neurodegenerative diseases for which there are currently no treatments able to stop either the progression or protect against neurodegeneration. The core of my thesis relies on a better understanding of α-synuclein and its effect on the progression of synucleinopathies.

My PhD work had for objective to better understand the underlying mechanisms behind the modeling and progression of synucleinopathies. Another complementary objective was to investigate potential therapeutic strategies to alter the progression of these diseases. Given the current misunderstanding of synucleinopathies and the mechanisms involved in their development, I focused my interests on studying and targeting α-syn accumulation in different projects using varying approaches. These projects aimed at: (i) using patient-derived α-syn extracts to induce a novel model of synucleinopathies in NHP; (ii) using different potential therapeutic strategies to decrease neurodegeneration and α-syn accumulation through the use of a zinc ionophore, an anti-aggregative molecule, or a transcription factor implicated in neuroprotection. These two axes will be detailed in the Results section of this manuscript.

In the first axis, we wanted to determine the effects of patient-derived α-synuclein containing extracts in NHP. Currently, very few *in vivo* models exist to study MSA, and even less use patient-derived brain extracts or NHP. For this project, we questioned the ability of patient-derived brain extracts from MSA patients to induce pathology in both mice and monkeys. We followed monkeys for two years after their injection to determine their effect on neurodegeneration, demyelination, α-syn accumulation and inflammation. This project is in line with the concept or notion of "strains" hypothesis of α-syn and allowed to determine if extracts from patient brains could reproduce the same neuropathological hallmarks in NHP.

The interest of my second axis was to focus on potential therapeutic strategies to induce neuroprotection in one progressive PD mouse model. For this, multiple angles of investigation were taken with three different sub-projects targeting both direct and indirect pathways of α-syn targeting. Firstly, given the importance of zinc in the brain, we focused our interest on its effect on neurodegeneration. By modulating the levels of zinc using a ionophore, we wanted to determine what consequences this would have on neurodegeneration and α-syn accumulation. This project's objective was also to determine the cellular

mechanisms implicated in the effects of zinc modulation in mice. This study allowed for a better determination of the role that zinc plays in the progression of PD and the mechanisms altered by zinc in this PD mouse model. Secondly, we tried to restore dopaminergic neuron survival by using a transcription factor implicated in proteasomal degradation and oxidative stress. By targeting survival and increased α-syn clearance, the goal was to assess the impact of an increased expression of this gene on neurodegeneration. Finally, we first used a small molecule targeting α-synuclein aggregation specifically to determine its ability to reduce neurodegeneration and aggregate formation. This project aimed at assessing whether the beneficial effects of this molecule were the same at two different time points. Through these three projects targeting various mechanisms affected by PD, I wanted to understand the implications of these molecules or genes on a singular and progressive PD mouse model.

Altogether, these projects I worked on allowed me to work on different aspects of synucleinopathies. First, to determine a new potential model for the study of MSA pathology in both mice and NHP, using a transmission model of the disease. Second, to assess the therapeutic effects of various molecules and genes through different routes of administration on the progression of PD pathology in the same PD mouse model.

RESULTS

CHAPTER 1: EVALUATING THE EFFECTS OF INTRACEREBRAL INJECTIONS OF MULTIPLE SYSTEM ATROPHY PATIENT-DERIVED BRAIN EXTRACTS IN MICE AND MONKEYS

PROJECT 1

Brain injections of glial cytoplasmic inclusions induce a multiple system atrophy-like pathology

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***Brain*, accepted for publication.**

As described in the introduction, transgenic mouse and recently viral-mediated mouse and NHP models of MSA already exist that allow an overexpression of wild-type α-syn specifically in oligodendrocytes. These models demonstrate neurodegeneration over time, α-syn accumulation in oligodendrocytes and some behavioral alterations with order of magnitude to the considered species, i.e., mouse and monkey. The use of these models has been beneficial in the study of MSA, but they are not able to address certain aspects of synucleinopathies: the cell-to-cell propagation of the pathology. Transmission models of MSA are rare, and only a few studies have focused on the ability of α-syn to propagate *in vivo*. One team has done experiments using patient-derived extracts but was only successful in transgenic mice.

In synucleinopathies, the injection of α-syn has shown the ability to propagate to other cells and template its pathological properties. Recently, a large cohort of *Papio papio* monkeys has been used to confirm the effects of these same patient-derived extracts *in vivo* (Arotcarena *et al.*, 2020; Bourdenx *et al.*, 2020). Given the effect of intracerebral injections of PD-purified brain fractions in mice and monkeys, it was also important to assess whether this could be the same for MSA pathology. In fact, as stated previously, similar patient-derived MSA patient homogenates were injected in transgenic mice and showed the induction of an MSA-like pathology. These homogenates were composed of homogenized brain samples that were diluted before injection, compared to our extractions which are purified by sucrose gradient (Watts *et al.*, 2013; Prusiner *et al.*, 2015; Woerman *et al.*, 2020). However, this was not the case in wild-type mice. In addition, the extractions of these patient-derived extracts were not done using the same technique as previously.

In this Axis I study and part of the large cohort of *Papio papio* animals previously studied, we used the same protocol of extractions from our team using a sucrose gradient to separate the density of aggregates. These GCI fractions were then injected in both mice and monkeys for 4 and 24 months, respectively. Using biochemical and histological analyses, we quantified the effect of these GCI fractions on neurodegeneration, oligodendroglial loss, demyelination and synucleinopathy.

This article demonstrates the ability of MSA-patient brain extracts to induce an MSA-like pathology recapitulating most of the aspects observed in the human pathology. This first is the first proof that MSA extracts can induce pathology in NHP and wild-type mice (**Figure 25**). However, after 4 and 24 months, we did not observe the presence of GCI inclusions in these *in vivo* models.

In conclusion, we demonstrate the interest in using patient extracts from MSA patients as a novel model for this pathology. Given the current interest in spreading models and their importance in the field, it is of crucial importance to have models for MSA that recapitulate most aspects of the disease. In addition, between LB and GCI fractions, we demonstrate that the injection of GCI can induce demyelination and loss of oligodendrocytes, as well as classic neurodegeneration. Similarly, the pattern of α-syn accumulation via heat map comparisons seems different. This contributes to the potential notion of strains associated with each pathology. This project and its impact will be further assessed in the global discussion of this thesis.

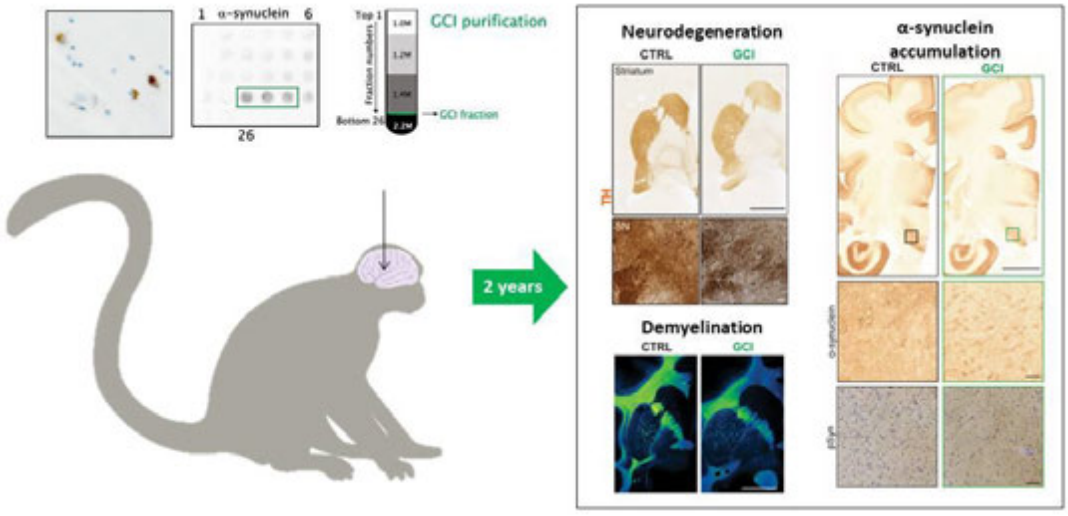

Figure 25: Intracerebral GCI injection induces an MSA-like pathology in non-human primates. In MSA patients, GCI were extracted and purified using a sucrose gradient. GCI were then injected in the striatum of non-human primates. Two years post-injection, these GCI induced neurodegeneration, demyelination and α-synuclein accumulation.

Brain injections of glial cytoplasmic inclusions induce a multiple system atrophy-like pathology

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Abstract

Synucleinopathies encompass several neurodegenerative diseases, which include Parkinson's disease, Dementia with Lewy Bodies, and Multiple system atrophy. These diseases are characterized by the deposit of α-synuclein aggregates in intracellular inclusions in neurons and glial cells. Unlike Parkinson's disease and Dementia with Lewy bodies, where aggregates are predominantly neuronal, multiple system atrophy is associated with α-synuclein cytoplasmic inclusions in oligodendrocytes. Glial cytoplasmic inclusions are the pathological hallmark of multiple system atrophy and are associated with neuroinflammation, modest demyelination, and, ultimately, neurodegeneration. To evaluate the possible pathogenic role of glial cytoplasmic inclusions, we inoculated glial cytoplasmic inclusion-containing brain fractions obtained from multiple system atrophy patients into the striatum of non-human primates. After a 2-year *in vivo* phase, extensive histochemical and biochemical analyses were performed on the whole brain. We found loss of both nigral dopamine neurons and striatal medium spiny neurons, as well as loss of oligodendrocytes in the same regions, which are characteristics of multiple system atrophy. Furthermore, demyelination, neuroinflammation, and α-synuclein pathology were also observed. These results show that the α-synuclein species in multiple system atrophy-derived glial cytoplasmic inclusions can induce a pathological process in non-human primates, including nigrostriatal and striatofugal neurodegeneration, oligodendroglial cell loss, synucleinopathy, and gliosis. The present data paves the way for using this experimental model for MSA research and therapeutic development.

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Running title: Injected GCIs induce MSA pathological features

Keywords: α-synuclein; Multiple system atrophy; non-human primates; neurodegeneration

Abbreviations: MSA = multiple system atrophy; α-syn = α-synuclein; GCI= glial cytoplasmic inclusion; NHP = non-human primate; SN = substantia nigra

Introduction

Multiple system atrophy (MSA) is a rare, rapidly progressive neurodegenerative disease. It is clinically characterized by parkinsonism, cerebellar impairment, autonomic dysfunction, and pyramidal signs in various combinations.[1] MSA patients are divided into the cerebellar subtype, MSA-C, where the olivopontocerebellar system is predominantly affected, and the parkinsonian subtype, MSA-P, with predominant involvement of the nigrostriatal system. The overlapping clinical features with Parkinson's Disease (PD), particularly at early disease stages, can challenge the differential diagnosis.

MSA belongs to the family of neurodegenerative diseases termed synucleinopathies, along with PD and Dementia with Lewy Bodies (DLB). The pathological hallmark of synucleinopathies is the formation of α-synuclein (α-syn)-positive inclusions in brain cells. In PD and DLB, these inclusions are primarily localized in neurons and named Lewy bodies (LB). In MSA, these inclusions mostly occur in oligodendrocytes, the myelin-producing cells of the central nervous system, and are called glial cytoplasmic inclusions (GCI).[2] The neuropathology of MSA includes neuronal and axonal degeneration, mild oligodendroglial loss,[3,4] myelin degeneration,[5] astrogliosis, and microgliosis.[6]

Few animal models exist to study MSA pathology.[7] These models mainly rely on the oligodendroglial overexpression of α-syn, thus leaving a need for models that induce (i) pathology by respecting the stoichiometric balance of α-syn expression (ii) without imposing an oligodendroglial α-syn expression. Transgenic mouse models have been developed that overexpress human α-syn under different oligodendrocyte-specific promoters: proteolipid promoter[8,9], myelin basic protein[10] and 2',3'–cyclic nucleotide 3'-phosphodiesterase.[11] More recently, viral-mediated overexpression of α-syn in rats and non-human primates has been proposed for modeling MSA pathology.[12,13] The injection of pathological forms of α-syn obtained from brain homogenates of a diseased animal or human patient has been used to model synucleinopathies across species. To date, the toxic effects of human-derived samples from patients with various synucleinopathies have been assessed mostly for PD, where LB-enriched fractions derived from patients have been used to induce a PD-like pathology in both mice and non-human primates.[14-16] Until now, only a few studies have aimed at using MSA patient-derived brain homogenates to evaluate the possible pathogenic role of GCIs. While intracerebral injections of MSA patient-derived brain into transgenic α-syn overexpressing mice have yielded pathological outcomes, no pathology was observed when these brain homogenates were injected into wild-type mice.[17,18] Importantly, no such study

has yet been conducted in non-human primates (NHPs).[18-21] NHPs present the benefit of being the closest species to humans in terms of their similarities of dopaminergic neuron physiology (i.e., age-dependent accumulation of neuromelanin), cerebral anatomy, glia versus neuron stoichiometry, overall physiology, and cognitive and social behaviors[22-25] as well as sensitivity to small patient-derived aggregates, at odds with rodents.[15] In this pilot study, we extracted MSA-derived GCI fractions, demonstrated their toxicity in an *in vitro* assay, and injected them in both wild-type mice and NHPs. We show that, two years after intrastriatal GCI injection, baboon monkeys exhibited marked nigral and striatal neurodegeneration and a loss of oligodendrocytes leading to demyelination and inflammation. Altogether, intracerebral injection of GCI material in NHPs recapitulated key aspects of MSA neuropathology.

Materials and methods

Ethics Statement

Experiments were performed following the European Union directive of September 22, 2010 (2010/63/EU) on the protection of animals used for scientific purposes. The Institutional Animal Care and Ethical Committee of Bordeaux University (CE50, France) approved mice experiments under the license number #24246-2020021916287618. The Institutional Animal Care and Ethical Committee of Murcia University (Spain) approved NHP experiments under the license number REGA ES300305440012.

Purification of GCIs from Human MSA Brains

GCI purification was conducted as previously described in detail for PD LBs.[14-16,26] The samples were obtained from brains collected in a Brain Donation Program of the Brain Bank "GIE NeuroCEB" run by a consortium of Patients Associations: ARSEP (association for research on multiple sclerosis), CSC (cerebellar ataxias), France Alzheimer, and France Parkinson. The consents were signed by the patients themselves or their next kin in their name, following the French Bioethical Laws. The Brain Bank GIE NeuroCEB (Bioresource Research Impact Factor number BB-0033-00011) has been declared at the Ministry of Higher Education and Research and has received approval to distribute samples (agreement AC-2013-1887). Human putamen was dissected from fresh frozen postmortem midbrain samples from 2 patients with MSA-P exhibiting conspicuous striatal GCI pathology on

neuropathological examination (mean age at death: 67.5 ± 3.5 years; frozen postmortem interval: 17 ± 4h; GIE Neuro-CEB BB-0033-00011). Tissue was homogenized in 9 vol (w/v) ice-cold MSE with protease inhibitor cocktail (Complete Mini; Boehringer Mannheim) with 12 strokes of a motor-driven glass/Teflon homogenizer. For GCI purification, a sucrose step gradient was prepared by overlaying 2.2M with 1.4M and finally with 1.2M sucrose in volume ratios of 3.5:8:8 (v/v).[27] The homogenate was layered on the gradient and centrifuged at 160,000xg for 3h using an SW32.1 rotor (Beckman). Twenty-six fractions of 500µl were collected from each gradient from the top (fraction 1) to bottom (fraction 26) and analyzed for the presence of α-syn aggregates by filter retardation assay, as previously described, with 45µl of each fraction deposited.[14] GCI-containing fractions from MSA patients were those between fractions 21 and 23 (Fig. 1A). The amount of α-syn in the GCI fractions was quantified using a human α-syn ELISA kit (#KHB0061; Invitrogen/Life Technologies, Carlsbad, CA, USA). Of the total fractions, the first patient represented around 60% and the second represented around 40%. Quantification by ELISA indicated that the GCI mix contained ~24pg of α-syn per microliter. In all cases, samples were bath-sonicated for 5 min before *in vitro* and *in vivo* injections.

Rat Ventral Midbrain Primary Cultures

Postnatally derived ventral midbrain cultures were prepared as described.[28] Briefly, cultures were prepared in two steps. In the first step, rat astrocyte monolayers were generated as follows. The entire cerebral cortex from a single rat pup (postnatal days 1-2) was removed, diced, and then mechanically dissociated by gentle trituration. The isolated cells were plated at a concentration of 80,000 cells onto wells under which a laminin-coated coverslip was affixed. The cells were housed at 37°C in an incubator in 5% CO_2 and were fed on glial media. Once confluence had been attained, fluorodeoxyuridine (6.7 mg/mL) and uridine (16.5 mg/mL) were added to prevent additional proliferation. In the second step, rat pups aged between 1 and 2 days were anesthetized, and 1-mm^3 blocks containing ventral midbrain neurons were dissected from 1-mm-thick sagittal sections taken along the midline of the brain. Tissues were collected immediately into cold phosphate buffer and were treated enzymatically using papain (20 U/mL) with kynurenate (500 µM) at 37°C under continuous oxygenation with gentle agitation for 2h. A dissociated cell suspension was achieved by gentle trituration and was then plated onto the pre-established glia wells at a density of 0.5-1.7 million neurons per well. Cultures were maintained in specially designed neuronal media

containing 27 µM fluorodeoxyuridine and 68 µM uridine to control glial outgrowth and in 10 ng/mL glial cell-derived neurotrophic factor. They were incubated for a further 7-8 days before GCI-derived exposure. Primary culture dopaminergic cells were exposed to 1µl of GCI fractions for 24 hours. Then, primary cultures were fixed in 4% paraformaldehyde, blocked and incubated with a primary rabbit anti-TH (1:1000, Calbiochem, Cat#657012) and corresponding fluorescence-conjugated secondary antibodies. All TH-positive neurons were analyzed by fluorescence microscopy and counted on each plate.

Animals and Stereotactic Injections

Mice

Wild-type C57BL/6J mice (3 months old) received 2µl of either GCI fractions (n=10) or sucrose (n=5) by stereotactic delivery to the region immediately above the right substantia nigra, to avoid mechanical lesion and to cover the whole SN, (SN, coordinates from Bregma: AP=-2.9, L= +1.3, DV=-4.5) at a flow rate of 0.4µl/min and the pipette was left in place for 5 min after injection to avoid leakage as previously described.[14,15,26,29] Mice were perfused four months after injection using 0.9% NaCl followed by 4% paraformaldehyde. Brains were then post-fixed for 24 hours in 4% paraformaldehyde at 4°C, cryoprotected in gradient 20% sucrose in PBS before being frozen by immersion in a cold isopentane bath (-60°C), and stored immediately at -80°C until sectioning for histochemical analysis.

NHPs

Experiments were conducted as previously described[15,16] at the research animal facility of the University of Murcia (Murcia, Spain). Adult female and male olive baboons (n= 8; *Papio papio*) ranging from 3 to 14 years of age were housed in two multi-male multi-female exterior pens. Animals were fed fruits, vegetables, and dry food pellets twice a day before 9 am and after 5 pm. Water was available ad libitum. Allocation to experimental groups was randomized. Three baboons were used for GCI injections, and five were untreated control animals. Intrastriatal injections of GCI fractions were performed at two rostrocaudal levels of the motor striatum (anterior commissure [AC], -1mm and -5mm) under stereotactic guidance as previously described.[15,16] The total injected volume per hemisphere was 100µl (2 injection sites with 50µl each at 3µl/min at each location site). After each injection, the syringe was left in place for 10 min to prevent leakage along the needle track. Several parameters were monitored during the two-year study, including survival and clinical observations. At the end of the experiment, all baboons were terminated with pentobarbital overdose (150 mg/kg i.v.),

followed by perfusion with room-temperature 0.9% saline solution (containing 1% heparin) following accepted European Veterinary Medical Association guidelines. Brains were removed quickly after death. Each brain was then dissected along the midline, and each hemisphere was divided into three parts. The left hemisphere was immediately frozen by immersion in a cold isopentane bath at -50°C for at least 5 min and stored at -80°C for biochemistry investigations. The right hemisphere was post-fixed one week in 10 vol/tissue of 4% paraformaldehyde at 4°C, cryoprotected in two successive gradients of 20 then 30% sucrose in phosphate-buffered saline (PBS) before being frozen by immersion in a cold isopentane bath (-50°C) during at least 5 min and stored immediately at -80°C until sectioning. No sample was excluded from analysis in these studies.

NHP Behavioral Assessment

Following a 4-hour habituation phase performed one day before the beginning of the observations, baboon behavior was observed outside the feeding and cleaning times, in random order at two-time points (morning and afternoon), over 4 to 9 days (8 sessions per group) as previously described.[15] On the first observational time point (i.e., 1-month post-surgery), the habituation phase was performed over three days, allowing the observer to recognize the animals individually. We used a scan-sampling method, appropriate for time budgeting, in which behavioral parameters were assessed every 5 minutes during 2-hour sessions, resulting in 192 scans per individual.[30-32] A unique trained observer blind to the experimental conditions (SC; intra-observer reliability: Spearman rank-order correlation R=0.987) collected the data live on the study's 2-time points: at 1- and 24-months post-surgery. The observer stood 1 meter away from the outdoor cages and focused on behavioral profiles rather than single items. Two repertoires were used: one reports the interaction with the environment, and one describes the position within the environment, according to published protocols and data collection by the very same observer.[30-32] We investigated the percentages of each item's occurrence about the total number of scans to obtain mean behavioral and postural time budgets, body orientation, and location profiles.

Histological Analysis

Neurodegeneration

To assess the effect of GCI injections in both mice and monkeys on dopaminergic neurons and fibers, tyrosine hydroxylase (TH), AADC (Aromatic L-amino acid decarboxylase) and

DAT (Dopamine Transporter) immunohistochemistry were performed on striatal and/or substantia nigra pars compacta (SNpc) sections as previously described.[15,16] For TH staining sections were selected in the posterior striatum and serial sections (1 to 12 for monkeys and 1 to 6 for mice) of whole SNpc, AADC and DAT in the medial striatum at the level of the anterior commissure. Sections were incubated with rabbit monoclonal TH antibody (Millipore, MAB318, 1:5000), rabbit polyclonal AADC antibody (Merck AB136, 1:1000) or rat monoclonal DAT antibody (Merck MAB369, 1:1000) for one night at room temperature (RT) and revealed the next day with the corresponding peroxidase EnVision secondary antibody, followed by 3,3′-diaminobenzidine (DAB) visualization. SNpc sections were mounted on gelatin-coated slides, counterstained with 0.1% cresyl violet solution, dehydrated and cover-slipped. Striatal sections were mounted on gelatin-coated slides, dehydrated and cover-slipped. Striatal sections were analyzed by optical density (OD) in the caudate nucleus and putamen. The slides were scanned using Epson expression 10000XL high-resolution scanner, and images were analyzed using ImageJ open-source software (version 1.53) to compare mean grey levels in the caudate nucleus and putamen. TH-positive neurons of the SNpc were counted by stereology blind about the experimental condition using a Leica DM6000B microscope coupled with the Mercator software (Explora Nova, France). The SNpc was delineated for each slide, and probes for stereological counting were applied to the map obtained. Each TH-positive cell with the neuron included in the probe was counted. The total number of TH-positive neurons in the SNpc per hemisphere was then assessed using the optical fractionator method.

Oligodendroglial loss

Olig2 (Oligodendrocyte transcription factor 2) and myelin immunohistochemistry were performed as previously described with minor changes[12] to assess oligodendroglial loss in the striatum of mice and monkeys. Sections were selected in the medial to posterior striatum and were incubated with mouse monoclonal Olig2 antibody (Merck, MABN5044, 1:1000) or rabbit monoclonal Myelin Basic protein antibody (Abcam, ab218011, 1:5000) for one night at RT and revealed the next day by an anti-mouse or anti-rabbit peroxidase EnVision secondary antibody respectively, followed by DAB visualization. Free-floating Olig2 stained sections were mounted on gelatin slides, counterstained with 0.1% cresyl violet solution, dehydrated, and cover-slipped. Free-floating myelin-stained sections were mounted on gelatin slides, dehydrated and cover-slipped. Slides were scanned using the Panoramic Scanner and analyzed using the Mercator software (Explora Nova, France). Olig2 slides were

analyzed using a surface threshold, where the baseline detection signal was determined and then applied on all slides. Myelin slides were analyzed two ways: using surface threshold similarly to Olig2 staining and using OD measuring the mean grey staining in the white matter in striatal sections. Besides, we measured the Nissl-cell count in the putamen on Olig2-counterstained sections using stereology.

Synuclein staining

Pathological handling of synuclein was assessed in monkeys with a mouse monoclonal antibody raised against human α-syn (syn211, ThermoFisher, MA5-12272, 1:1000) and another one against phosphorylated α-syn (clone11A5, Elan, 1:5000), as previously reported.[14,15] Briefly, selected sections at two rostrocaudal levels were specifically identified and incubated in the same well to allow direct comparison of immunostaining intensity. Sections were incubated overnight at RT with the aforementioned antibodies. The following day, the revelation was performed with anti-species peroxidase EnVision system (DAKO) followed by DAB incubation. Sections were then mounted on gelatinized slides, dehydrated, counterstained, if necessary, and cover-slipped until further analysis. Grey level quantification or immunostaining-positive surface quantification in twenty-nine brain regions were performed as previously described.[15,16] A heat map was generated using the data from grey-level quantifications in the different brain regions.[33]

Secondly, to assess the accumulation of total α-syn in cells in the SNpc, staining was performed on serial sections (1 to 12) of SNpc, corresponding to the whole SNpc.[16] Sections were incubated in monoclonal mouse Syn211 antibody for one night at RT and revealed the next day using an anti-mouse peroxidase EnVision secondary antibody followed by DAB visualization. Syn211-positive cells were counted using the Mercator software (Explora Nova, France). The SNpc was delineated for each slide, and probes for stereological counting were applied to the map obtained. Each Syn211-positive cell with the nucleus included in the probe was counted. The total number of Syn211-positive neurons in the SNpc was then assessed using the optical fractionator method.

Inflammation

Inflammatory processes in striatal sections were measured as previously described[15,16] through GFAP/S-100 (DAKO, Z0334/Abnova, PAP11341) and Iba1 (Abcam, ab5076) immunohistochemistry. Striatal sections were incubated overnight with a mix of rabbit antibodies raised against GFAP and S-100 for the astroglial staining (respective dilutions 1:2000 and 1:1000), and with a goat anti-Iba1 antibody for the microglial staining (dilution

1:1000). These signals were revealed with anti-species peroxidase EnVision system (DAKO) followed by DAB incubation. GFAP-S100 sections were mounted on slides, counterstained in 0.1% cresyl violet solution, dehydrated and cover-slipped. Sections stained by Iba1 were mounted on slides, dehydrated and cover-slipped. Sections stained by GFAP-S-100 were numerized at x20 magnification with a NanoZoomer (Hamamatsu, France), and Iba1 sections were numerized using the Panoramic Scanner. All quantifications were estimated by immunostaining-positive surface quantification at regional levels with the Mercator software (Explora Nova, France).

Biochemical Analysis in NHPs

Total protein extraction

Tissue patches collected on 300µm-thick cryostat-cut sections of caudate nucleus and putamen (n=5 patches per structure and animal) were extracted on ice using 100µl of RIPA buffer with a protease and phosphatase inhibitor cocktail as previously described.[15,16] Lysates were incubated for 20 minutes and then centrifugated at 14,000 rpm for 15 minutes at 4˚C. Supernatants were collected, and the total amount of protein in the lysates was assessed by Bicinchoninic Acid (BCA) assay before storage at -80˚C.

Based on total protein concentrations from the BCA assays, aliquots of tissue lysates corresponding to known amounts of total protein per sample were prepared for each animal in Laemmli buffer (Tris-HCl 25mM pH=6.8, Glycerol 7.5%, SDS 1%, DTT 250mM, and Bromophenol Blue 0.05%) for immunoblotting experiments.

Sequential protein extraction

Tissue patches, collected as above, (n=10 patches per structure and per animal) were homogenized in Triton-X (TX) extraction buffer (50 mM Tris-base pH 7.6, 150 mM NaCl, 1% Triton-X-100, 2 mM EDTA) containing protease and phosphatase inhibitors as previously described.[15,16,34] The lysate was sonicated and then centrifuged (120,000 x g for 60 min at 4 °C), and the supernatant was collected (TX-soluble fraction). The pellet was then washed 3 times with 1 M PBS/1% TX, centrifuged (13,000 x g for 15 min) and re-suspended in SDS extraction buffer (50 mM Tris pH 7.6, 150 mM NaCl, 1% Triton-X-100, 0.5% Na-deoxycholate, 1% SDS), sonicated, and centrifuged (120,000 x g for 60 min at 4 °C) and the supernatant was collected (TX-insoluble fraction).

Immunoblotting

Western blots were run in all conditions using 20µg of protein separated by SDS-PAGE and transferred to nitrocellulose membranes, as previously described.[15,16] Incubation of the primary antibodies was performed overnight at 4°C with rabbit anti-phosphorylated-α-syn at Ser129 (1:5000, Abcam EP1536Y), Syn1 (1:1000, BD Biosciences), Syn211 (1:1000, ThermoFisher), TPPP/p25a (1:5000, Sigma). Anti-β-actin (1:2000, Sigma) was used to control equal loading. Appropriate secondary antibodies coupled to peroxidase were revealed using a Super Signal West Chemiluminescent kit (Immobilon Western, Chemiluminescent HRP substrate, Millipore). Chemiluminescence images were acquired using the ChemiDoc+XRS system measurement (BioRad). Signals per lane were quantified using ImageJ (version 1.53). A ratio (protein of interest normalized to β-Actin protein levels, then to Control values) of the signal on loading per animal was performed and used in statistical analyses.

Neurotransmitter analysis in NHPs

Tissue patches collected on 300µm-thick cryostat-cut sections of Globus pallidus *pars internalis* (n=5 patches per structure and animal) were prepared and analyzed as previously described[15], homogenized in methanol/water (50:50% v/v), then centrifuged at 14000 rpm for 15 min at 4°C.[35] The supernatant was aliquoted and stored at -80°C until amino acid derivatization. Glutamate and γ-aminobutyric acid (GABA) content in the samples was measured by HPLC coupled with fluorometric detection (FP-2020 Plus fluorimeter, Jasco, Tokyo, Japan) after precolumn derivatization with o-phthaldialdehyde/mercaptoethanol (OPA) reagent.[36] Thirty microliters of OPA reagent were automatically added to 28 µL sample by a refrigerated autosampler kept at 4°C (Triathlon, Spark Holland, Emmen, The Netherlands). Fifty microliters of the mixture were injected onto a 5-C18 Hypersil ODS column (3 X 100 mm; Thermo-Fisher, USA) perfused at 0.48 mL/min (Jasco PU-2089 Plus Quaternary Pump; Jasco, Tokyo, Japan) with a mobile phase containing 0.1 M sodium acetate, 10% methanol, 2.2% tetrahydrofuran (pH 6.5). Chromatograms were acquired and analyzed using ChromNav software (Jasco, Tokyo, Japan). Under these conditions, the limits of detection for glutamate and GABA were ~1 nM and ~0.5 nM, and their retention times ~3.5 min and ~18.0 min, respectively.

Immunofluorescent Labeling in NHPs

Double-immunofluorescent labeling was performed on striatal sections to localize the accumulation of α-syn. Sections were permeabilized and blocked using normal donkey serum or normal goat serum diluted in PBS 1X-Saponine 0.2% for one hour before being incubated overnight in primary antibodies [mouse anti-CNPase (1:1000, ab237961, Abcam) or mouse anti-NeuN (1:1000, MAB377, Merck) and rabbit anti-α-syn (1:5000, MJFR1, ab209420, Abcam) or rabbit anti-phosphorylated α-syn at Ser129 (1:2000, EP1536Y, ab51253, Abcam)]. The next day, sections were incubated in secondary antibodies sequentially: first with donkey anti-mouse Alexa 488 secondary antibodies, then with donkey anti-rabbit Alexa 568 (α-syn) or Alexa 647 (pSyn) secondary antibodies. After washing, sections were incubated with 10µM of Hoechst staining for 8 min to color the nuclei. Sections were mounted on non-gelatinized slides and cover-slipped using fluorescent mounting media without DAPI (Vector Labs). Images were acquired using a Zeiss SP5 confocal microscope (x63). Analysis of images was done using ImageJ (version 1.53), with a macro designed to delineate individual cells, analyze particles within the cell and extrapolate the count and size of puncta.

Statistical Analysis

Principal component analysis (PCA) was performed in Python (Python software foundation v.3.7.4 available at https://www.python.org/) and the scientific python stack: scipy (v.1.4.1)[37], numpy (v.1.18.1)[38], matplotlib (v.3.3.3)[39] and scikit-learn (v.0.22.1).

For all experiments, comparisons among means were performed using raw data by using Student's one-tailed unpaired t-test (GraphPad Prism 9.0, San Diego, CA). Correlations between variables were assessed with Spearman's correlation analysis. Statistical significance was set at $p<0.05$.

The debate about the need to move beyond *p* value is raging. Data must now be analyzed further with estimation graphics[40] that put the emphasis on the effect size. Therefore, all data appear as estimation graphics called ‘Gardner–Altman plots’: on the left of each graph, data of vehicle (mice) or controls (NHP) and GCI groups are presented as scatter plot showing the observed values along with above defined descriptive statistics (mean ± standard deviation). On the right of each graph, a contrast graph using the difference axis to display an effect size, here mean difference. Horizontally aligned with the mean of the test group, the mean difference is indicated by the black circle. The 95% confidence interval (CI) of the mean

difference is illustrated by the black vertical line. The grey curve indicates the resampled distribution of the effect size, given the observed data.

Results

Qualification of GCIs-containing fractions from human MSA Brains

GCI-enriched fractions were obtained from two MSA patient brains, containing conspicuous α-syn-positive and ubiquitin-positive aggregates (Fig. 1A). They were used to evaluate the pathological consequences of injecting α-syn-containing putaminal GCI extracts purified from MSA-P patients into the brains of wild-type mice and NHPs. Fresh-frozen postmortem striatal samples were purified through differential ultracentrifugation using a sucrose step gradient and tested for their α-syn immunoreactivity using filter retardation assay (Fig. 1A), as previously described.[14-16]

GCI fractions were first tested *in vitro* on rat mesencephalic primary cultures. GCI fraction administration induced a significant loss of TH-positive cells by over 50% (Fig. 1B), thereby demonstrating the toxicity of GCI fractions. This raised the question of whether these fractions have also harmful effects *in vivo*. To test this, wild-type mice received intranigral injections of GCI fractions. Four months after administration, mice presented a significant 39 ± 4.5% loss of dopaminergic neurons in the SN (Fig. 1C), without losing dopaminergic fibers projecting to the striatum (Mean difference lower than 5% - Fig. 1D). Possibly owing to the relatively short live phase, no significant loss of striatal oligodendrocytes was observed, although a trend towards a decrease was noticeable with an effect size of ~20% in Olig2 staining (Fig. 1E). Overall, these results confirmed the toxicity of putaminal-derived GCI fractions extracted from MSA patients onto SN cells in wild-type mice and grounded the NHP experiment.

GCIs-containing fractions induce nigral and striatal degeneration in NHPs

Following our *in vitro* and *in vivo* mice experiments, we aimed to determine whether GCI fractions affected NHPs. GCI-injected baboons were ethologically followed during two years after administration. We observed motor difficulties with an increase in the time spent with their body oriented in the open environment, indicating anxiety-like behavior and decreased time spent in the corridor connecting the main aviary to the secondary, indicating decreased motivation/locomotion[31] (Supplementary Fig. 1A-B). These subtle phenotypes are reminiscent of those displayed by LB-injected monkeys[15] and suggest that GCI-injected

monkeys might have been in a prodromal phase. Postmortem examination two years after injection unraveled that GCI injection induced a significant impairment of the nigrostriatal pathway with TH immunoreactivity losses of 36% in the caudate nucleus (Fig. 2A and B-D), 18% in the putamen (Fig. 2A and E) and 40% in the SN (Fig. 2A and F-G). AADC, the enzyme that decarboxylates L-Dopa into dopamine, (Fig. 2H) and striatal DAT immunostaining were also reduced (Fig. 2I) with a mean difference between groups of ~50% for both markers in caudate and putamen. GCI-injected monkeys also presented a significant decrease of the density of Nissl-positive cells in the putamen (Fig. 2J), suggesting a loss of striatal medium spiny neurons. Together, these results reflect an impairment and degeneration of the nigrostriatal pathway and putamen.

Next, we performed dimensionality reduction using principal component analysis (PCA – a multivariate analysis method). Projection of individual points along PC1-PC2 demonstrated that neurodegeneration-related variables allowed clustering of controls and GCI-injected animals in two opposite parts of the PC1-PC2 space with no overlap (Fig. 2K). Most of the variance was captured by PC1 (70.3%), which showed high correlation values with most neurodegeneration-related variables (Supplementary Table 1 features all raw data). Analysis of individual scores along the PC1 axis showed significant differences between controls and GCI-injected animals (Fig. 2L). Altogether, these results confirmed the harmful impact of GCI fractions on the nigro-striatal pathway integrity in monkeys.

Since both the SN and the striatum exhibited neurodegeneration, we wondered whether these changes resulted into functional changes into the basal ganglia network. Glutamate levels slightly increased (~20%) while GABA content decreased in GCI-injected monkeys (Supplementary Fig. 2A-B). This is consistent with (i) an increased activity of the glutamatergic projection from subthalamic nucleus, i.e. the last relay nucleus of the indirect pathway and (ii) a decreased drive from the GABAergic projection that composes the direct pathway.[41] Both changes sign an imbalance of striatofugal pathways, resulting from neurodegeneration.

GCIs-containing fractions induce oligodendrocyte loss in NHPs

Given that MSA pathology is not only characterized by the loss of striatal and dopaminergic neurons but also by oligodendrocyte dysfunction and consequently myelin disruption, we evaluated whether this could be observed at the striatal and white matter levels in GCI-injected monkeys. Histochemical analysis of Olig2 staining showed a significant decrease in

the caudate nucleus and the putamen, in terms of both total surface and cell size (Fig. 3A-F). No significant changes, but trends, due to the small sample size, were observed in the white matter, with a mean difference in Olig2 staining of 60% (Fig. 3G-I). Our results suggested a decrease in the total number and the size of striatal oligodendrocytes in GCI-injected monkeys. We next examined the myelination, one of the crucial roles of oligodendrocytes, using myelin immunostaining in the putamen of GCI-injected and control animals. We observed a decrease in myelin immunoreactivity in the white matter of GCI-injected monkeys (Fig. 3J-L).

Interestingly, the loss of putaminal oligodendrocytes correlated positively with the loss of striatal dopamine fibers, DAT and AADC staining (Supplementary Fig. 3A-B), and striatal cell density (Supplementary Fig. 3C). Moreover, the oligodendroglial loss in the white matter also significantly and positively correlated with dopaminergic degeneration (i.e., loss of TH and DAT-positive fibers in the putamen and caudate nucleus) (Supplementary Fig. 3D-G). These associations suggested the existence of striatal neurodegeneration affecting both the incoming dopamine fibers and the striatofugal neurons concomitant with oligodendroglial neurodegeneration, thereby corroborating neurochemistry findings.

In MSA, TPPP/p25α is relocated from myelin sheaths to oligodendrocyte cytoplasm and is a significant component of GCIs.[1,5,42,43] We characterized variations in TPPP/p25α protein levels in the putamen and caudate of GCI-injected monkeys, particularly in the putamen with an increase of 25% (Fig. 3M-N).

In addition to oligodendrogliopathy, increased inflammation via astrogliosis and microgliosis has been observed in human MSA neuropathology.[44] We observed that they did not present a marked inflammatory response as indicated by the absence of astrocytic reaction, using the marker GFAP, and microglial proliferation, using Iba1, in the striatum and the white matter (Supplementary Fig. 4A-B). While GFAP staining also remained unaffected in the entorhinal cortex, we observed local increase of microglial surface staining in the entorhinal cortex of GCI-injected monkeys (Supplementary Fig. 4A-B). Altogether these results suggested that, in addition to the mesencephalic and striatal degeneration, GCI-injected monkeys present an MSA-like oligodendroglial loss and demyelination but only modest neuroinflammation.

GCIs-containing fractions induce synuclein pathology in NHPs

To evaluate if GCI-induced neurodegeneration and oligodendrogliopathy were associated with synuclein pathology, we quantified the levels of α-syn staining in twenty-nine different regions of the basal ganglia and cortex (Fig. 4A, Supplementary Fig. 5-6). While total α-syn accumulation was modestly affected in the investigated regions (Fig. 4A top, Supplementary Fig. 5), α-syn phosphorylated at S129 (pSyn – a pathogenic form)[45] accumulated differently in GCI-injected baboons compared to controls (Fig. 4A bottom, Fig. 4B bottom inset, Supplementary Fig. 6). However, when looking more closely at α-syn signal in the SN, we observed a re-localization from a diffuse staining (typical of the physiological presynaptic localization of the protein) to a cellular-like staining in soma, represented by a significant increase (~50% effect size) in α-syn positive cells (Fig. 4B-C). Of interest, the number of nigral α-syn-positive cells significantly and negatively correlated with TH-positive cell surface staining in the SN, indicating that the decrease in TH-positive cells is associated with an increase in the number of α-syn-positive cells in the SN (Fig. 4D). Biochemical investigations corroborated an increase of pSyn in the putamen and caudate nucleus in GCI-injected monkeys (Fig. 5A-B). Furthermore, using sequential extraction of Triton-X (TX) soluble and insoluble α-syn[34], we detected higher levels of TX-insoluble monomeric and high molecular weight forms of total α-syn (Fig. 6A-B) and pSyn (Fig. 6C-D) in the putamen, but not the caudate nucleus. TX-soluble fractions were also enriched in high molecular weight forms of α-syn in the putamen (Supplementary Fig. 7A-B). Concerning pSyn, monomeric forms were enriched in both the putamen and caudate nucleus, whereas high molecular weight forms were only increased in the caudate nucleus (Supplementary Fig. 7C-D). Altogether, these results indicate α-syn buildup with both a change in cellular staining and increased TX-insoluble and soluble protein expression in the striatum two years after GCI injection.

One of the hallmarks of MSA is the accumulation of α-syn in oligodendrocytes. We determined the cell-type specificity and distribution of α-syn expression via immunofluorescence confocal microscopy with the well-established oligodendrocyte marker CNPase and the neuronal marker NeuN. In both controls and GCI-injected monkeys, α-syn colocalized with CNPase in putaminal oligodendrocytes (Fig. 7A), with a significant increase in the number and a decrease in the size of α-syn-positive puncta in GCI-injected monkeys. This suggests that α-syn accumulates in oligodendrocytes of GCI-injected primates, with more abundant smaller puncta distributed in the oligodendrocyte. On the contrary, α-syn puncta were rare in neurons, both in controls and GCI-injected monkeys (Fig. 7B). In the remaining oligodendrocytes, colocalization of pSyn and CNPase was observed in control and

GCI-injected animals, with a surprising decrease in pSyn puncta in GCI-injected baboon monkeys (Fig. 7C). In neurons, pSyn was also found in the cytoplasm of control and GCI-injected baboons (Fig. 7D). Altogether, these results suggest an accumulation of α-syn in oligodendrocytes of GCI-injected monkeys, indicating a potential first phase of MSA-related α-syn pathology induction.

Discussion

In this pilot study, we show that intracerebral injections of GCI-enriched fractions in NHPs induce most of the disease-specific aspects of MSA neuropathological features. Two years after stereotactic striatal injections, baboon monkeys inoculated with GCIs demonstrated subtle behavioral changes and neuropathological evidence of MSA pathology, including loss of both dopaminergic and striatal neurons, loss of oligodendrocytes in the putamen along with demyelination, slight neuroinflammation, and accumulation of α-syn in oligodendrocytes compared to age-matched control monkeys. These observations suggest that, two years after injection, the GCI-exposed baboon monkeys were in an initial phase of MSA-like condition.

Mice experiments were aimed at qualifying the GCI fractions based on dopaminergic neuron-induced toxicity. After 4 months post-injection, the supranigral injection of GCI in C57BL6/J wild-type mice led to dopamine neuron loss. Despite meeting the qualification criterion set before NHP experimentation, one may wonder why other parameters were not affected at 4 months in wild-type mice. There are few previous reports of MSA-derived extracts injected in wild-type and transgenic mice. Interestingly, a recent report showed that detergent-insoluble α-syn isolated from MSA patients could also induce neuronal inclusions in C57BL6/C3H wild-type mice at 3 months after striatal injection but failed to generate an oligodendroglial pathology.[19] Other types of extracts, i.e., brain homogenates instead of our GCI extraction procedure, have also been tested, but they were administered to transgenic α-syn-overexpressing mice. In this regard, intracerebral injections of brain extracts from MSA patients in homozygous and hemizygous TgM83 mice led to neurodegeneration and formation of α-syn-positive inclusions after 3 months.[17,18,46] Following this, another study used injections of brain homogenates from sick M83 transgenic mice or human MSA patient extracts in TgM83 mice.[47] This study demonstrated that pathological α-syn accumulation in transgenic mice was higher when inoculated with homogenates from sick M83 mice than

from MSA patient extracts. Unlike our GCI inoculations, none of these injections induced neurodegeneration or synucleinopathy in wild-type mice, regardless of the inoculum.

MSA extracts have been by far less used than PD extracts in an experimental setting. Notably, the α-syn spreading and toxicity efficiency observed in different laboratories depends strongly on several factors, including the biochemical preparation of the inoculum, the injected quantity (in the microgram-picogram range) and volume, the choice of mouse strain, the brain areas of injection, the post-injection observational period, and the potential species barrier.[17,18,46,47] Interestingly, the literature using transgenic MSA mouse models based on α-syn overexpression is more profuse and provides interesting timing considerations. The PLP-α-syn transgenic mouse model displays a loss of dopamine neurons (predominant between 2-4 months of age), together with age-related impairments that worsen when striatal atrophy comes into play around 10-12 months of age.[8,10,11] Besides, this model recapitulates the oligodendroglial accumulation of insoluble α-syn as well as some dysautonomic features of MSA only when mice reach 10-12 months of age.[8,48,49] Also, another recent study where preformed fibrils of α-syn were injected in both young and old WT C57Bl6/J mice showed the presence of oligodendroglial α-syn aggregates starting at 12 to 18 months.[20] Nonetheless, a recent study demonstrated the difference between the atomic structures of seeded assemblies of recombinant human α-syn fibrils compared to that of human α-syn extracts, also demonstrating the need for models using patient-derived extracts.[50] Although follow-up studies would be necessary to confirm this suggested time-related hypothesis, wild-type mice receiving GCI extracts might likely require a more extended survival period before manifesting signs of oligodendrogliopathy and demyelination in the striatum, as observed in NHPs.

One of the major unanswered questions in the field remains what triggers the localization of GCIs in oligodendrocytes compared to LB formation in neurons.[51-53] Follow-up questions are whether, why, and how much α-syn accumulation in oligodendrocytes would lead to neurodegeneration, as it undoubtedly occurs in MSA patients, in α-syn transgenic mice models and in the current set of GCI-exposed NHPs. Various non-exclusive mechanisms might be at work, such as (i) the relocalization of TPPP/p25α in oligodendrocytes from the myelin sheath to the cytoplasm, which appears to be an early pathologic event that precedes α-syn aggregation[5,43]; (ii) the α-syn release from neurons to oligodendrocytes through active or passive mechanisms[54]; and (iii) non-cell autonomous factors such as pro-inflammatory or environmental factors.[55,56] In light of the ongoing debate, these cells may produce much of the protein[57-60] and the intracellular and the extracellular factors responsible for α-syn

expression in oligodendrocytes. In addition, further studies are needed to determine to what extent conformational status and accumulation of α-syn and pSyn end up in oligodendrocytes. No genuine GCIs were observed in the present NHP experiments. Given the otherwise convincing reported pathology, we posit that actual formation of GCIs would take longer than the 2-year survival period imposed by the experimental design. Redistribution of pSyn accumulation towards oligodendrocytes is however present and, being concomitant to other aspects of MSA neuropathology, including TPPP/p25α accumulation[61], we strongly suggest these animals model a pre-GCI phase. This observation raises two consequences: it suggests that MSA-derived extracts can induce oligodendroglial α-syn pathology, irrespective of the controversy on the expression of α-syn itself by oligodendrocytes. It further suggests that nigral and striatal degeneration precedes GCI formation although linked to α-syn redistribution in oligodendrocytes. Importantly, such redistribution was not observed in NHPs injected with LB. Taken together, this provides additional pieces to the concept of disease-related strains, which could discriminate between the occurrence of PD or MSA pathologies.
Relying on a single snapshot at two years however precludes a dynamic understanding of the process. It prevents us from defining whether cell death promotes accumulation of α-syn in oligodendrocytes or the contrary. What we can affirm, however, is that abnormal handling of α-syn propagates from the putamen, i.e. the location of intracerebral injection, to other brain regions, as can be seen in the heat-map (Fig. 4A) with progression to cortical areas and the lateral dorsal nucleus. This suggests that GCI-induced α-syn pathology can propagate in NHPs, as shown previously for PD-derived α-syn species.[14-16]

While acceptable for an exploratory study, further studies would require larger NHP cohorts to reach adequate statistical power for all variables. An additional question deals with the nature of the control arm of this exploratory study. We had to predetermine the experimental groups with a known number of animals and make choices regarding the control groups at the beginning of this large-scale study. Before our experiments on olive baboons[15,16], only two studies reported on using this species to model synucleinopathy and neurodegeneration.[62,63] We thus faced a lack of baseline parameters, for instance, references of dopaminergic-related parameters.
The present study is the MSA component of a large effort to explore the impact of PD and MSA patient-derived α-syn aggregates in NHPs.[15,16,21] Uninjected animals were therefore used as controls for multiple extracts, preventing the use of other several additional control individuals, in agreement with the 3R principle. This choice was further validated *a*

posteriori by the discovery that, in NHPs but not in mice, a small amount of singular small PD-derived α-syn aggregates is as toxic as larger PD-derived amyloid fibrils present in the LB[15]. Relevant to this issue, we did not inoculate animals with immunodepleted α-syn or α-syn fractions denatured with formic acid, as we previously showed that inoculation with α-syn immunodepleted fractions did induce neither neurodegeneration nor synuclein pathology.[14]

Overall, we here provide the proof-of-concept that MSA-derived GCI fractions trigger a pathogenic process in rodents and NHPs. This study widens exciting perspectives for understanding human MSA pathology *per se* and across species within the normal α-syn expression context. Further studies with more monkeys and different time points of postmortem analysis are needed to extend our observations and appreciate the full scope of the phenotypes following GCI injections. These could also give insights into the contribution of the oligodendroglial synucleinopathy in MSA neurodegeneration to help solve the perplexing dichotomy of PD/DLB vs. MSA.

Acknowledgements

The authors thank Carmen Lagares Martínez (Head, Veterinary Service, University of Murcia, Murcia, Spain) for administrative assistance; Maria Fermina Ros Romero and Josefa Martínez Rabadán (University of Murcia) for veterinary and husbandry support; Ana Luisa Gil, Lorena Cuenca, and Ignacio Mascarell from Clinical and Experimental Neuroscience group (University of Murcia) for their technical help with various parts of the In Vivo part of these complex experiments. We would like to thank Philippe Hantraye (MIRCen, Fontenay-aux-Roses, France) for providing a baboon stereotactic frame. The University of Bordeaux and the Centre National de la Recherche Scientifique provided infrastructural support. This work was supported by a grant from the Michael J Fox Foundation (Project Grant No. 2013-8499), Fundacion de Investigacion HM Hospitales (Madrid, Spain), the Fundación Séneca (Project Grant No: FS19540/PI/14), the TARGET PD ANR grant and The Simone and Cino Del Duca Prize from French Academy of Sciences. MT, MB, and MLA were supported by a Minist re de l'Enseignement S rieur et de la Recherche fellowship (France). MB and MLA were also supported by the France Parkinson Foundation (France). The sequential α-synuclein extraction was performed in the Biochemistry and Biophysics Platform of the Bordeaux Neurocampus at the Bordeaux University funded by the LABEX BRAIN (ANR-10-LABX-43) with the help of Y. Rufin. The samples were obtained from the Brain Bank GIE NeuroCEB (BRIF number 0033-00011), funded by the patients' associations France Alzheimer, France Parkinson, ARSEP, and "Connaître les Syndromes Cérébelleux" to which we express our gratitude.

Author contributions

MV, JAO, EB, and BD conceived and designed the study. MB, GP, I.T-D., CE, NGC, MTH, EB and BD performed surgeries. SC performed behavioral analysis. MT and SD performed histological and immunohistochemical analysis of the data. MT and MLA performed imaging experiments. MT and MLT performed biochemistry experiments. MM performed the neurochemistry experiments. CP performed and analyzed primary culture experiments. MM performed the HPLC analysis. MT, SD, MB, MLA, SC, CP, WGM, MTH, MV, JAO, EB and BD analyzed the data. MT, WGM, MV, JO, EB and BD wrote the paper. MTH supervised the *in vivo* phase of the experiments, while BD and EB supervised the whole

project. All authors discussed the results, assisted in the preparation, and contributed to the manuscript. All authors approved the final version of the document.

Competing interests

EB is a director and a shareholder of Motac neuroscience Ltd. WGM has served as an advisor for Lundbeck and Biohaven and has received teaching honoraria from UCB. Other authors declare no conflict of interest.

Supplementary material

Supplementary material is available in the online version of the paper.

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Figure legends

Fig. 1. Purified Glial Cytoplasmic Inclusions (GCI) from Multiple System Atrophy (MSA) patient brains induce neurodegeneration in mice. (**A**) *(left)* α-synuclein and ubiquitin immunohistochemistry of striatal postmortem brain samples unravelling GCIs. Scale bar = 200µm. *(middle)* Schematic representation of the sucrose gradient fractionation procedure used to purify GCI-containing fractions from freshly frozen postmortem nigral brain tissue of two MSA-P patients. *(right)* Filter retardation assay probed with a human specific α-synuclein antibody to assess the presence of insoluble α-synuclein aggregates in the different fractions obtained by sucrose gradient fractionation from freshly frozen postmortem striatal brain tissue of MSA patients. The green rectangle indicates the GCI-containing fractions selected to prepare the mixture used for GCI injections. (**B**) Scatter plots of Tyrosine hydroxylase (TH) immuno-positive rat mesencephalic cells treated with 1µl of GCI fractions for 24 hours and expressed as a percentage of controls; p=0.0002, t=5.872. (**C**) Illustrative image *(left),* and quantification *(right)* of TH immuno-positive cells by stereological counting in the substantia nigra pars compacta (SNpc) of GCI-injected and control mice; p<0.0001, t=6.581. Scale bar = 100µm (**D**) Illustrative image *(top)* and quantification *(bottom)* of TH immunostaining measured by optical density in the striatum of GCI-injected and control mice, normalized by controls; p=0.2818, t=0.5926. Scale bar = 500µm (**E**) Illustrative image *(top)* and quantification *(bottom)* of Olig2 immunostaining in the striatum of control and GCI-injected mice expressed as surface threshold occupied by Olig2 normalized by controls; p=0.0720, t=1.555. Scale bar = 50µm. The horizontal line indicates the average value per group ± standard deviation. Each dot represents one mouse of the control (black) and GCI-injected group (green). Bootstrapped mean difference with 95% CI (error bar) is shown on the right side of each graph. Comparisons were made using an unpaired t-test. *p<0.05 compared to control animals.

Fig. 2. Intrastriatal injection of GCI-derived fractions induces nigrostriatal neurodegeneration in the NHP brain. (**A**) TH immunostaining at striatum and SNpc levels in control (n=5) and GCI-injected baboon monkeys (n=3). Scale bars = 5mm (striatum) and 200µm (SNpc). (**B-E**) Scatter plots of mean grey TH immunostaining in the caudate nucleus; p=0.0098, t=3.159 (**B**), in the head of the caudate nucleus; p=0.0042, t=3.849 (**C**), in the tail of the caudate nucleus; p=0.0248, t=2.452 (**D**), and in the putamen; p=0.0488, t=1.961 (**E**) in Control vs. GCI-injected baboon monkeys, expressed as a percentage of controls. (**F-G**)

Scatter plots of TH staining in the substantia nigra by quantification of surface staining, expressed as a percentage of controls; p=0.0054, t=3.648 (**F**) and the number of TH-positive cells; p=0.1475, t=1.147 (**G**). (**H**) Illustrative images *(left)* and scatter plots *(right)* of Aromatic L-Amino acid Decarboxylase (AADC) immunostaining measured by optical density in the caudate nucleus; p=0.0187, t=3.573 and in the putamen; p=0.0236, t=3.259 of control and GCI-injected NHPs and expressed as a percentage of controls. A green fire blue LUT (lookup table) was used to enhance contrast and highlight the difference. Scale bar = 5mm. (**I**) Illustrative images *(left)* and scatter plots *(right)* of Dopamine Transporter (DAT) immunostaining measured by optical density in the caudate nucleus; p=0.0147, t=3.929 and in the putamen; p=0.0105, t=4.456 of control and GCI-injected NHPs and expressed as a percentage of controls. A green fire blue LUT (lookup table) was used to enhance contrast and highlight the difference. Scale bar = 5mm. **(J)** Scatter plot representing the density of Nissl-positive cells in the putamen of control and GCI-injected NHPs, expressed as a percentage of controls. p=0.0405, t=2.095. The horizontal line indicates the average value per group ± standard deviation. Each dot represents one NHP of the control (black) and GCI-injected baboon monkeys (green). Bootstrapped mean difference with 95% CI (error bar) is shown on the right side of each graph. Comparisons were made using an unpaired t-test. *p<0.05 compared to control animals. (**K-L**) Principal Component (PC) analysis was applied on 11 neurodegenerative parameters (above). Each animal is represented by a dot in the new space created by PC1-2 **(K)**. Histogram plot depicts mean values of PC1 scores for each experimental group; p=0.0160 **(L)**. Each dot represents one NHP of the control (black) and GCI-injected baboon monkeys (green).

Fig. 3. Intrastriatal injection of GCI-derived fractions induces oligodendroglial loss and demyelination in the NHP brain. (**A-C**) Illustrative images **(A)** and quantification **(B-C)** of Olig2 immunostaining measured by surface threshold; p=0.0221, t=3.346 **(B)** or surface size; p=0.0310, t=0.2911 **(C)** expressed as a percentage of controls, in the caudate nucleus of control and GCI-injected NHPs. Scale bar = 40µm. (**D-F**) Illustrative images **(D)** and quantification **(E-F)** of Olig2 immunostaining measured by surface threshold; p=0.0043, t=6.154 **(E)** or surface size; p=0.0124, t=4.190 **(F),** expressed as a percentage of controls, in the putamen of control and GCI-injected NHPs. Scale bar = 40µm. (**G-I**) Illustrative images **(G)** and quantification **(H-I)** of Olig2 immunostaining measured by surface threshold; p=0.0552, t=2.245 **(H)** or surface size; p=0.1370, t=1.336 **(I)**, expressed as a percentage of controls, in the white matter of control and GCI-injected NHPs. Scale bar = 40µm. (**J-L**)

Illustrative images **(J)** and quantification **(K-L)** of myelin immunostaining measured by optical density; p=0.0140, t=4.004 **(K)** and surface threshold; p=0.0036, t=6.572 **(L)**, expressed as a percentage of controls, in the striatum of control and GCI-injected NHPs. Scale bar = 5mm. The horizontal line indicates the average value per group ± standard deviation. Each dot represents one monkey of the control (black) and GCI-injected baboon monkeys (green). Comparisons were made using an unpaired t-test. *p<0.05 compared to control animals. (**M**) Representative images *(left)* and quantification *(right)* of TPPP/p25α immunoblotting in the rostral putamen of control and GCI-injected NHPs; p=0.0648, t=2.075. (**N**) Representative images *(left)* and quantification *(right)* of TPPP/p25α immunoblotting in the rostral caudate nucleus of control and GCI-injected NHPs; p=0.2264, t=0.8607. Each dot represents one NHP of the control (black) and GCI-injected NHPs (green). Bootstrapped mean difference with 95% CI (error bar) is shown on the right side of each graph. Comparisons were made using an unpaired t-test. *p<0.05 compared to control animals.

Fig. 4. Intrastriatal injection of GCI-derived fractions induces a specific pattern of S129 phosphorylated α-syn levels in the NHP brain and a specific accumulation of total α-synuclein in the NHP SN. (**A**) Heat map representing the surface threshold of α-syn (Syn211) and S129 phosphorylated α-syn (Elan) immunostaining intensity in the brain of control and GCI-injected NHPs. The heat maps show all brain regions measured. From top to bottom, for total α-syn: amygdala (amyg), caudate nucleus (cd), cingulate cortex (ctx cing), entorhinal cortex (ctx ent), insular cortex (ctx ins), premotor cortex (ctx pm), sensorimotor cortex (ctx sm), external globus pallidus (gpe), internal globus pallidus (gpi), putamen, white matter (wm). From top to bottom for phosphorylated α-synuclein: fornix (frx), head of the caudate nucleus (cd head), claustrum (clstrm), geniculate body (gen body), corpus callosum (corcal), posterior cingulate cortex (ctx cing post), entorhinal cortex (ctx ent), parahippocampal cortex (ctx phipp), retroinsular cortex (ctx retins), posterior sensorimotor cortex (ctx sm post), hippocampus (hipp), lateral dorsal nucleus (ldn), red nucleus (rn), subthalamic nucleus (stn), posterior putamen (put post), substantia nigra (sn), white matter (wm), ventral tegmental area (vta). The color bars represent the log2 value of the ratio of each brain region. **(B)** Representative coronal brain sections of endogenous α-syn immunostaining *(top panel)* and illustrative photomicrographs of endogenous *(middle inset)* and phosphorylated *(bottom inset)* α-syn in the SNpc of control and GCI-injected NHPs. Scale bars = 5mm (top panel) and 50µm (insets). (**C**) Scatter plot of the number of α-syn-positive cells in the substantia nigra in control and GCI-injected NHPs; p=0.0048, t=3.746. The

horizontal line indicates the average value per group ± standard deviation. Each dot represents one NHP of the control (black), GCI-injected NHPs (green). Comparisons were made using an unpaired t-test. *p<0.05 compared to control animals. (**D**) Linear regression between surface threshold value of TH immunostaining and the number of α-syn-positive cells in the susbtantia nigra; F=6.669, p=0.0416, r^2=0.5264 for control or GCI-injected NHPs. Each dot represents one monkey of the control (black) and GCI-injected NHPs (green). Bootstrapped mean difference with 95% CI (error bar) is shown on the right side of each graph. Comparisons were made using an unpaired t-test. *p<0.05 compared to control animals.

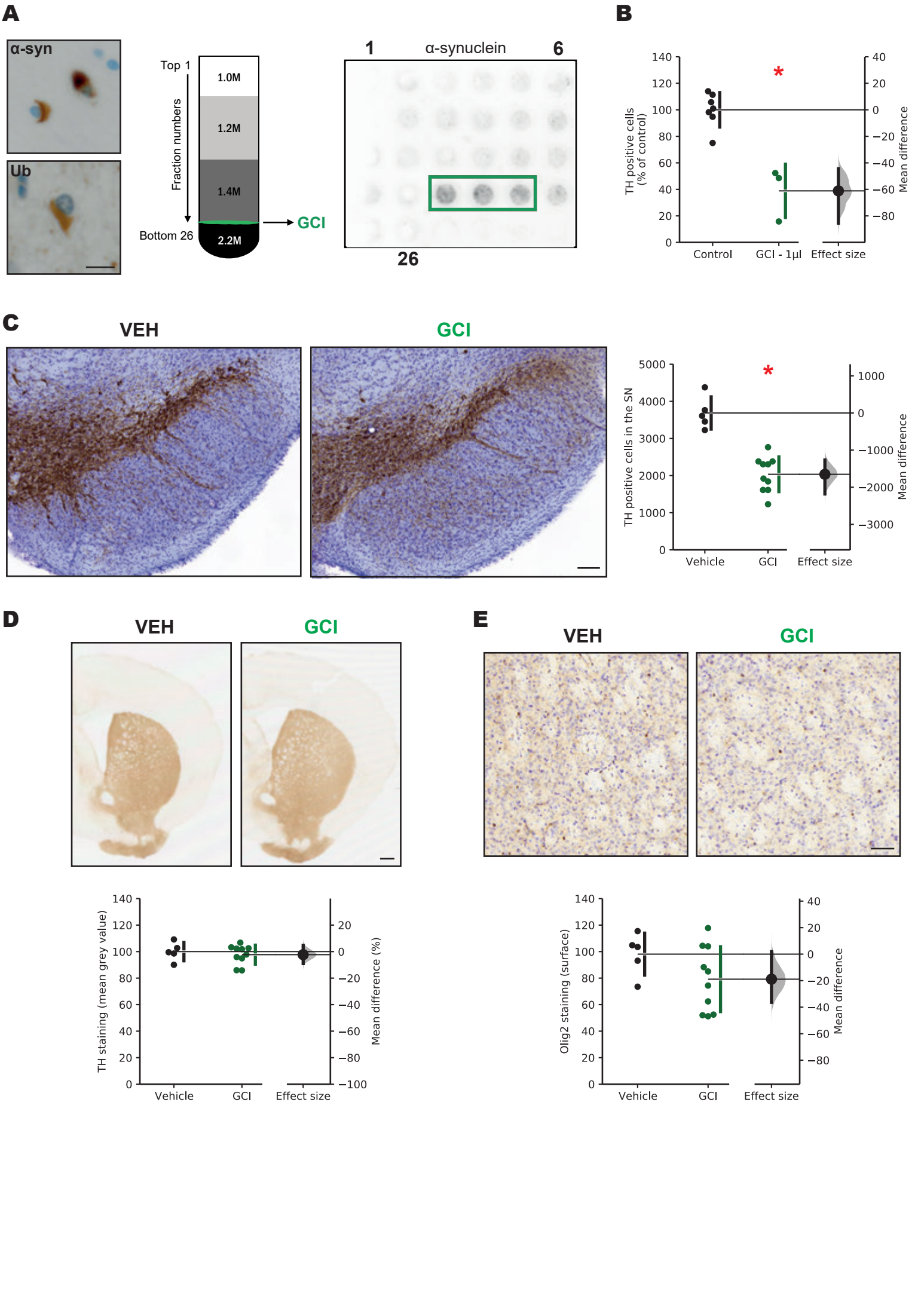
Fig. 5. Total protein extracts of putamen and caudate nucleus reveal an accumulation of S129-phosphorylated α-syn after GCI injections. (**A**) Representative images *(top)* and quantification *(bottom)* of total protein extracts of α-syn and S129-phosphorylated α-syn (pSyn) immunoblotting using Syn1 and EP1536Y antibodies in the rostral, medial and caudal putamen (respectively PuR, PuM, PuC) of control and GCI-injected NHPs (pSyn PuR: p=0.0040, t=2,607; pSyn PuM: p=0.1263, t=1.413; pSyn PuC: p=0.3830, t=0.3258; α-syn PuR: p=0.1336, t=1.359; α-syn PuM: p=0.4120, t=0.2426; α-syn PuC: p=0.2169, t=0.9016). (**B**) Representative images *(top)* and quantification *(bottom)* of total protein extracts of α-syn and S129-phosphorylated α-syn (pSyn) immunoblotting using Syn1 and EP1536Y antibodies in the rostral, medial and caudal caudate nucleus (respectively CdR, CdM, CdC) of control and GCI-injected NHPs (pSyn CdR: p=0.0010, t=10.27; pSyn CdM: p=0.0016, t=8.663; pSyn CdC: p=0.4845, t=0.0422; α-syn CdR: p=0.2579, t=0.7345; α-syn CdM: p=0.3378, t=0.4618; α-syn CdC: p=0.3660, t=0.3759). The horizontal line indicates the average value per group ± standard deviation. Each dot represents one monkey of the control (black), GCI-injected NHPs (green). Bootstrapped mean difference with 95% CI (error bar) is shown on the right side of each graph. Comparisons were made using unpaired t-test. *p<0.05 compared to control animals.

Fig. 6. Subcellular fractionation of putamen and caudate nucleus reveals an accumulation of triton insoluble forms of total and S129-phosphorylated α-syn after GCI injections (**A**) Representative images *(left)* and quantification *(right)* of TX-insoluble monomeric and high molecular weight α-syn immunoblotting using Syn211 antibody in the rostral, medial and caudal putamen of control and GCI-injected baboon monkeys (14kDa α-syn PuR: p=0.0675, t=2.032; 14kDa α-syn PuM: p=0.1816, t=1.069; 14kDa α-syn PuC:

p=0.2444, t=1.17; 55kDa α-syn PuR: p=0.1010, t=1.628; 55kDa α-syn PuM: p=0.2606, t=0.7242; 55kDa α-syn PuC: p=0.0088, t=4.769). (**B**) Representative images *(left)* and quantification *(right)* of TX-insoluble monomeric and high molecular weight α-syn immunoblotting using Syn211 antibody in the rostral, medial and caudal caudate nucleus of control and GCI-injected baboon monkeys (14kDa α-syn CdR: p=0.1769, t=1.095; 14kDa α-syn CdM: p=0.0615, t=2.13; 14kDa α-syn CdC: p=0.0882, t=1.762; 55kDa α-syn CdR: p=0.0907, t=1.733; 55kDa α-syn CdM: p=0.0865, t=1.781; 55kDa α-syn CdC: p=0.4864, t=0.03697). (**C**) Representative images *(left)* and quantification *(right)* of TX-insoluble monomeric and high molecular weight pSyn immunoblotting using EP1536Y antibody in the rostral, medial and caudal of control and GCI-injected baboon monkeys (14kDa α-syn PuR: p=0.1113, t=1.534; 14kDa α-syn PuM: p=0.1089, t=1.555; 14kDa α-syn PuC: p=0.4868, t=0.0358; 100kDa α-syn PuR: p=0.0057, t=5.571; 100kDa α-syn PuM: p=0.2647, t=0.7092; 100kDa α-syn PuC: p=0.1188, t=1.472) (**D**) Representative images *(left)* and quantification *(right)* of TX-insoluble monomeric and high molecular weight pSyn immunoblotting using EP1536Y antibody in the rostral, medial and caudal caudate nucleus of control and GCI-injected baboon monkeys (14kDa α-syn CdR: p=0.2203, t=0.8865; 14kDa α-syn CdM: p=0.0456, t=2.457; 14kDa α-syn CdC: p=0.1114, t=1.533; 100kDa α-syn CdR: p=0.0007, t=11.35; 100kDa α-syn CdM: p=0.2397, t=0.8054; 100kDa α-syn CdC: p=0.2089, t=0.9373). The horizontal line indicates the average value per group ± standard deviation. Each dot represents one monkey of the control (black), GCI-injected baboon monkeys (green). Bootstrapped mean difference with 95% CI (error bar) is shown on the right side of each graph. Comparisons were made using unpaired t-test. *p<0.05 compared to control animals.

Fig. 7. Injection of GCI induces colocalization of total and S129-phosphorylated α-syn in oligodendrocytes and S129-phosphorylated α-syn exclusively in NHP neurons. (A) Representative confocal microscopy images (*left*) and quantifications (*right*) of oligodendrocytes and α-syn immunostaining, using CNPase and MJFR1 antibodies, respectively, in the putamen of control and GCI-injected NHPs. Scale bar = 20μm, inset scale bar = 10 μm. Quantifications measured puncta of α-syn staining in oligodendrocytes (*top*) p=0.007, t=3.426; and the average size of these puncta (*bottom*) p=0.003, t=3.690. **(B)** Representative confocal microscopy images (*left*) and quantifications (*right*) of neuronal and α-syn immunostaining, using NeuN and MJFR1 antibodies, respectively, in the putamen of control and GCI-injected NHPs. Scale bar = 20μm, inset scale bar = 10 μm. Quantifications measured puncta of α-syn staining in neurons (*top*) p=0.2270, t=0.7528; and the average size

of these puncta (*bottom*) p=0.2386, t=0.7146. (**C**) Representative confocal microscopy images (*left*) and quantification (*right*) of oligodendrocytes and phosphorylated α-syn immunostaining, using CNPase and EP1536Y antibodies, respectively, in the putamen of control and GCI-injected NHPs. Scale bar = 20μm, inset scale bar = 10 μm. Quantifications measured puncta of pSyn staining in oligodendrocytes (*top*) p=0.0043, t=2.788; and the average size of these puncta (*bottom*) p=0.4757, t=0.06138. (**D**) Representative confocal microscopy images (*left*) and quantification (*right*) of neuronal and phosphorylated α-syn immunostaining, using NeuN and EP1536Y antibodies, respectively, in the putamen of control and GCI-injected NHPs. Scale bar = 20μm, inset scale bar = 10 μm. Quantifications measured puncta of pSyn staining in neurons (*top*) p=0.2297, t=0.7435; and the average size of these puncta (*bottom*) p=0.4788, t=0.05344. The horizontal line indicates the average value per group ± standard deviation. Each dot represents one cell of the control (black) or GCI-injected NHPs (green). Bootstrapped mean difference with 95% CI (error bar) is shown on the right side of each graph. Comparisons were made using unpaired t-test. *$p<0.05$ compared to control animals.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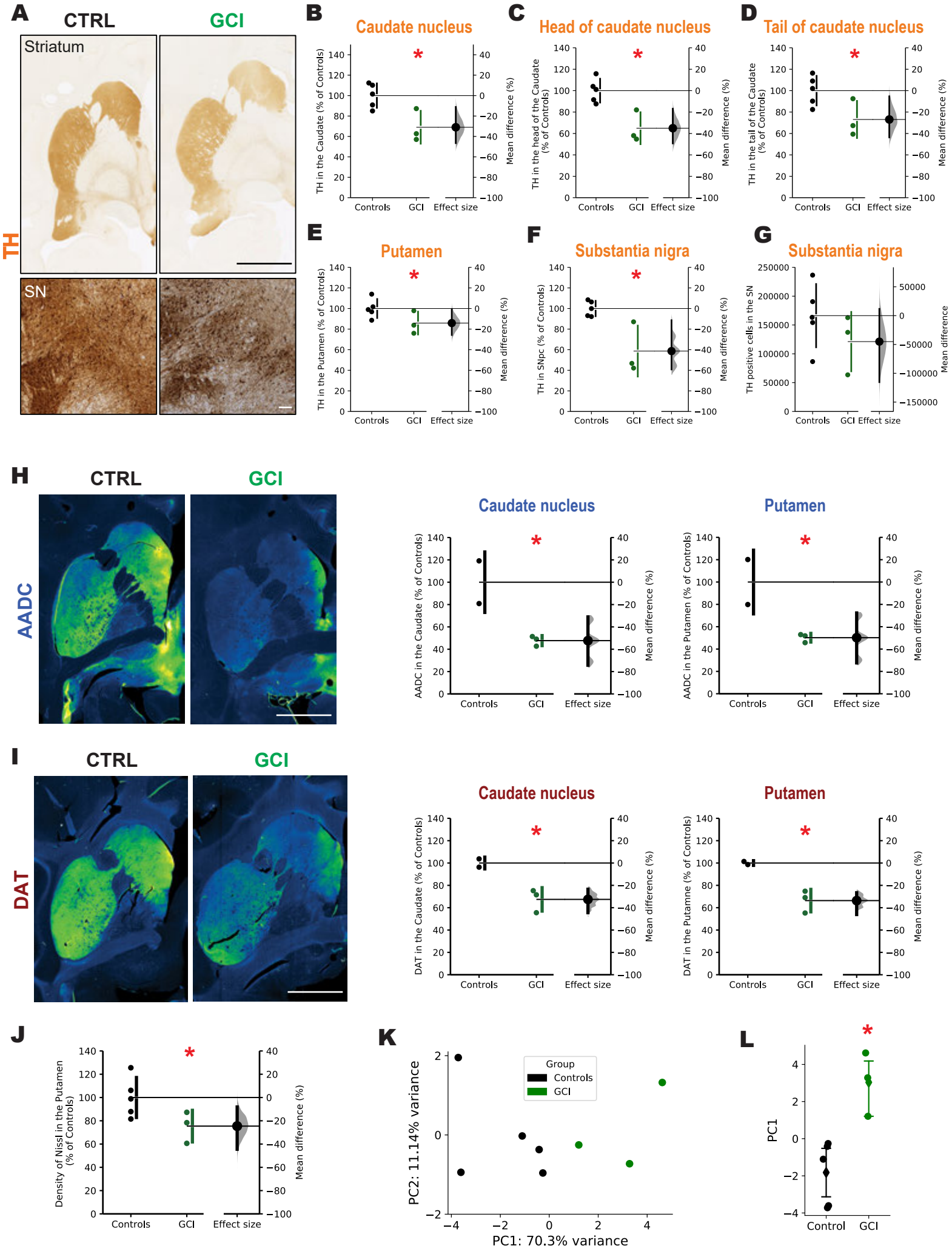
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TH in SNpc (% of Controls)
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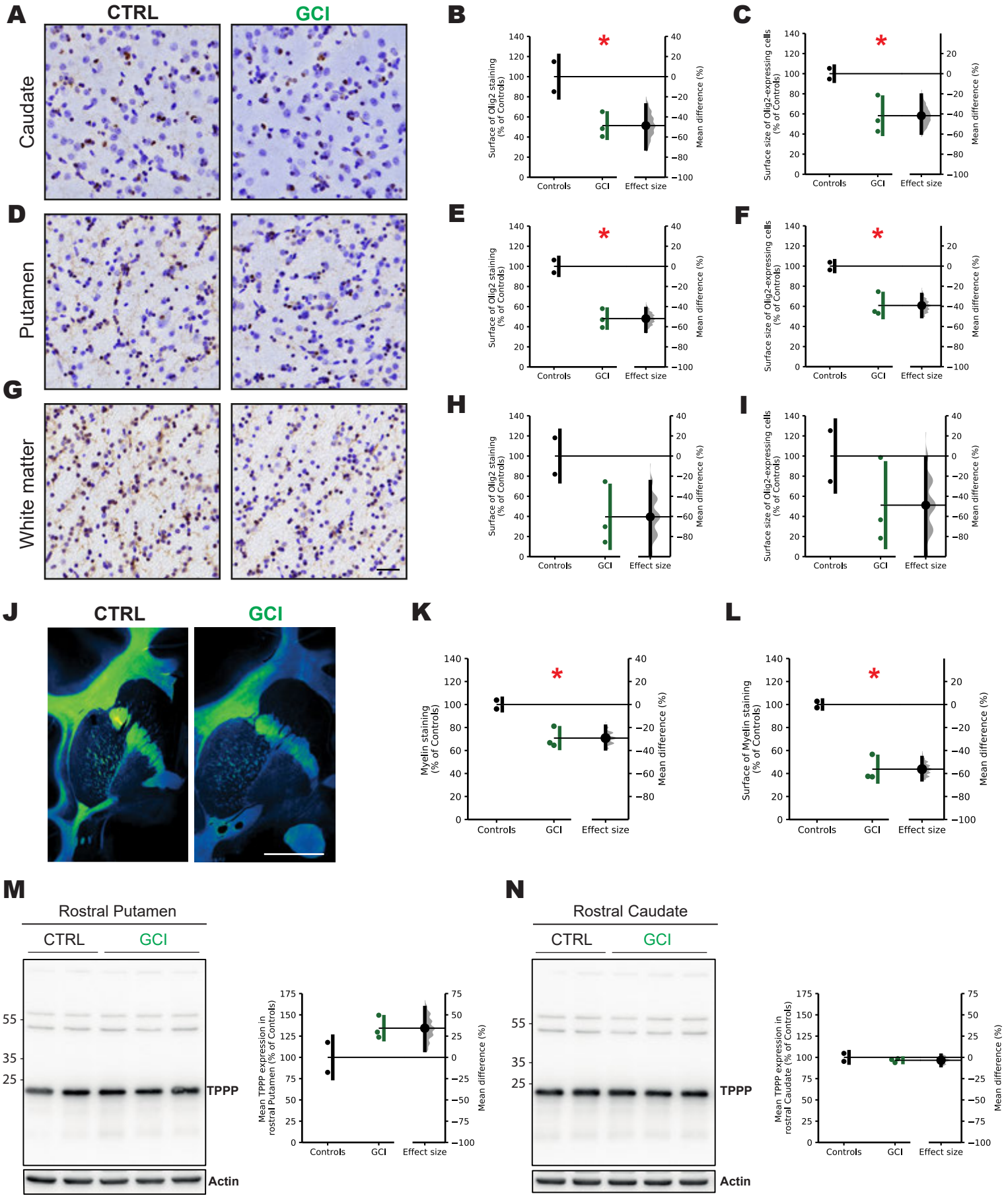
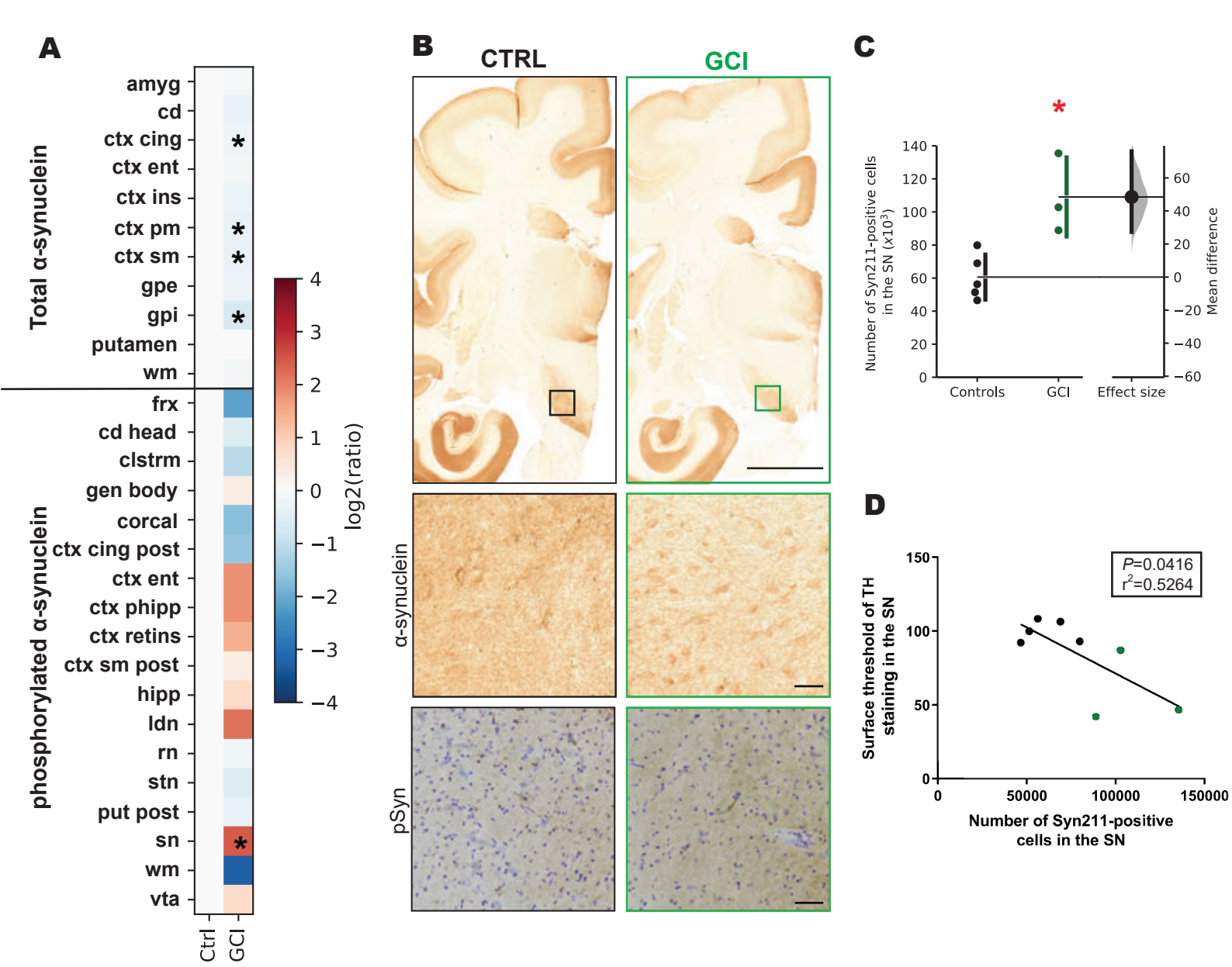
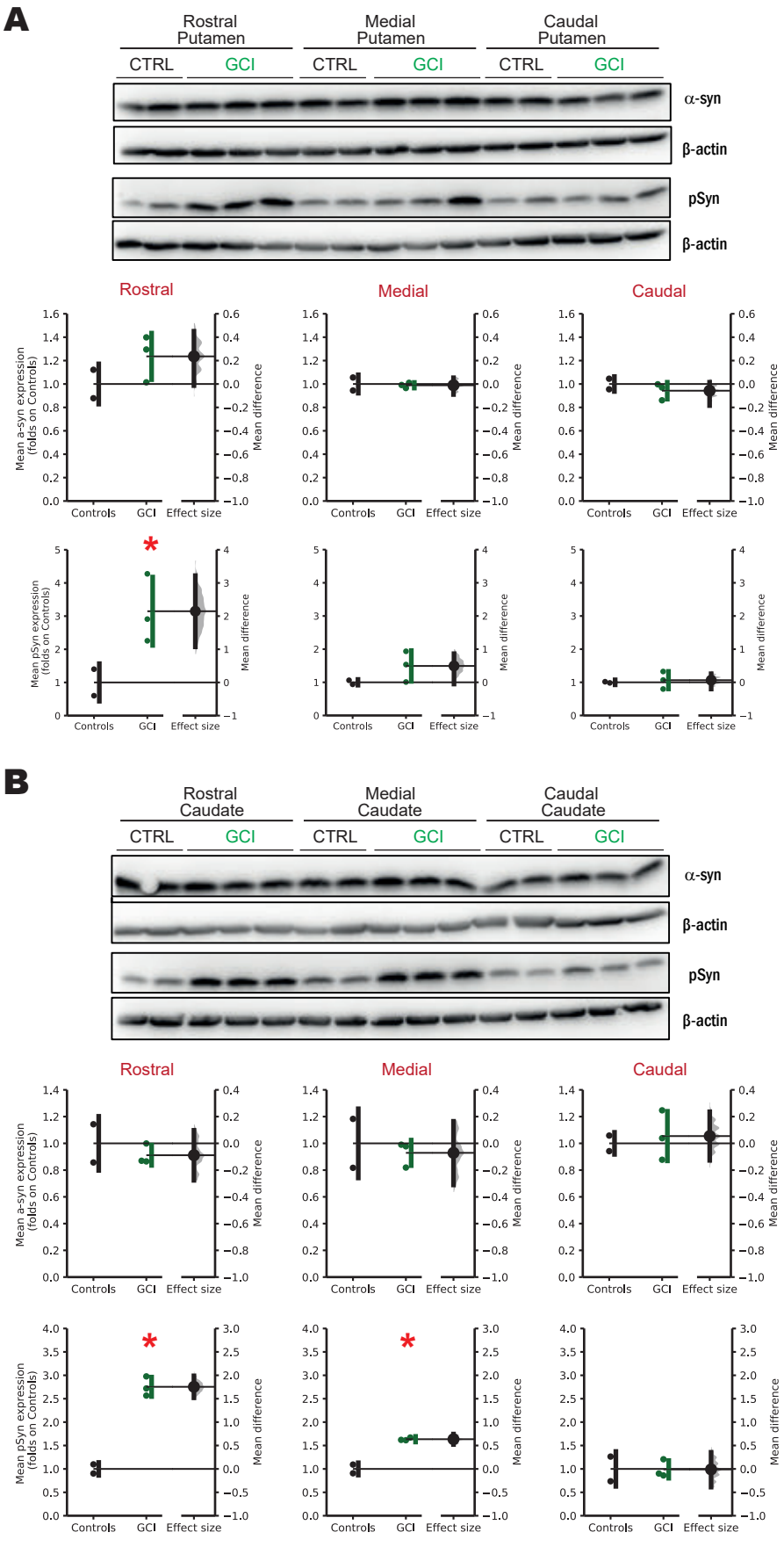
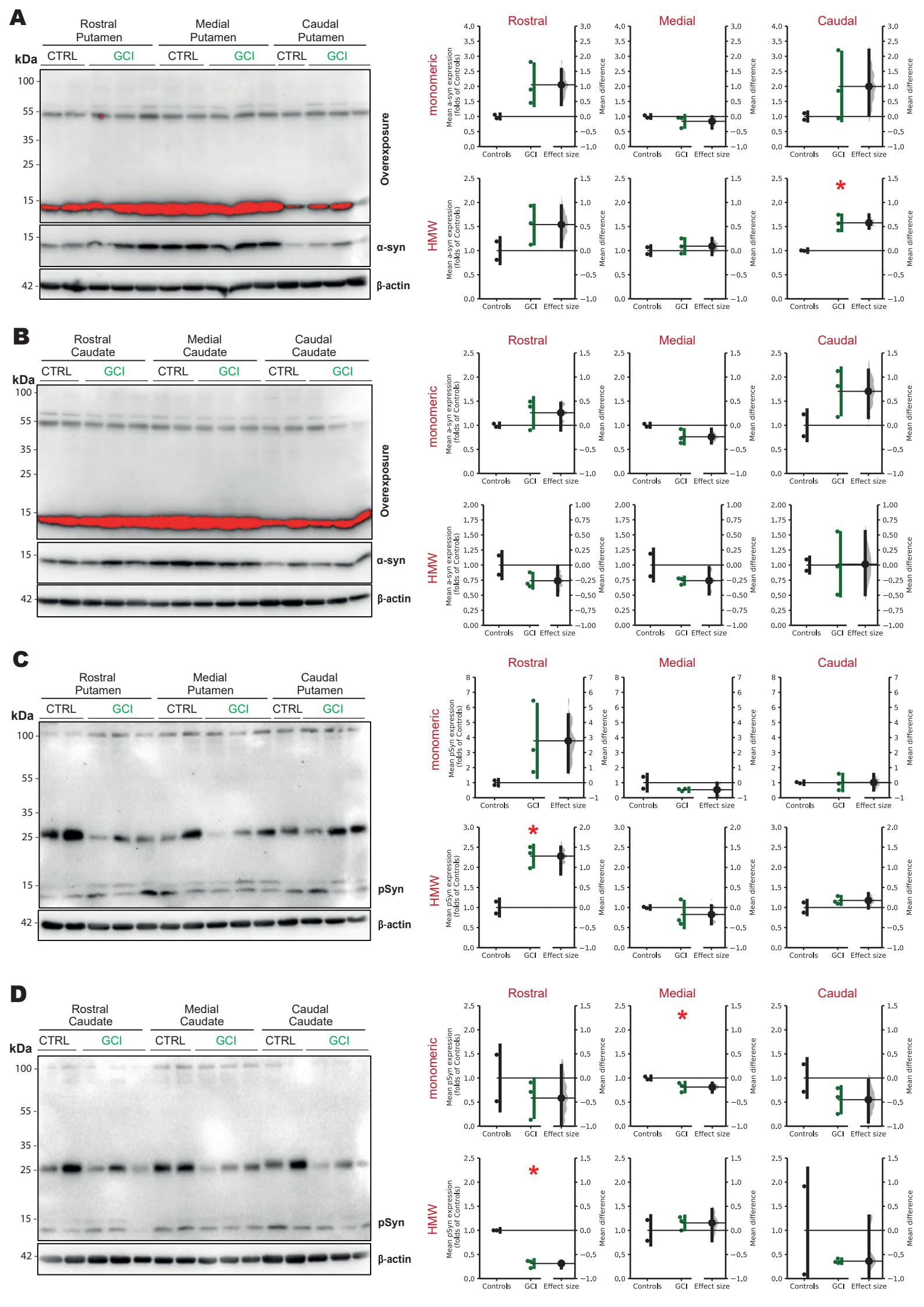
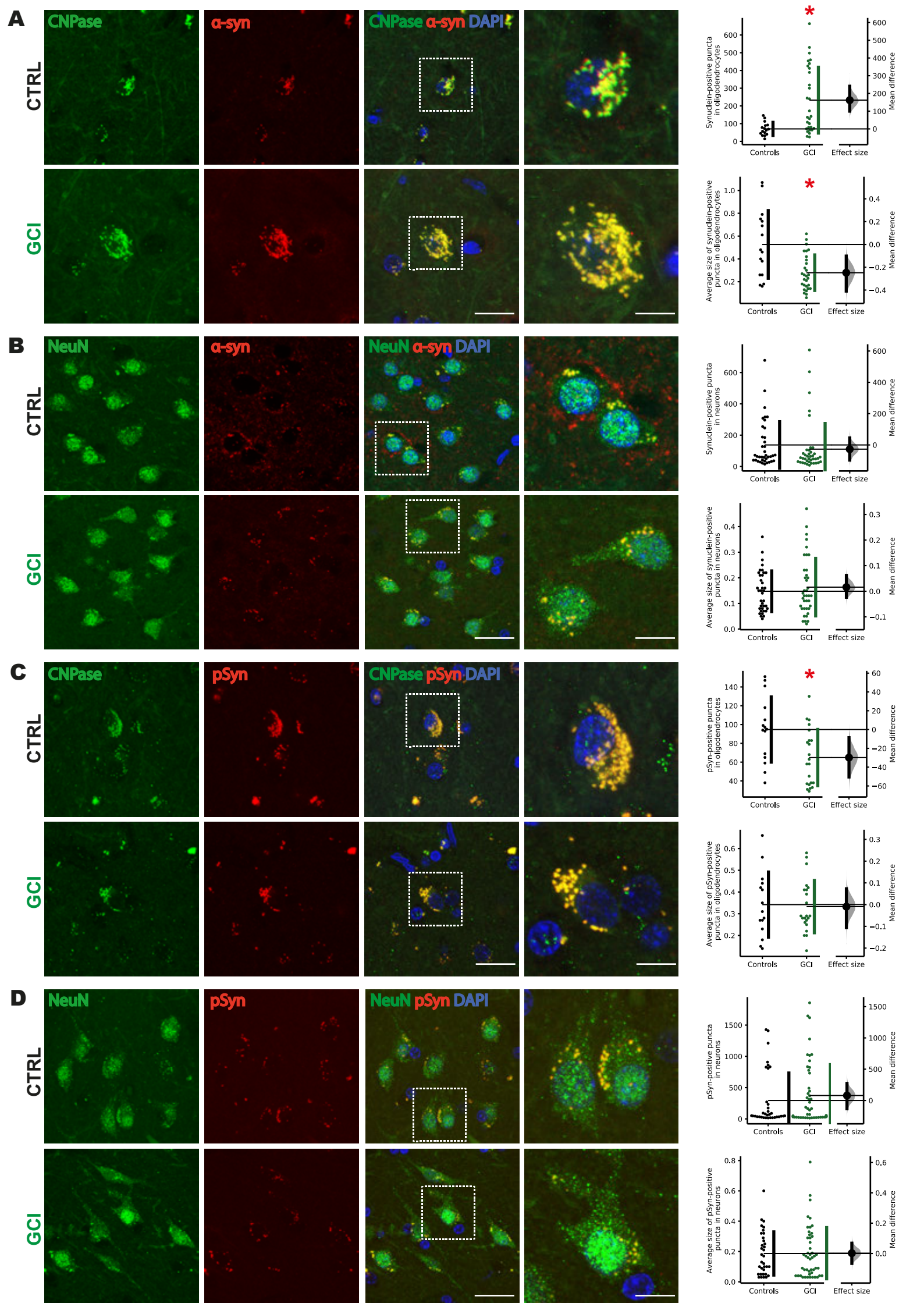
AADC
Caudate nucleus
AADC in the Caudate (% of Controls)
Putamen
AADC in the Putamen (% of Controls)
I
CTRL
GCI
DAT
Caudate nucleus
DAT in the Caudate (% of Controls)
Putamen
DAT in the Putamne (% of Controls)
J
Density of Nissl in the Putamen (% of Controls)
K
Group
Controls
GCI
PC2: 11.14% variance
PC1: 70.3% variance
L
PC1
Control
GCI


A
CTRL
GCI
Caudate
B
C
D
Putamen
E
F
G
White matter
H
I
Surface of Olig2 staining (% of Controls)
Surface size of Olig2-expressing cells (% of Controls)
Mean difference (%)
Controls
GCI
Effect size
J
CTRL
GCI
K
L
Myelin staining (% of Controls)
Surface of Myelin staining (% of Controls)
M
Rostral Putamen
CTRL
GCI
55
35
25
TPPP
Actin
Mean TPPP expression in rostral Putamen (% of Controls)
N
Rostral Caudate
Mean TPPP expression in rostral Caudate (% of Controls)

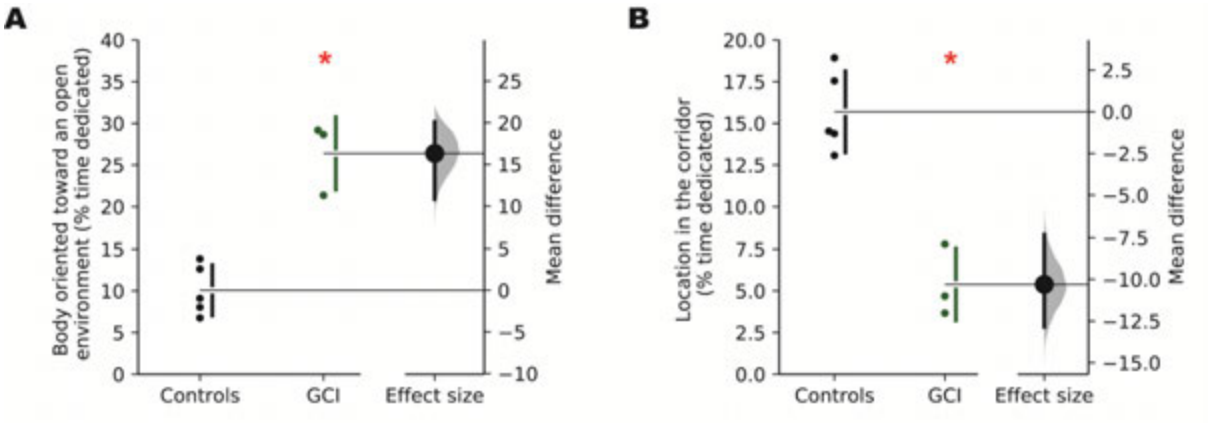

A
Total α-synuclein
amyg
cd
ctx cing
ctx ent
ctx ins
ctx pm
ctx sm
gpe
gpi
putamen
wm
phosphorylated α-synuclein
frx
cd head
clstrm
gen body
corcal
ctx cing post
ctx ent
ctx phipp
ctx retins
ctx sm post
hipp
ldn
rn
stn
put post
sn
wm
vta
Ctrl
GCI
log2(ratio)
4
3
2
1
0
−1
−2
−3
−4
B
CTRL
GCI
α-synuclein
pSyn
C
Number of Syn211-positive cells in the SN (x10³)
Mean difference
Controls
GCI
Effect size
D
Surface threshold of TH staining in the SN
Number of Syn211-positive cells in the SN
P=0.0416
r²=0.5264


A
Rostral Putamen
Medial Putamen
Caudal Putamen
CTRL
GCI
α-syn
β-actin
pSyn
β-actin
Rostral
Medial
Caudal
Mean a-syn expression (folds on Controls)
Mean difference
Controls
GCI
Effect size
Mean pSyn expression (folds on Controls)
*
B
Rostral Caudate
Medial Caudate
Caudal Caudate
CTRL
GCI
α-syn
β-actin
pSyn
β-actin
Rostral
Medial
Caudal
Mean a-syn expression (folds on Controls)
Mean difference
Controls
GCI
Effect size
Mean pSyn expression (folds on Controls)
*
*

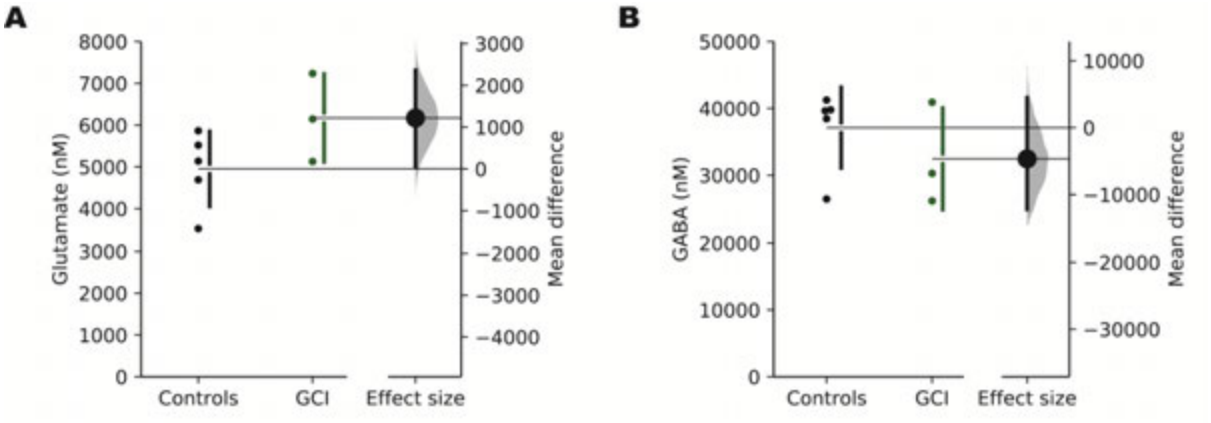

A
Rostral Putamen
Medial Putamen
Caudal Putamen
CTRL
GCI
kDa
100
55
35
25
15
15
42
Overexposure
α-syn
β-actin
Rostral
Medial
Caudal
monomeric
HMW
Mean a-syn expression (folds of Controls)
Mean difference
Controls
GCI
Effect size
*
B
Rostral Caudate
Medial Caudate
Caudal Caudate
C
Mean pSyn expression (folds of Controls)
pSyn
D


A
CNPase
α-syn
CNPase α-syn DAPI
CTRL
GCI
Synuclein-positive puncta in oligodendrocytes
Average size of synuclein-positive puncta in oligodendrocytes
B
NeuN
α-syn
NeuN α-syn DAPI
Synuclein-positive puncta in neurons
Average size of synuclein-positive puncta in neurons
C
CNPase
pSyn
CNPase pSyn DAPI
pSyn-positive puncta in oligodendrocytes
Average size of pSyn-positive puncta in oligodendrocytes
D
NeuN
pSyn
NeuN pSyn DAPI
pSyn-positive puncta in neurons
Average size of pSyn-positive puncta in neurons
Controls
GCI
Effect size
Mean difference
*

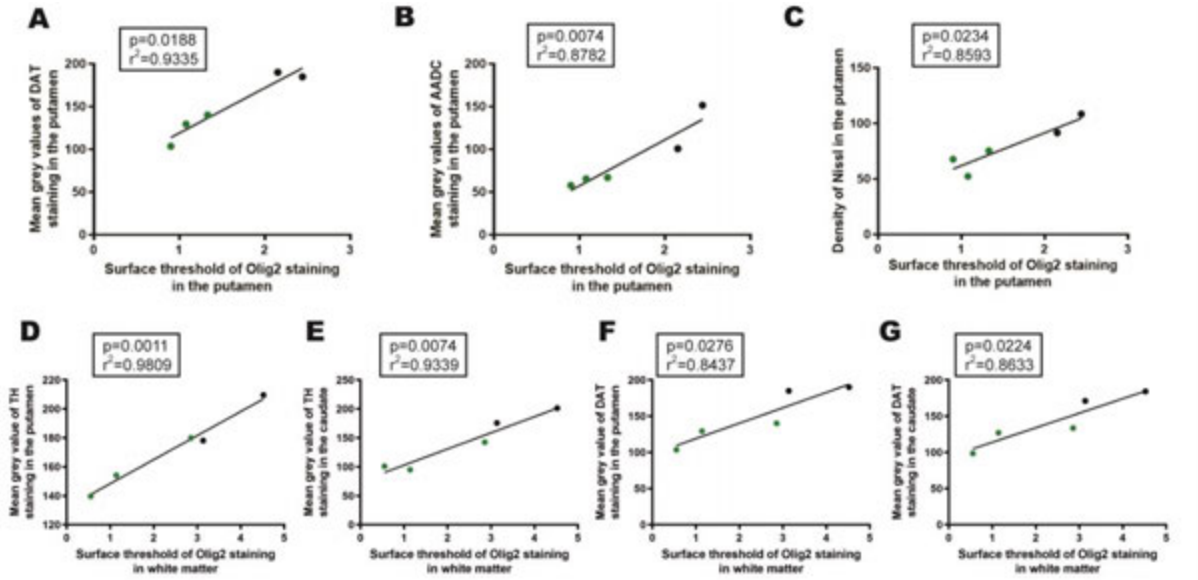
SUPPLEMENTARY FIGURES



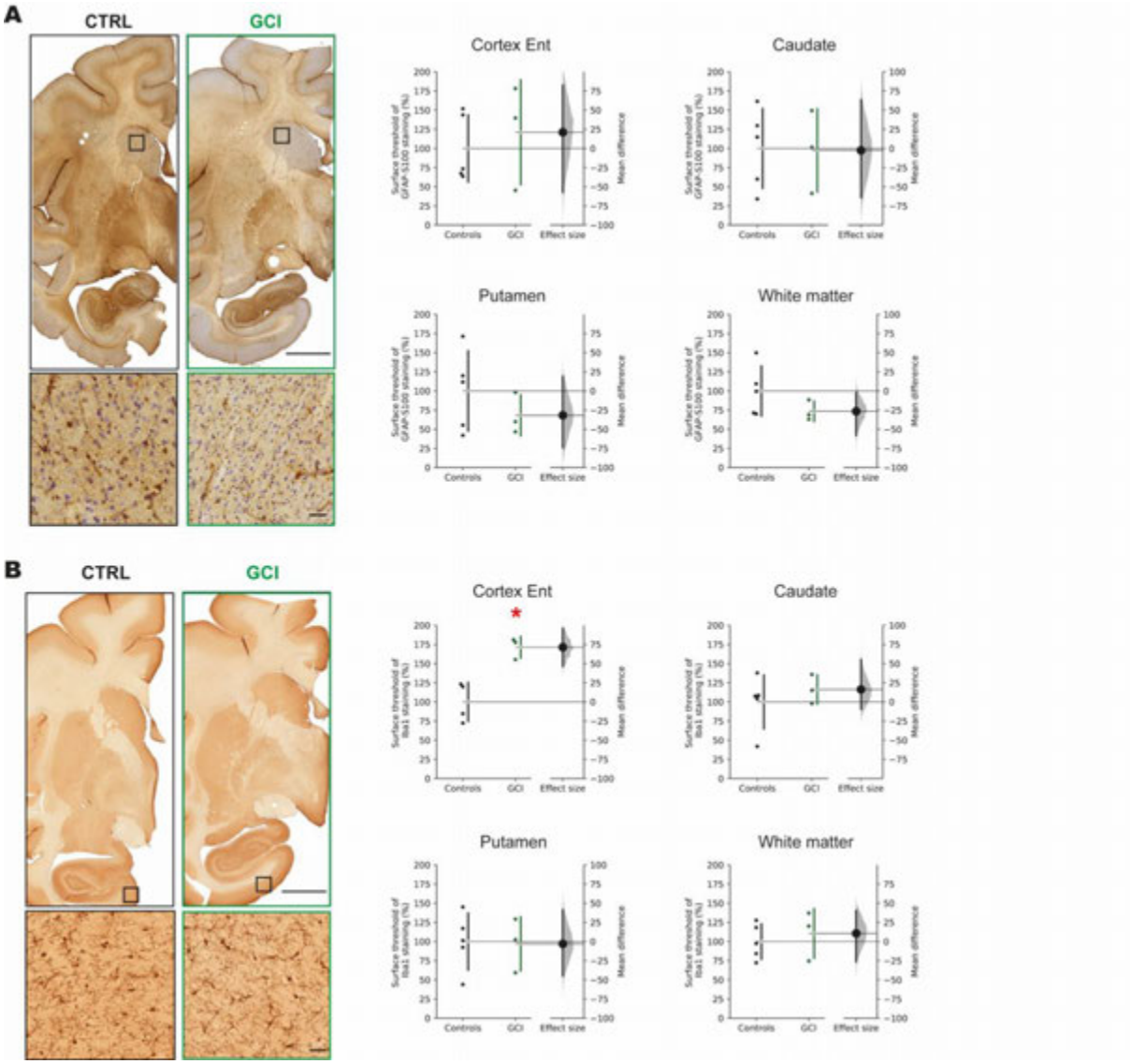
Supplementary Fig. 1. Intrastriatal injection of GCI-derived fractions in NHPs induces behavioral changes. (**A**) Scatter plot representing the time NHPS dedicated with their body oriented toward an open environment; p=0.0007, t=6.322. (**B**) Scatter plot depicting the time NHPs dedicated searching for the location in the corridor; p=0.0010, t=5.996. The horizontal line indicates the average value per group ± standard deviation. Each dot represents one NHP of the control (black), GCI-injected NHPs (green). Bootstrapped mean difference with 95% CI (error bar) is shown on the right side of each graph. Comparisons were made using an unpaired t-test. *p<0.05 compared to control animals.



Supplementary Fig. 2. Glutamate concentrations are modulated in GCI-injected NHPs. Nanomolar concentrations of Glutamate; p=0.0654, t=1.749 (**A**) and GABA; p=0.1856, t=0.9662 (**B**) in Control or GCI-injected NHPs. The horizontal line indicates the average value per group ± standard deviation. Each dot represents one monkey of the control (black), GCI-injected NHPs (green). Bootstrapped mean difference with 95% CI (error bar) is shown on the right side of each graph. Comparisons were made using an unpaired t-test. *p<0.05 compared to control animals.

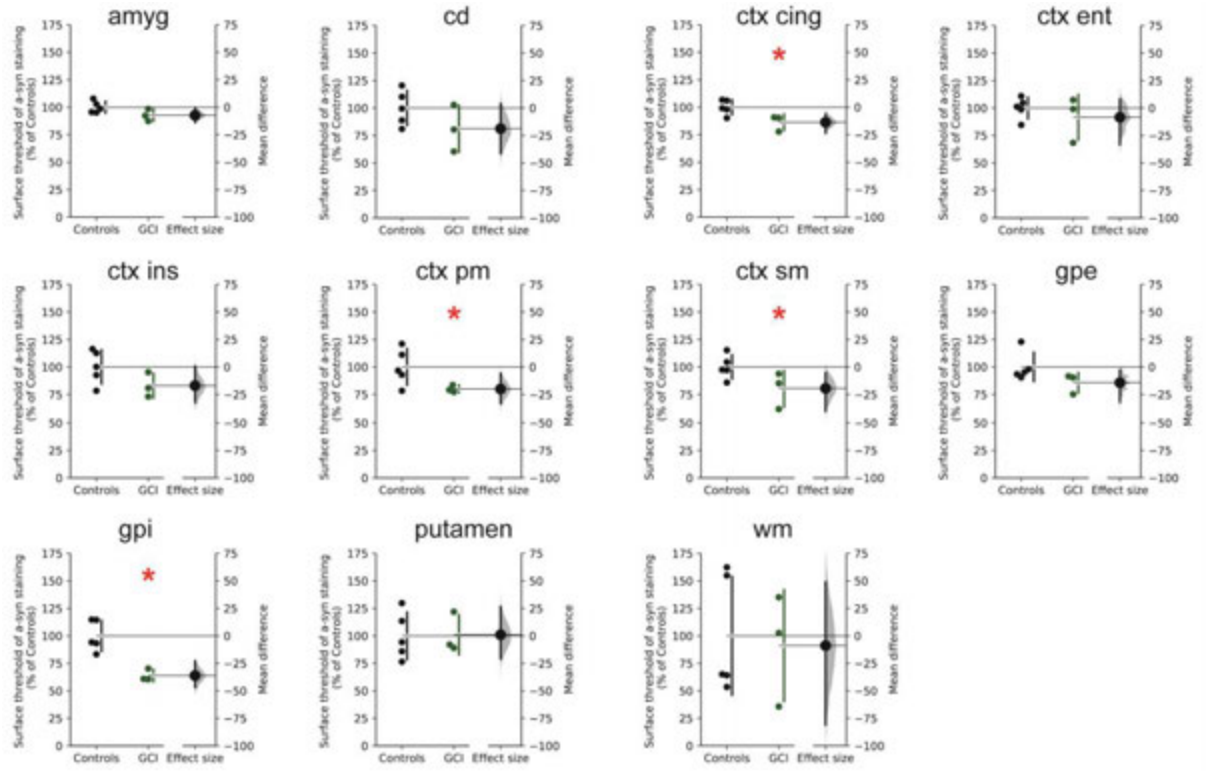


Supplementary Fig. 3. Intrastriatal injection of GCI-derived fractions in NHPs induces changes of Olig2 levels. (**A-C**) Linear regression between mean grey value of Olig2 immunostaining and DAT staining; F=42.10, p=0.0074, r^2=0.9335 (**A**), AADC staining; F=21.62, p=0.0188, r^2=0.8782 (**B**) and Nissl count; F=18.32, p=0.0234, r^2=0.8593 (**C**) in the putamen for control and GCI-injected NHPs. (**D-E**) Linear regression between mean grey value of Olig2 immunostaining in white matter and TH immunostaining in the putamen; F=154, p=0.0011, r^2=0.9809 (**D**) and in the caudate nucleus; F=42.31, p=0.0074, r^2=0.9338 (**E**) for control and GCI-injected NHPs. (**F-G**) Linear regression between mean grey value of Olig2 immunostaining in white matter and DAT immunostaining in the putamen; F=16.19, p=0.0276, r^2=0.8437 (**F**) and in the caudate nucleus; F=18.94, p=0.0224, r^2=0.8633 (**G**) for control and GCI-injected NHPs. Each dot represents one monkey of the control (black) and GCI-injected NHPs (green).

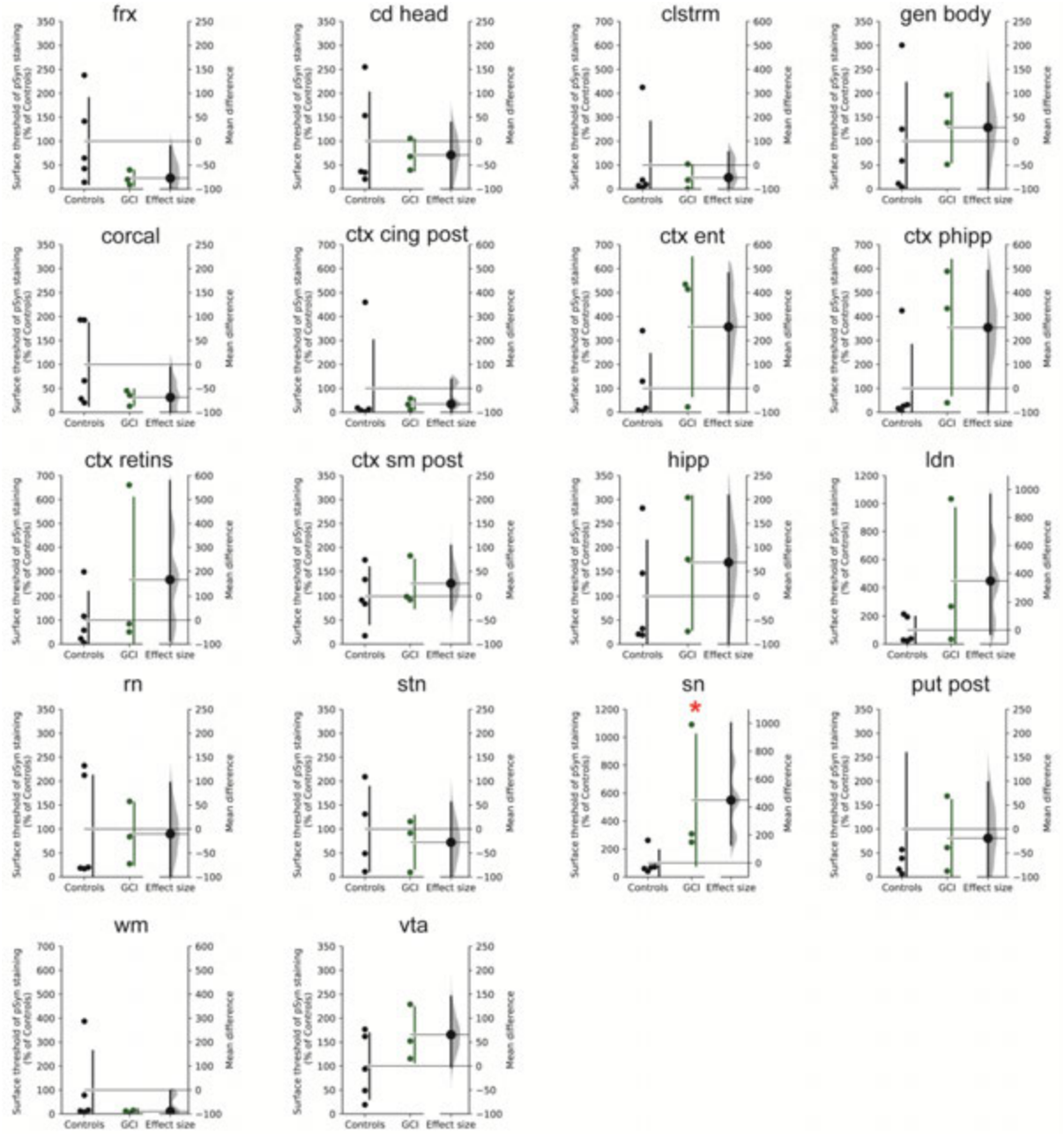


Supplementary Fig. 4. Intrastriatal injection of GCI-derived fractions in NHPs induces a microglial response. (**A**) Representative coronal brain sections of *(top)* and illustrative photomicrographs *(bottom)* of GFAP-S100 immunostaining in the caudate nucleus of control and GCI-injected NHPs. Scale bars = 5mm *(top)* and 50µm *(bottom inset)*. *(left)* Scatter plot of surface threshold GFAP-S100 immunostaining in the entorhinal cortex, caudate nucleus, putamen and white matter of control, and GCI-injected NHPs (Entorhinal ctx: p=0.3035; Caudate: p=0.4746; Putamen: p=0.1877; WM: p=0.1192). (**B**) Representative coronal brain sections *(top)* and illustrative photomicrographs *(bottom)* of Iba1 immunostaining in the entorhinal cortex of control and GCI-injected NHPs. Scale bars = 5mm *(top)* and 50µm *(bottom inset)*. *(left)* Scatter plot of surface threshold Iba1 immunostaining in the entorhinal cortex, caudate nucleus, putamen and white matter of control, and GCI-injected NHPs (Entorhinal ctx: p=0.0038; Caudate: p=0.2480; Putamen: p=0.4574; WM: p=0.3011). The horizontal line indicates the average value per group ± standard deviation. Each dot

represents one NHP of the control (black), GCI-injected NHPs (green). Bootstrapped mean difference with 95% CI (error bar) is shown on the right side of each graph. Comparisons were made using an unpaired t-test. *$p<0.05$ compared to control animals.

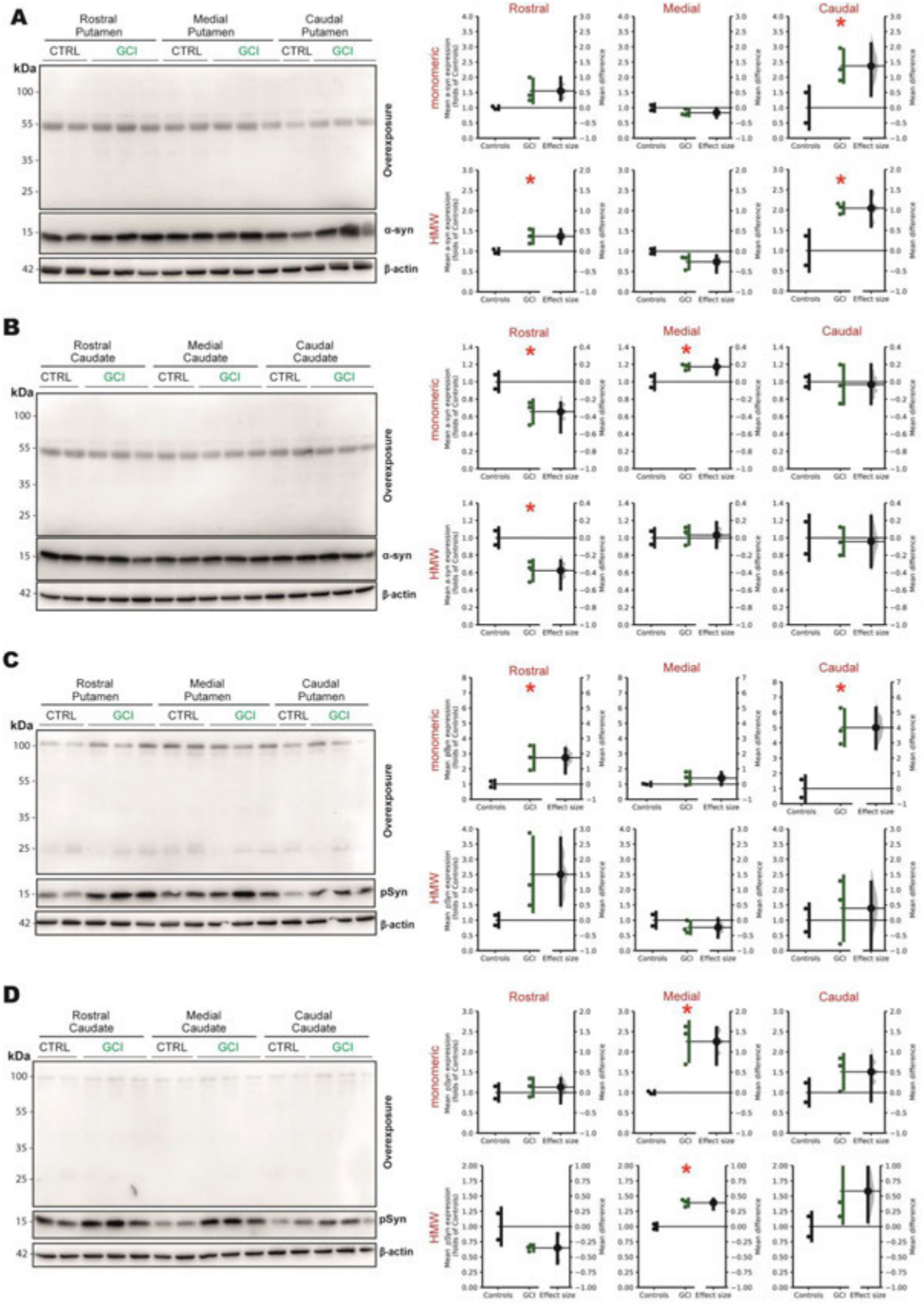


Supplementary Fig. 5. Intrastriatal injection of GCI-derived fractions induces a specific pattern of S129 phosphorylated α-syn levels in the brain. Scatter plots representing the surface threshold of α-syn (Syn211) immunostaining intensity in the brain of control and GCI-injected NHPs, represented in percentage compared to controls. Amygdala (amyg) p=0.0556, t=1.866; caudate nucleus (cd) p=0.1003, t=1.438; cingulate cortex (ctx cing) p=0.0178, t=2.70; entorhinal cortex (ctx ent) p=0.2252, t=0.807; insular cortex (ctx ins) p=0.0758, t=1.642; premotor cortex (ctx pm) p=0.0455, t=2.012; sensorimotor cortex (ctx sm) p=0.0412, t=2.082; external globus pallidus (gpe) p=0.0757, t=1.643; internal globus pallidus (gpi) p=0.003, t=4.161; putamen p=0.4744, t=-.06707; white matter (wm) p=0.4128, t=0.2302. The horizontal line indicates the average value per group ± standard deviation. Each dot represents one NHP of the control (black), GCI-injected NHPs (green). Bootstrapped mean difference with 95% CI (error bar) is shown on the right side of each graph. Comparisons were made using an unpaired t-test. *p<0.05 compared to control animals.



Supplementary Fig. 6. Intrastriatal injection of GCI-derived fractions induces a specific pattern of S129 phosphorylated α-syn levels in the brain. Scatter plots representing the surface threshold of S129 phosphorylated α-syn (Elan) immunostaining intensity in the brain of control and GCI-injected NHPs, represented in percentage compared to controls. Fornix (frx) p=0.1021, t=1.424; head of the caudate nucleus (cd head) p=0.3287, t=0.4663; claustrum (clstrm) p=0.3261, t=0.4742; geniculate body (gen body) p=0.3649, t=0.3619; corpus callosum (corcal) p=0.1174, t=1.320; posterior cingulate cortex (ctx cing post) p=0.3022, t=0.5465; entorhinal cortex (ctx ent) p=0.0682, t=1.719; parahippocampal cortex (ctx phipp) p=0.0830, t=1.576; retroinsular cortex (ctx retins) p=0.1721, t=1.027; posterior sensorimotor cortex (ctx sm post) p=0.2832, t=0.6066; hippocampus (hipp) p=0.2376,

t=0.7614; lateral dorsal nucleus (ldn) p=0.0896, t=1.520; red nucleus (rn) p=0.4454, t=0.1432; subthalamic nucleus (stn) p=0.3282, t=0.4726; posterior putamen (put post) p=0.4270, t=0.1922; substantia nigra (sn) p=0.0357, t=2.186; white matter (wm) p=0.1959, t=0.9224; ventral tegmental area (vta) p=0.1094, t=1.373. The horizontal line indicates the average value per group ± standard deviation. Each dot represents one NHP of the control (black), GCI-injected NHPs (green). Bootstrapped mean difference with 95% CI (error bar) is shown on the right side of each graph. Comparisons were made using an unpaired t-test. *$p<0.05$ compared to control animals.



Supplementary Fig. 7. Subcellular fractionation of putamen and caudate nucleus reveals an accumulation of triton soluble forms of total and S129-phosphorylated α-syn

after GCI injections. (**A**) Representative images *(left)* and quantification *(right)* of TX-soluble monomeric and high molecular weight α-syn immunoblotting using Syn211 antibody in the rostral, medial and caudal putamen of control and GCI-injected NHPs (14kDa α-syn PuR: p=0.0799, t=1.86; 14kDa α-syn PuM: p=0.1022, t=1.616; 14kDa α-syn PuC: p=0.0441, t=2.495; 55kDa α-syn PuR: p=0.0370, t=2.695; 55kDa α-syn PuM: p=0.0790, t=1.871; 55kDa α-syn PuC: p=0.018, t=3.633). (**B**) Representative images *(left)* and quantification *(right)* of TX-soluble monomeric and high molecular weight α-syn immunoblotting using Syn211 antibody in the rostral, medial and caudal caudate nucleus of control and GCI-injected NHPs (14kDa α-syn CdR: p=0.0310, t=2.908; 14kDa α-syn CdM: p=0.0261, t=3.126; 14kDa α-syn CdC: p=0.4323, t=0.1856; 55kDa α-syn CdR: p=0.020, t=3.483; 55kDa α-syn CdM: p=0.3806, t=0.3327; 55kDa α-syn CdC: p=0.4152, t=0.2335). (**C**) Representative images *(left)* and quantification *(right)* of TX-insoluble monomeric and high molecular weight pSyn immunoblotting using EP1536Y antibody in the rostral, medial and caudal putamen of control and GCI-injected NHPs (14kDa pSyn PuR: p=0.0339, t=2.799; 14kDa pSyn PuM: p=0.1524, t=1.235; 14kDa pSyn PuC: p=0.0658, t=4.035; 100kDa pSyn PuR: p=0.1006, t=1.632; 100kDa pSyn PuM: p=0.1688, t=1.139; 100kDa pSyn PuC: p=0.4967, t=0.4676) (**D**) Representative images *(left)* and quantification *(right)* of TX-insoluble monomeric and high molecular weight pSyn immunoblotting using EP1536Y antibody in the rostral, medial and caudal caudate nucleus of control and GCI-injected NHPs (14kDa pSyn CdR: p=0.2814, t=0.6488; 14kDa pSyn CdM: p=0.0213, t=3.396; 14kDa pSyn CdC: p=0.1292, t=1.391; 100kDa pSyn CdR: p=0.0624, t=2.114; 100kDa pSyn CdM: p=0.0027, t=7.279; 100kDa pSyn CdC: p=0.1279, t=1.401). The horizontal line indicates the average value per group ± standard deviation. Each dot represents one NHP of the control (black), GCI-injected NHPs (green). Bootstrapped mean difference with 95% CI (error bar) is shown on the right side of each graph. Comparisons were made using an unpaired t-test. *p<0.05 compared to control animals.

Supplementary Table 1. Raw data that served for the non-human primate study analyses for all histological and biochemical approaches.

CHAPTER 2: THERAPEUTIC APPROACHES TO INHIBIT THE PROGRESSION OF PARKINSON'S DISEASE PATHOLOGY

Context

The three subprojects of the Axis II that will be discussed hereafter encompass multiple therapeutic approaches to inhibit neurodegeneration and decrease α-syn accumulation in a PD mouse model. Given the variety of models used in the field, I chose to focus the study of multiple therapies on one same model to assess their effects.

The LB model is based on the unilateral injection of PD-derived brain fractions in the SN of wild-type mice. Sporadic PD patient brains presenting LB pathology are processed and purified using a sucrose gradient. The fractions used were previously determined by the presence of α-syn aggregates using filter retardation assay. These fractions are then injected using stereotaxic surgeries in the SN. After four months of inoculation, these LB mice demonstrate a loss of around 30% of dopaminergic neurons in the SN. With this modest neurodegeneration, we do not consistently observe the significant loss of dopaminergic fiber in the striatum, or behavioral deficits. When assessing the effect of these injections on α-syn pathology, we can observe the presence of proteinase K-resistant α-syn species with the rare presence of phosphorylated α-syn species (Bengoa-Vergniory *et al.*, 2020; Bourdenx *et al.*, 2020; Soria *et al.*, 2020) (**Figure 26**).

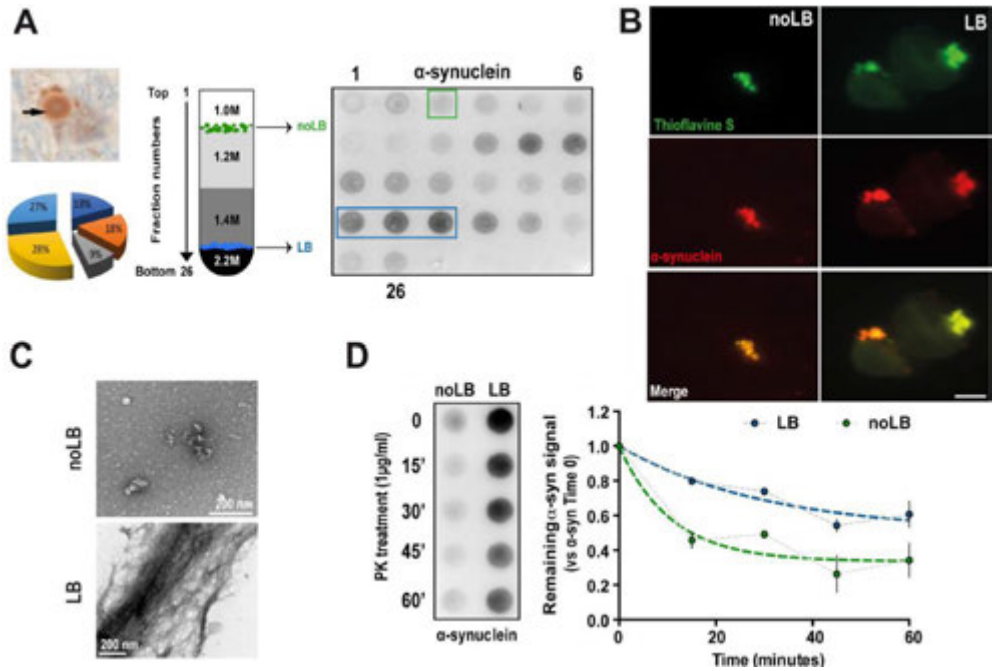


Figure 26: Purification and characterization of Lewy Bodies (LB) and noLB fractions from Parkinson's Disease patient brains. (A) Purification of LB from PD-patient brains using a sucrose gradient, and confirmation of the presence of α-synuclein using filter retardation assay. (B) Co-localization of α-synuclein and Thioflavin S stainings in noLB and LB fractions. (C) Ultrastructural analysis of fractions using electron microscopy. (D) Resistance of α-syn contained in fractions to proteinase K (PK) treatment. *Adapted from Bourdenx et al. 2020, Science Advances*.

In order to assess the effects of three different molecules, the injections of LB were done either concomitantly to the treatment delivery or with an additional delayed start design for one of the strategies. These molecules were either administrated in the diet of mice for two molecules or through viral vector-mediated administration (**Figure 27**).

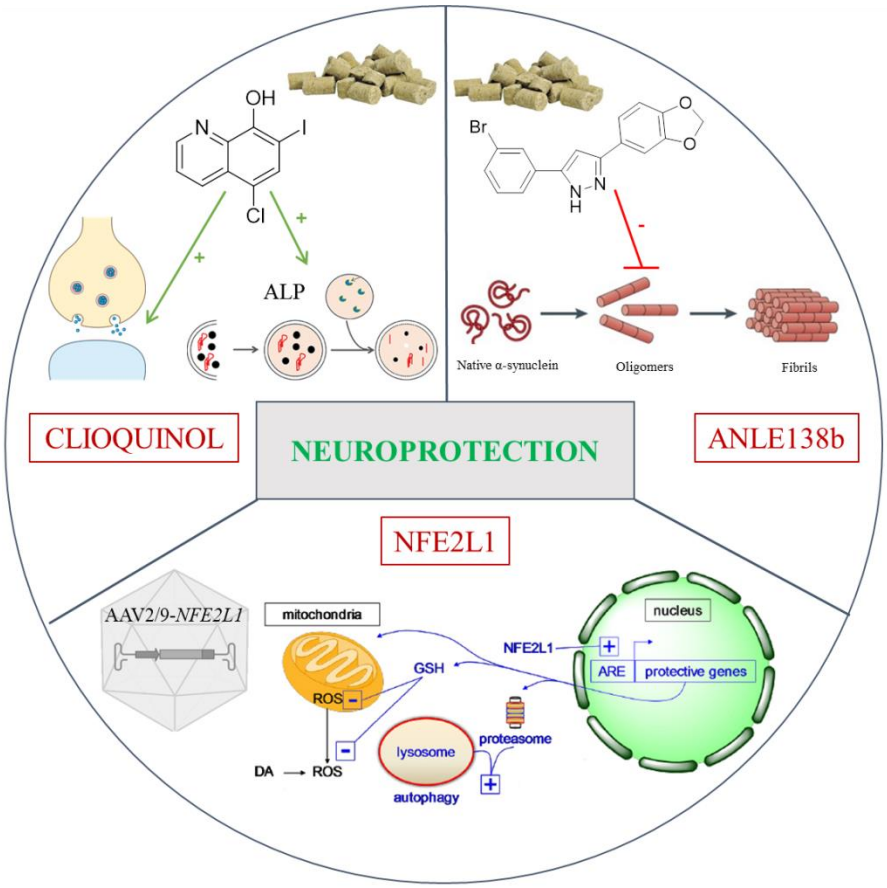


Figure 27: Therapeutic approaches used to induce neuroprotection in PD. (1) Clioquinol was added to the diet of mice and could induce neuroprotection via the activation of the autophagy-lysosomal pathway (ALP) or by activating synaptic transmission. (2) NFE2L1 was delivered via adeno-associated virus (AAV). This transcription factor acts by inducing the transcription of protective genes, which in turn activate the proteasome, increasing clearance, and activate glutathione (GSH), inhibiting the formation of reactive oxygen species (ROS). (3) Anle138b, also added to the diet of mice, acts by inhibiting the formation of oligomeric species, the potential toxic species in PD.

Altogether, this chapter 2 aimed to challenge the LB mouse model to determine the efficacy of these molecules to slow the progress of LB-induced neuropathology in this progressive model of PD. These therapeutic strategies are different as they target different aspects of PD pathology. First, Clioquinol (ClQ) is a zinc ionophore that has been shown to target synaptic transmission and the ALP. Second, NFE2L1, a transcription factor, increases the transcription of protective genes able to reduce oxidative stress and increase protein clearance. Finally,

Anle138b is a small molecule that inhibits protein aggregation, and in particular, the formation of oligomeric species of α-syn.

Methods

Mice Stereotaxic injections and diet

Wild-type C57BL/6J mice (3 months old) received 2µl of either LB fractions, noLB fractions, or sucrose by stereotactic delivery to the region immediately above the right SN (coordinates from Bregma: AP=-2.9, L= +1.3, DV=-4.5) at a flow rate of 0.4µl/min and the pipette was left in place for 5 min after injection to avoid leakage as previously described. Mice were perfused four months after injection using 0.9% NaCl followed by 4% paraformaldehyde. Brains were then post-fixed for 24 hours in 4% paraformaldehyde at 4°C, cryoprotected in gradient 20% sucrose in PBS before being frozen by immersion in a cold isopentane bath (-60°C), and stored immediately at -80°C until sectioning for histochemical analysis.

Purification of Lewy bodies from human PD Brains

The samples were obtained from brains collected in a Brain Donation Program of the Brain Bank "GIE NeuroCEB" run by a consortium of Patients Associations: ARSEP (association for research on multiple sclerosis), CSC (cerebellar ataxias), France Alzheimer and France Parkinson. The consents were signed by the patients themselves or their next of kin in their name, in accordance with the French Bioethical Laws. The Brain Bank GIE NeuroCEB (Bioresource Research Impact Factor number BB-0033-00011) has been declared at the Ministry of Higher Education and Research and has received approval to distribute samples (agreement AC-2013-1887). Human SNpc was dissected from fresh frozen postmortem midbrain samples from 5 patients with sporadic PD exhibiting conspicuous nigral LB pathology on neuropathological examination (mean age at death: 75 ± 2.75 years; frozen post-mortem interval: 31.8 ± 7.45h; GIE Neuro-CEB BB-0033-00011). Tissue was homogenized in 9 vol (w/v) ice-cold MSE buffer (10 mM MOPS/KOH, pH 7.4, 1M sucrose, 1mM EGTA, and 1mMEDTA) with protease inhibitor cocktail (Complete Mini; Boehringer Mannheim) with 12 strokes of a motor-driven glass/teflon homogenizer. For LB purification, a sucrose step gradient was prepared by overlaying 2.2 M with 1.4 M and finally with 1.2 M sucrose in volume ratios of 3.5:8:8 (v/v). The homogenate was layered on the gradient and

centrifuged at 160,000 x g for 3 h using a SW32.1 rotor (Beckman). Twenty-six fractions of 1500 µl were collected from each gradient from top (fraction 1) to bottom (fraction 26) and analyzed for the presence of α-synuclein aggregates by filter retardation assay, as previously described (Recasens *et al.*, 2014). Further characterization of LB fractions was performed by immunofluorescence, α-synuclein ELISA quantification and electron microscopy as previously described (Recasens *et al.*, 2014). For stereotactic inoculations, LB-containing fractions from PD patients were mixed together in the same proportion (PD#1, fractions 19 and 20; PD#2, fractions 19 and 20; PD#3, fraction 22; PD#4, fractions 17,18 and 19; PD#5, fractions 20, 21 and 23). NoLB-containing fractions (i.e. fraction 3, at the beginning of the 1,2M interface) derived from the same PD patients (which contain soluble or finely granular α-synuclein) but lacks large LB-linked α-synuclein aggregates were obtained from the same sucrose gradient purification. LB fractions were adjusted to ~24 pg α-synuclein per microliter of injected samples, as measured by a specific enzyme-linked immunosorbent assay (ELISA) kit against human α-synuclein (Invitrogen, #KHB0061) according to the manufacturer's instructions. In all cases, samples were bath-sonicated for 5 min prior to *in vitro* and *in vivo* inoculations.

Histological Analysis

Neurodegeneration. To assess the effect of LB injections on dopaminergic neurons and fibers, tyrosine hydroxylase (TH) immunohistochemistry was performed on striatal and SN sections as previously described. Briefly, sections were selected in the posterior striatum. Serial sections (1 to 6 slices) corresponding to the whole SNpc were incubated with rabbit monoclonal TH antibody (Abcam, EP1532Y, 1:5000) for one night at room temperature (RT) and revealed the next day by an anti-mouse peroxidase EnVision secondary antibody, followed by 3,3′-diaminobenzidine (DAB) visualization. Free-floating SNpc sections were mounted on gelatin-coated slides, counterstained with 0.1% cresyl violet solution, dehydrated and cover-slipped. Striatal sections were mounted on gelatin-coated slides, dehydrated and cover-slipped. Striatal sections were analyzed by optical density (OD). The slides were scanned using Epson expression 10000XL high-resolution scanner, and images were analyzed using ImageJ open-source software (version 1.53) to compare mean grey levels in the caudate nucleus and putamen. TH-positive neurons of the SNpc were counted by stereology blind about the experimental condition using a Leica DM6000B microscope coupled with the Mercator software (Explora Nova, France). The SNpc was delineated for each slide, and probes for stereological counting were applied to the map obtained. Each TH-

positive cell with the neuron included in the probe was counted. The total number of TH-positive neurons in the SNpc per hemisphere was then assessed using the optical fractionator method.

α-synuclein pathology. Synucleinopathy has been assessed with a mouse monoclonal antibody raised against murine α-syn (BD Biosciences, Syn1, 1:1,000) immunostaining, as we previously reported. Briefly, selected sections of 1 rostro-caudal level of SN and striatum were specifically identified and incubated in the same well to allow direct comparison of immunostaining intensity. For pretreatment with proteinase K (PK), sections were incubated first with PK at 10 μg/ml in PBS before long sequential washes in distilled water and then in PBS. Sections were incubated overnight at room temperature with the aforementioned antibodies. The following day, revelation was performed with anti-species peroxidase EnVision system (DAKO) followed by DAB incubation. Sections were then mounted on gelatinized slides, dehydrated, counter-stained if necessary, and cover-slipped until further analysis. Syn1-positive dots in SN were counted by stereology, blind with regard to the experimental condition using a Leica DM6000B motorized microscope coupled with the Mercator software (Explora Nova). The SN was delineated for each slide, and probes for stereological counting were applied to the map obtained (size of probes was 60 × 80 μm) and transformed as object after counting. The optical fractionator method was finally used to estimate the total number of Syn1-positive dots per μm^2 in the SN of each mouse. Syn1 immunostaining–positive surface in the striatum and phosphorylated α-syn immunostaining–positive surface quantification in the SN and the striatum were performed as previously.

Biochemical Analysis

Total protein extraction. Tissue patches collected on 300μm-thick cryostat-cut sections of SN and striatum were extracted on ice using 100μl of RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1.0% Triton X-100, 0.5% Na-deoxycholate, 0.1% sodium dodecyl sulfate) with a protease and phosphatase inhibitor cocktail as previously described. Lysates were incubated for 20 minutes and then centrifugated at 14,000 rpm for 15 minutes at 4˚C. Supernatants were collected, and the total amount of protein in the lysates was assessed by Bicinchoninic Acid (BCA) assay before storage at -80˚C.

Based on total protein concentrations from the BCA assays, aliquots of tissue lysates corresponding to known amounts of total protein per sample were prepared for each animal in Laemmli buffer (Tris-HCl 25mM pH=6.8, Glycerol 7.5%, SDS 1%, DTT 250mM, and Bromophenol Blue 0.05%) for immunoblotting experiments.

Immunoblotting. Western blots were run in all conditions using 10µg of protein separated by SDS-PAGE and transferred to nitrocellulose membranes, as previously described. Incubation of the primary antibodies was performed overnight at 4°C and Anti-β-actin (1:10000, Sigma) was used to control equal loading. Appropriate secondary antibodies coupled to peroxidase were revealed using a Super Signal West Chemiluminescent kit (Immobilon Western, Chemiluminescent HRP substrate, Millipore). Chemiluminescence images were acquired using the ChemiDoc+XRS system measurement (BioRad). Signals per lane were quantified using ImageJ (version 1.53). A ratio (protein of interest normalized to β-Actin protein levels, then to Control values) of the signal on loading per animal was performed and used in statistical analyses.

PROJECT 2: MODULATING ZINC LEVELS IN LB-INJECTED MICE

Zinc chelation reduces α-synuclein-induced neurodegeneration in a mouse model of Parkinson's disease.

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Manuscript in preparation.

As previously discussed in the introduction, heavy metals seem to play an important role in the initiation and progression of PD pathology. Until now, the studies on the implication of iron and copper have been prioritized over the study of zinc given their binding properties to α-syn. Nevertheless, high zinc diets have been shown to be able to induce a PD-like pathology that is responsive to L-Dopa treatments (Kumar *et al.*, 2018). This, combined with the implication of ATP13A2 in genetic PD cases, seems to indicate that zinc's role in PD could be more important than we believe. In fact, with an artificial neuronal network used in machine learning with variables measured from NHP models injected with LB and noLB patient-derived fractions, it has been shown that zinc levels among the five other metals (Cu, Fe, Mn, Ca, S) measured in the SN could be a good predictor of neurodegeneration (Bourdenx *et al.*, 2020).

Knowing that zinc has been implicated in PD, and in particular through various models such as the 6-hydroxydopamine toxin mouse model, ATP13A2 mutant cells, and LB-injected animals, we wanted to decipher the impact of this ion in the pathological process and assess whether modulating these zinc levels could have a beneficial impact on the progression of LB-induced neuropathology. To test this, we used two conditions in our control or LB-injected mice: either to increase zinc levels by adding zinc sulfate to the diet of the mice, or decrease this zinc by using ClQ, a zinc ionophore. Modulating zinc through the diet has been commonly used in studies and presented the less invasive route of administration. In addition, given the ability of ClQ to cross the blood-brain barrier, it presented no challenges for the efficacy of the drug.

ClQ is a ionophore that has already been used to modulate the concentration of heavy metals in models of PD, AD, and Huntington's disease (Cherny *et al.*, 2001; Nguyen *et al.*, 2005; Wilkins *et al.*, 2009; Lei *et al.*, 2015; Finkelstein *et al.*, 2016). In all cases, it has shown

beneficial effects on the progression of the disease through various mechanisms. However, none of these studies have been conducted in models using LB-injected mice, and little is known of the mechanisms underlying these changes.

To better understand how zinc is implicated and its role, it is crucial to understand the mechanisms it impacts in PD pathology. Given previous studies undertaken concerning ClQ, it is possible that ClQ could act on the activation of the ALP or on increasing synaptic transmission. With this in mind, we focused on observing the potential variations in autophagy in this LB mouse model to determine if ClQ was indeed increasing autophagy-lysosomal clearance within the brain.

Objective

The objective of this study was to determine the implication of zinc in an LB-injected mouse model and assess the therapeutic potential of ClQ in halting neurodegeneration.

Experimental procedures

Diet

One week after their stereotaxic surgeries, mice were divided into three different groups, depending on the received diet, until the end of the experiment: ClQ supplemented diet (30mg/kg/day), zinc sulfate supplemented diet (180mg/kg/day), or a standard diet.

Immunofluorescent Labeling

Double-immunofluorescent labeling was performed on nigral sections to localize lysosomes in dopaminergic neurons. Sections were permeabilized and blocked using normal goat serum diluted in PBS 1X-Saponine 0.2% for one hour before being incubated overnight in primary antibodies rabbit anti-TH (1:1000, ab237961, Abcam) and rat anti-LAMP2 (1:1000, ab25339, Abcam). The next day, sections were incubated in secondary antibodies sequentially: first with goat anti-rabbit Alexa 488 secondary antibodies, then with goat anti-rat Alexa 568 secondary antibodies. After washing, sections were incubated with 10µM of Hoechst staining for 8 min to color the nuclei. Sections were mounted on non-gelatinized slides and cover-slipped using fluorescent mounting media without DAPI (Vector Labs). Images were acquired using a Zeiss SP5 confocal microscope (x63). Analysis of images was done using

ImageJ (version 1.53), with a macro designed to delineate individual cells, delineate the nucleus of each cell and localize the perinuclear and cytosolic LAMP2 puncta within the cell.

Immunoblotting

For immunoblotting, the experiments were done as previously stated. The following primary antibodies were used: LC3-IIB (Novus Biologicals, 1:1000), LAMP2 (Abcam, 1:1000), p62 (Progen, 1:1000), ZnT3 (Abcam, 1:1000), ZnT4 (Invitrogen, 1:1000), SOD1 (R&D Systems, 1:2000).

Results

After the injection of LB or Vehicle, the diet of these mice was altered for the remaining 4 months of the experiment. We aimed to determine whether these different diets had an effect on neurodegeneration in the SN in Vehicle and LB-injected mice. We observed a 30% loss of survival of TH-positive neurons in LB injected mice compared to Vehicle mice (**Figure 28**). However, we did not observe this same loss in LB mice having received altered diets. In fact, in mice treated with ClQ (blue groups), there was a significant increase in the survival of dopaminergic neurons in the SN of LB-injected mice compared to untreated LB mice. In the case of zinc supplemented LB mice (green groups), no significant difference was observed in the loss of dopaminergic neurons, but the number of TH-positive neurons remained similar to the untreated LB-injected mice. This result suggests a potential neuroprotective effect of ClQ on LB-induced dopaminergic neuron loss, which is not observed with a zinc-supplemented diet. To evaluate the effect of zinc modulation on the accumulation of α-syn in mice, we quantified the levels of both total α-syn and proteinase K (PK)-resistant α-syn (**Figure 29**). PK treatment reveals the accumulation of misfolded PK-resistant aggregates. When comparing groups, we observed that untreated LB-injected mice had a higher amount of PK-resistant α-syn (**Figure 29A**). With ClQ treatment, the amount of PK-resistant α-syn puncta was decreased in LB-injected mice compared to untreated mice. This alteration in PK-resistant puncta was not observed in zinc supplemented LB-injected mice, once more indicating the effect of ClQ treatment compared to zinc in these mice. When quantifying total α-syn levels, without PK treatment, using immunoblotting, no significant differences were observed between groups (**Figure 29B**). This lack of variation in total α-syn could indicate

the potential change in type of α-syn species present in LB-injected mice, which is not the case in mice having received ClQ.

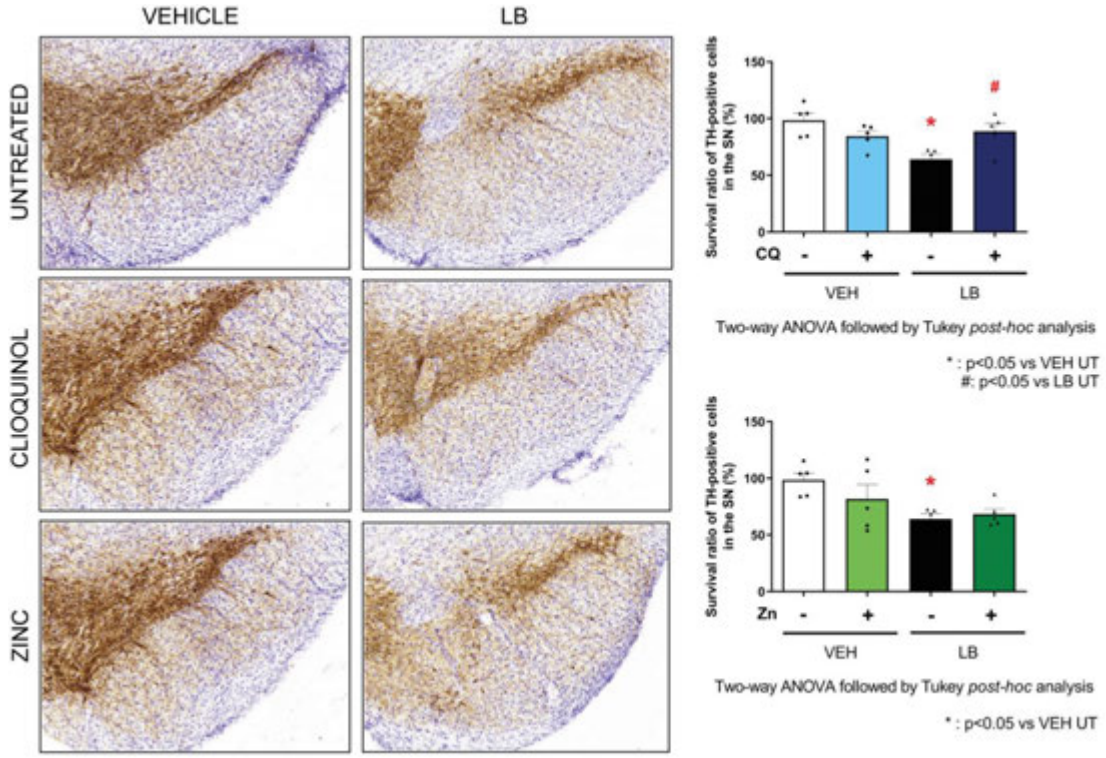

Figure 28: Determining the effect of zinc modulation on neurodegeneration in the substantia nigra of Vehicle and LB-injected mice. Illustrative images and quantifications of TH staining in the right SN in mice injected with Vehicle or LB fractions that received one of three diets: (i) standard diet, (ii) Clioquinol supplemented diet, or (iii) Zinc supplemented diet. Comparisons were made using two-way ANOVA followed by Tukey's post-hoc analysis, n=5 per group. * $p<0.05$

When zinc homeostasis is disturbed in the cell, the expression of zinc transporters is altered to restore zinc levels in the cytosol and avoid zinc-associated toxicity. Knowing this, we wanted to assess the expression levels of zinc transporters associated with synaptic vesicles and the lysosome, as well as a zinc-binding protein (**Figure 30**). In synaptic vesicles, the only zinc transporter present at the membrane is ZnT3. When measuring striatal ZnT3 levels, we observed a decrease in ZnT3 levels in LB-injected mice having received ClQ, but not in any other groups (**Figure 30A**). This could indicate a reorganization of cellular zinc concentrations away from the synapse, leading to less zinc released at the synapse. Superoxide dismutase 1 (SOD1) is a zinc-binding protein present in the cytosol that has been shown to be dysregulated in multiple neurological disorders, in particular amyotrophic lateral sclerosis. In vehicle and LB-injected mice, SOD1 showed no changes in expression in the

striatum of mice treated with ClQ or zinc (**Figure 30B**). This could indicate that this protein is not implicated in zinc binding, at least at the striatal level.

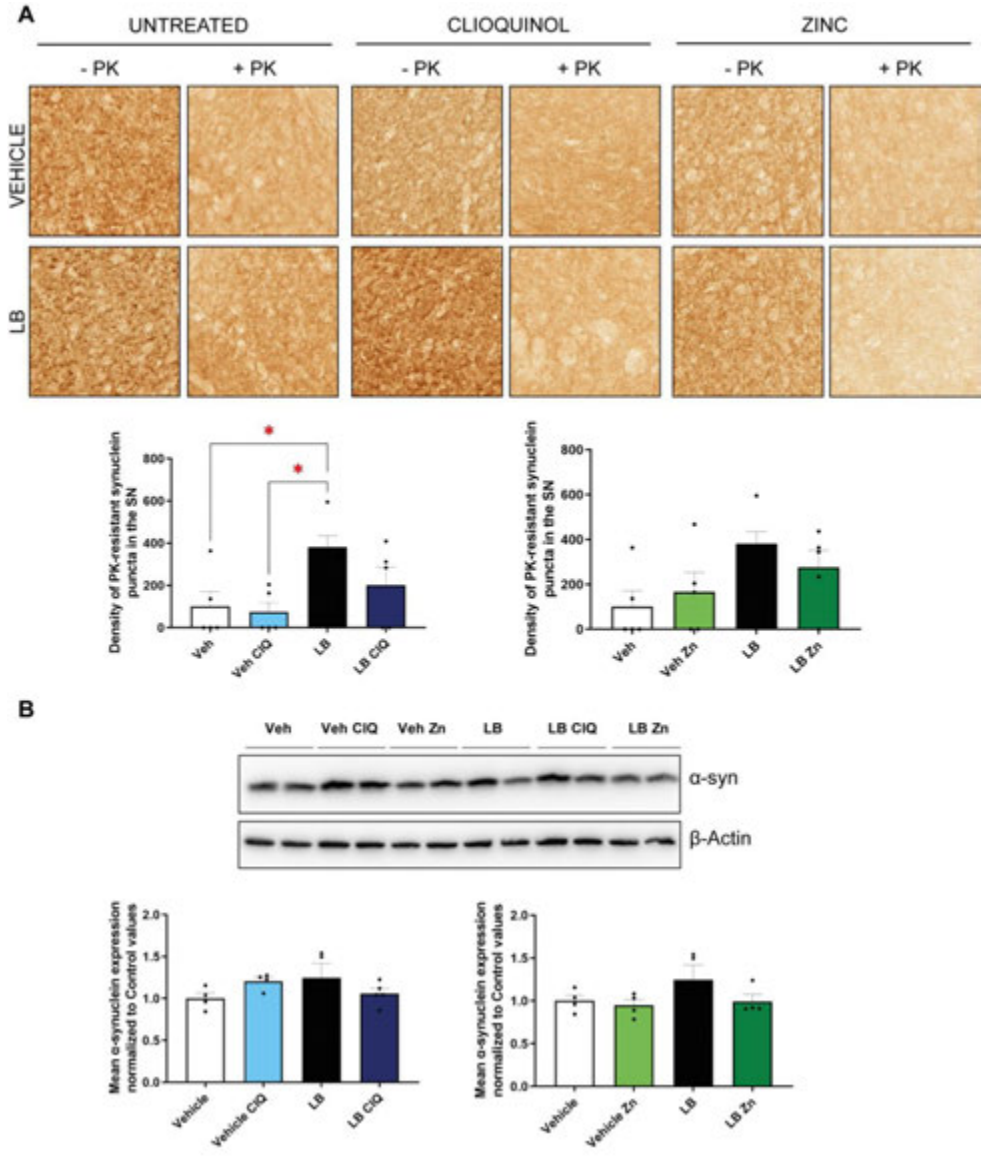

Figure 29: Evaluation of the effect of zinc modulation on α-synuclein accumulation in the substantia nigra of LB-injected mice. (**A**) Illustrative images and quantification of proteinase-K (PK) resistant α-synuclein in Vehicle and LB-injected mice treated with Clioquinol or Zinc. (**B**) Immunoblot and quantification of total α-syn expression in Vehicle and LB mice. Comparisons were made using one-way ANOVA followed by Tukey's post-hoc analysis, n=5 per group. * $p<0.05$

Concerning lysosomal transporters, we assessed the levels of ZnT4 and ZIP8, the transporters responsible for the export and import of zinc from the cytosol to the lysosome, respectively. In the striatum, the levels of ZnT4 remained generally unchanged, with a trend towards a decrease of ZnT4 in untreated LB-injected mice (**Figure 30C**). However, ZIP8 levels were increased in LB mice having received a zinc diet, suggesting that there is an increase in

cytosolic zinc in LB mice treated with zinc (**Figure 30D**). With an increase of zinc in the cytosol, this could be a potential explanation for the unchanged toxicity of LB in zinc-treated mice. Altogether, these striatal transporters and zinc-binding proteins showed little alterations, with the particular exception of ZnT3 in ClQ treated LB-injected mice and ZIP8 in zinc supplemented LB mice.

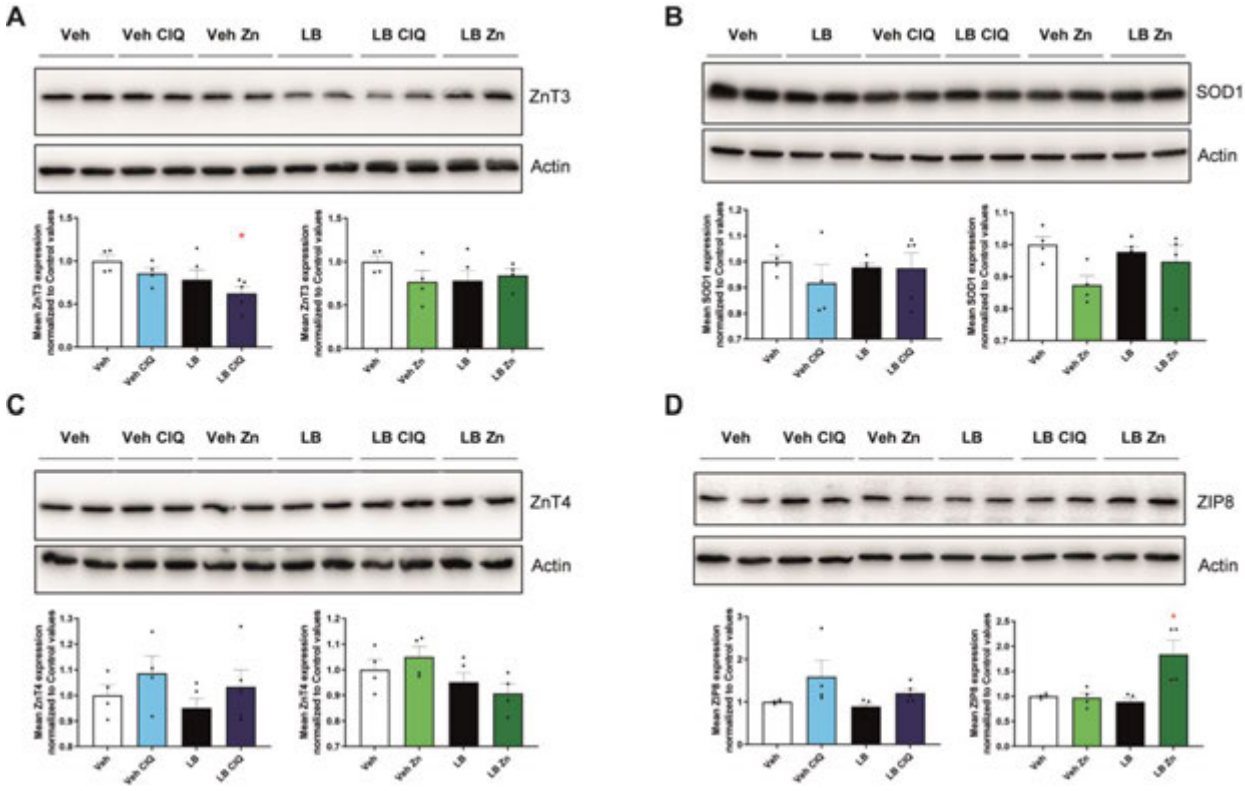

Figure 30: Zinc Transporters are altered in mice with modulated zinc diets. Representative immunoblots and histograms of ZnT3 (A), SOD1 (B), ZnT4 (C), and ZIP8 (D) expressions in the striatum. Histograms represent the mean protein expression normalized by β-Actin levels in Vehicle and LB mice treated with clioquinol or zinc. Comparisons were made using one-way ANOVA followed by Tukey's post-hoc analysis, n=5 per group. * $p<0.05$

Given the implication of zinc in autophagy and lysosomal function, we next wanted to determine whether ClQ or zinc diets altered the general function of autophagy (**Figure 31**). For this, we first measured the expression of autophagy-related genes, including LC3-II and p62, in the SN of vehicle and LB-injected mice. LC3-II is present at the autophagosome membrane and binds to p62, a cargo protein that brings misfolded proteins for their degradation. Concerning LC3, we observed a slight decrease in LB mice having received both ClQ and zinc diets, indicating a potential decrease in autophagosome accumulation

(**Figure 31A**). However, p62 levels were shown to be increased only in LB-injected mice having received a zinc diet. This could indicate a higher amount of autophagic cargo to be delivered for clearance compared to other groups.

With this in mind, we have previously seen that lysosomal degradation is impaired in PD pathology and that this impairment can be translated by a change in cellular localization of lysosomes. In fact, functional lysosomes have been shown to locate in the perinuclear region, whereas dysfunctional lysosomes are further from the nucleus. To assess this lysosome function, we used co-immunofluorescence to determine LAMP2 position in TH-positive neurons (**Figure 31B**). We found that in untreated LB-injected mice, the percentage of perinuclear LAMP2 puncta in TH-positive neurons was decreased. This decrease in perinuclear lysosomes indicates lysosomal impairment (Johnson *et al.*, 2016; Pu *et al.*, 2016). However, in LB mice treated with ClQ, the number of perinuclear lysosomal puncta was significantly increased compared to untreated LB mice. The restoration of the percentage of perinuclear fractions of LAMP2 was not observed in LB-injected mice treated with zinc. Taken together, this demonstrated a potential neuroprotective effect of ClQ in LB-injected mice through the restoration of correct lysosomal function.

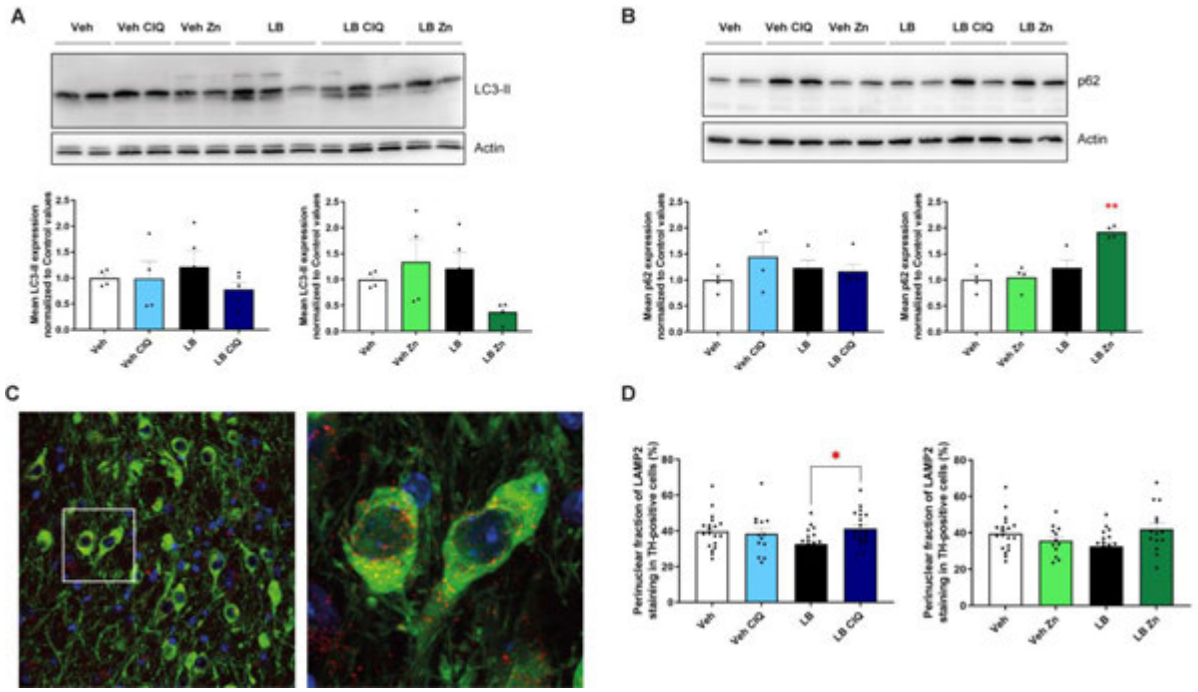

Figure 31: Clioquinol and Zinc induce autophagy-lysosomal pathway changes in the brain of LB-injected mice. (**A-B**) Representative images and quantification of LC3-II (**A**) and p62 (**B**) immunoblotting in the different experimental groups. n=5 per group. (**C-D**) Representative images of TH and LAMP2 immunofluorescence in the SN (**C**) and quantification (**D**) to examine lysosomal distance to the nucleus. LAMP2-positive lysosomes were segmented, and the distance to the border of the nucleus was calculated for each lysosome. Comparisons were made using one-way ANOVA followed by Tukey's post-hoc analysis, n=5 per group. * $p<0.05$

Discussion

Altogether, we have seen that LB-injected mice treated with ClQ have an increased survival of dopaminergic neurons in the SN. In addition, there is a reduction in the number of α-syn PK-resistant puncta in these mice and a change in the expression of the ZnT3 transporter. These ClQ-treated LB mice also have lysosomes that are closer to the nucleus, indicating their correct function compared to LB-injected mice without treatment. Overall, these results suggest the potential therapeutic potential of ClQ. As seen in the introduction, ClQ seems to act either on the ALP or on synaptic transmission. Through this study, we have seen that zinc synaptic transmission seems to be modified, through the changes in expression of ZnT3, and that lysosomal activity is improved in LB-injected treated mice. Furthermore, both of these changes in activity seem to decrease the loss of dopaminergic neurons in the SN, proving its potential neuroprotective effects. The effect of zinc treatment, however, is less clear as neurodegeneration is not inhibited significantly. Nonetheless, zinc seems to increase autophagy, seen through its impact on p62 and LC3 expressions. Zinc also increases ZIP8 expression, indicating a modulation in lysosomal and cytosolic zinc in neurons.

Given that our goal was to alter neuronal zinc through the use of ClQ and a supplemented zinc diet, it is equally important that we assess whether or not this modulation was successful. To determine this, we used Synchrotron X-Ray fluorescence to measure zinc in the SN of the entire cohort of mice. Using this technique, we will be able to quantify the levels of zinc, and other heavy metals. This confirmation is of crucial importance to verify that it is in fact zinc modulation that induces these effects in LB-injected mice. Unfortunately, the measures were carried out just a few months ago and analyses are still ongoing. Once the analyses are done, we will confirm modulation in zinc levels and finish the final experiments for this project.

Conclusions

This project 2 aimed at demonstrating the potential role of ClQ as a neuroprotective drug in LB-injected mice. ClQ has shown its ability to modulate both synaptic transmission and lysosomal function in this model. Further studies will be required to confirm these effects and the mechanisms behind its action, but these preliminary results already show promise for the therapeutic potential of this molecule. We have seen that targeting zinc could be an interesting approach to induce neuroprotection of dopaminergic neurons in the SN, thus slowing the progression of PD pathology.

PROJECT 3: STUDY ON NEUROPROTECTION BY THE NFE2L1 TRANSCRIPTION FACTOR IN A PD MOUSE MODEL

Dopaminergic neuron loss is one of the main hallmarks of PD and is the cause for most of the motor symptoms associated with the pathology. As such, it is important for future therapies to be able to halt or prevent this dopaminergic loss. Given the different potential therapies that exist to target α-syn, it is primordial to also develop strategies that target dopaminergic cell survival.

With the vulnerability of dopaminergic neurons in PD pathology, they have been and are currently studied extensively. In the study of these neurons, the role played by various transcription factors has been subject to intense examination, in particular using mice models. Various signaling pathways control the development and survival of these neurons, including the WNT1 pathway, regulated by LMX1A, a LIM homeodomain transcription factor required for dopaminergic neuron specification. LMX1A and WNT1 regulate the expression of *Nurr1* and the paired-like homeodomain transcription factor 3 (*Pitx3*) genes, which are both required for the differentiation and survival of dopaminergic neurons. Pitx3 has previously been shown to be regulated by *Pbx1* homeobox gene, important for the specification and survival of dopaminergic neurons. PBX1 has also shown to regulate the expression of *Nfe2L1*, another transcription factor that regulates mitochondrial function and proteasomal activity. In dopaminergic neurons, PBX1 activation of NFE2L1, Nrf1, has also been shown to promote neuron survival (Villaescusa *et al.*, 2016). This study established that both PBX1 and NFE2L1 levels were decreased in the SN of PD patients. These decreased levels of NFE2L1 also induced a higher vulnerability to oxidative stress. Taken together, this amount of data established the potential therapeutic potential of both of these factors.

In addition to its function in dopaminergic neurons, transcription factor NFE2L1 has been shown to have multiple different roles within the cell. NFE2L1 has first been shown as an anti-oxidative stress factor through the modulation of antioxidant response element (ARE) expression and through its involvement in the glutathione (GSH) synthesis pathway. In addition, Nrf1 is involved in regulating the expression of other oxidative stress response genes, including metallothionein-1, implicated in heavy metal binding (Song 2014). Besides its role in oxidative stress reduction, NFE2L1 is implicated in protein homeostasis through the modulation of the UPS. Deletions in the *Nrf1* gene in neurons have demonstrated neurodegeneration and the accumulation of ubiquitinated proteins in mice (Lee 2011).

Similarly, knockdown of Nrf1 blocked protein turnover and had similar effects as rapamycin, an inhibitor of mTOR-mediated autophagy.

Given these essential functions in dopaminergic neuron survival, oxidative stress reduction, and protein clearance, it appears that NFE2L1 could be a very important therapeutic target to inhibit the progression of PD pathology. In this subproject, we strived to increase the expression of NFE2L1 in dopaminergic neurons of the SN in our LB mouse model, whereas our collaborator, Dr. Chris Rochet, assessed in a rat model of PD based on viral vector-mediated overexpression of human A53T α-syn. For this, we used a rAAV2/9-NFE2L1 that was injected simultaneously with Vehicle, LB, and noLB fractions in the right SN of wild-type mice. At 4 months post-injection, histological analyses were performed to determine the effect of NFE2L1 overexpression on neurodegeneration and α-syn pathology.

Objective

The objective of this study was to evaluate the effect of increased NFE2L1 expression on neurodegeneration and synucleinopathy in a PD mouse model.

Experimental procedures

Virus production

Recombinant AAV9-CMVie/SynP-NFE2L1-WPRE and AAV9-CMVie/SynP-WPRE vectors were produced by PEI-mediated triple transfection of low-passage HEK-293T/17 cells (ATCC, catalog CRL-11268). The AAV expression plasmids were cotransfected with the adeno helper pAd Delta F6 plasmid (Penn Vector Core, catalog PL-F-PVADF6) and AAV Rep Cap pAAV2/9 plasmid (Penn Vector Core, catalog PL-TPV008). AAV vectors were purified as previously described (59). Cells were harvested 72 hours after transfection, resuspended in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.5), and lysed by 3 freeze-thaw cycles (37°C/–80°C). The cell lysate was treated with 150 units/ml benzonase (MilliporeSigma) for 1 hour at 37°C, and the crude lysate was clarified by centrifugation. Vectors were purified by iodixanol step gradient centrifugation, and they were concentrated and buffer-exchanged into Lactated Ringer's solution (Baxter) using vivaspin20 100kDa cut-off concentrator (Sartorius Stedim). Titrations were performed at the platform study of the transcriptome (Neurocentre Magendie, INSERM U862). The genome-containing particle

(gcp) titer was determined by quantitative PCR (qPCR) using the Light Cycler 480 SYBR green master mix (Roche Diagnostics) with primers specific for the AAV2 ITRs (forward, 5′-GGAACCCCTAGTGATGGAGTT-3′; reverse, 5′-CGGCCTCAGTGAGCGA-3′) (60) on a Light Cycler 480 instrument. Purity assessment of vector stocks was estimated by loading 10 µl of vector stock on 10% SDS acrylamide gels; total proteins were visualized using the Krypton Infrared Protein Stain according to the manufacturer's instructions (Invitrogen). We obtained a titer of 3.3×10^{13} gcp/ml for the neuronal AAV9-CMVie/SynP-Nfe2L1-WPRE and a titer of 2×10^{13} gcp/ml for AAV9-CMVie/SynP-WPRE (Stuffer).

Stereotaxic injections

Mice received stereotaxic injections of Vehicle, LB and noLB fractions combined with one of two viruses. They received either 3.3×10^{12} gcp/ml of AAV9-CMVie/SynP-NFE2L1-WPRE or 2×10^{13} gcp/ml for AAV9-CMVie/SynP-WPRE (Stuffer). These concomitant injections were delivered immediately above the right SN.

Results

At four months post-injection, we determined whether there were signs of cell death in nigral dopaminergic neurons in between experimental groups having received either vehicle, LB or noLB fractions, combined with the presence of the stuffer or NFE2L1 virus. We observed variations in neurodegeneration first between the groups having received the stuffer virus between Vehicle, LB, and noLB mice, where LB mice demonstrated a loss of dopaminergic neurons in the right SN (**Figure 32**). In mice having received the NFE2L1 virus, the survival of TH-positive cells in LB-injected mice was restored in the SN. No significant differences were observed in the case of noLB injected mice having received the virus.

Following these results, we wanted to assess whether dopaminergic fibers were affected by these various treatments through the observation of TH-positive dopaminergic fibers in the striatum of mice. In these mice, we did not observe any significant differences between mice having received the stuffer virus and vehicle, LB or noLB fractions (**Figure 33**). However, we observed a significant loss of dopaminergic fibers in mice having received noLB injections combined with the NFE2L1 virus.

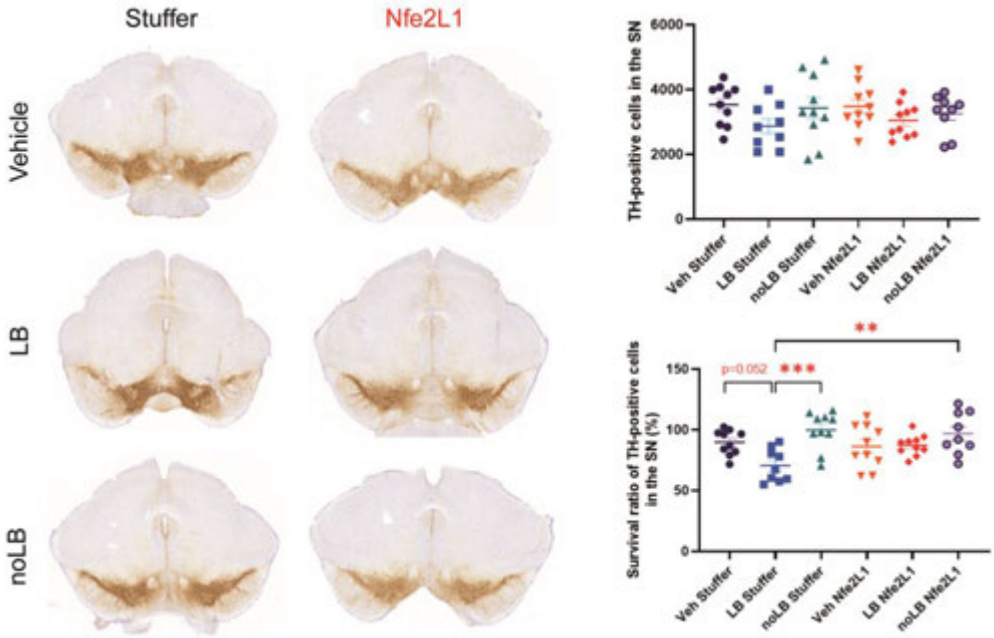

Figure 32: Assessing neurodegeneration in the substantia nigra of Vehicle, LB and noLB-injected mice after NFE2L1 overexpression. Illustrative images and quantifications of TH staining in the right SN in mice injected with Vehicle, LB, or noLB fractions that received either a stuffer or NFE2L1 virus. Comparisons were made using one-way ANOVA followed by Tukey's post-hoc analysis, n=9-10 per group. * p<0.05

These results suggest a modification of the toxicity of noLB fractions when combined with the NFE2L1 overexpressing virus. However, it seems that in LB-injected mice, there were no changes in striatal TH expression but a restoration of the number of TH-positive cells in the SN (**Figure 33**). The combination of the AAV-NFE2L1 with noLB fractions could induce a modification in TH fiber survival which is not the case for the other fractions.

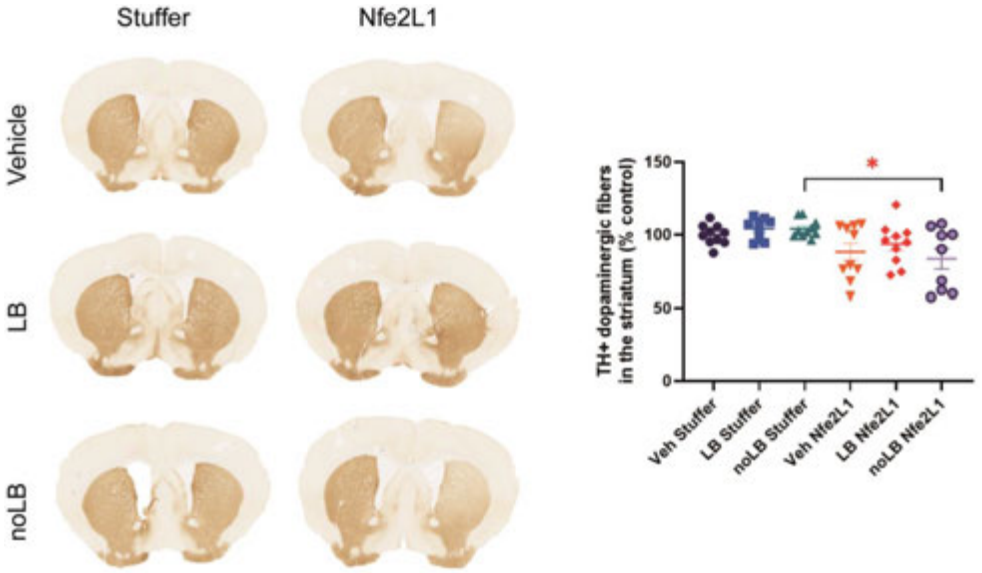

Figure 33: Assessing neurodegeneration in the striatum of Vehicle, LB and noLB-injected mice after NFE2L1 overexpression. Illustrative images and quantifications of TH staining in the striatum of mice injected with Vehicle, LB, or noLB fractions that received either a stuffer or NFE2L1 virus. Comparisons were made using one-way ANOVA followed by Tukey's post-hoc analysis, n=9-10 per group. * p<0.05

We next wanted to determine if there were any changes in the synucleinopathy induced by the injection of the LB or noLB fractions combined with both the stuffer and NFE2L1 viruses. For this, we used total α-syn staining in the SN of mice, which were treated or not with PK. With a lot of variability, we observed a potential increase in the mean grey intensity of α-syn staining in the SN, without PK staining, in stuffer LB-injected mice (**Figure 34**). However, in slices having received PK treatment, we observed that LB mice having received the stuffer virus had a trend towards PK-resistant α-syn accumulation compared to LB + NFE2L1-injected mice. This trend towards a decrease in PK-resistant α-syn in LB mice having received the NFE2L1 virus could indicate an inhibition of α-syn accumulation.

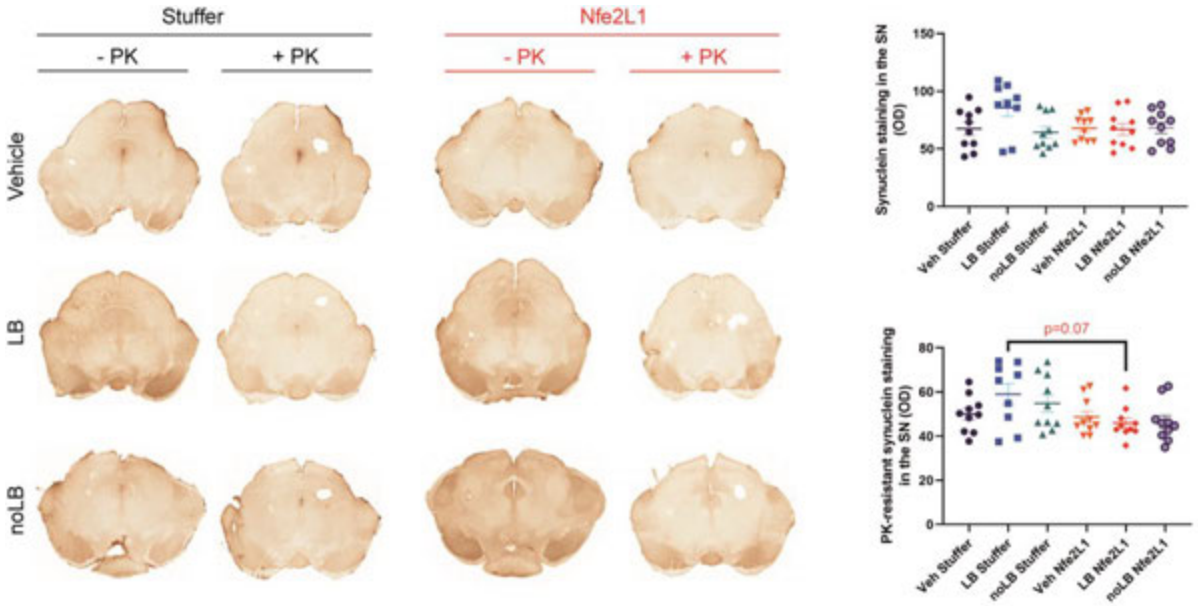

Figure 34: Determining the effect of NFE2L1 overexpression on α-synuclein accumulation in the substantia nigra of Vehicle, LB and noLB-injected mice. Representative images and quantifications of untreated (-PK) and treated (+PK) slices immunostained with α-syn in Vehicle, LB and noLB-treated mice with NFE2L1 overexpression or stuffer. Comparisons were made using one-way ANOVA followed by Tukey's post-hoc analysis, n=9-10 per group. * $p<0.05$

We can also notice that noLB-injected mice that received the NFE2L1 virus show no modulations in PK-resistant α-syn species (**Figure 34**). This result could suggest that the modulations seen in dopaminergic neurons could have no effect on α-syn accumulation and could be uniquely related to the toxicity of the virus on neurons in the SN.

To continue in the identification of synucleinopathy, we looked at α-syn accumulation in the striatum of these same mice. Once again, we observed no significant variations in the PK-

resistance of α-syn species. In slices untreated by PK, a slight increase in the intensity of α-syn staining was present in the stuffer group of LB-injected mice, which was not observed in the NFE2L1 group (**Figure 35**). Conversely, when slices were treated with PK, we observed a trend toward an increase in the stuffer LB-injected mice compared to the NFE2L1 LB-injected group, with a p=0.055. This data suggests once more a decrease in α-syn intensity when LB-mice received the NFE2L1 virus, thus indicating its potential to inhibit the accumulation of PK-resistant α-syn species.

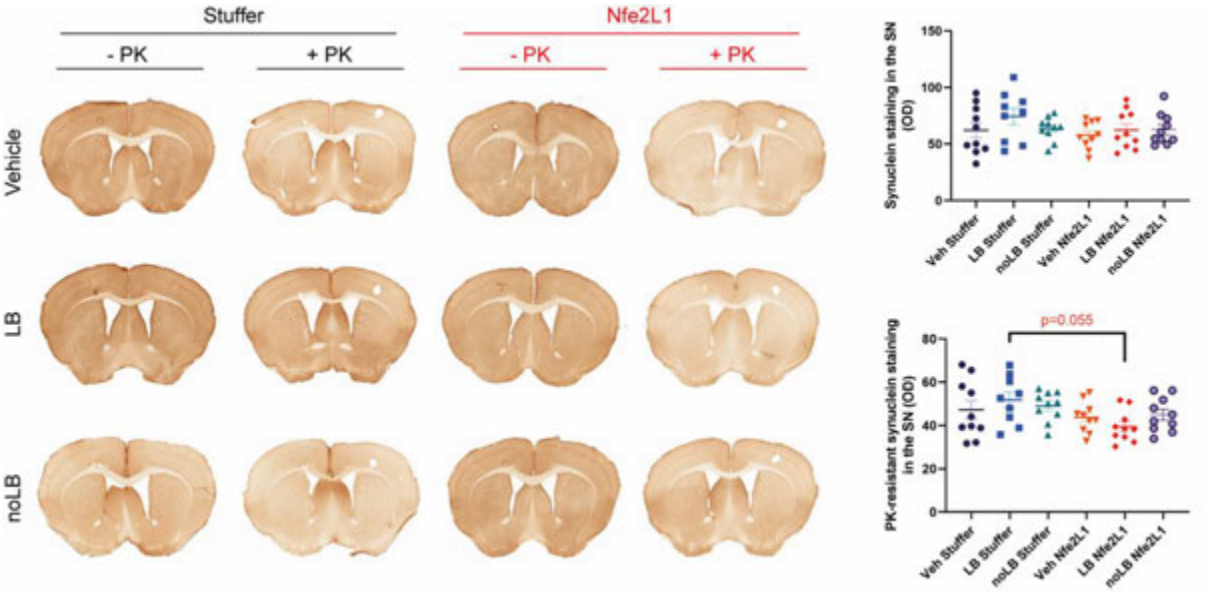

Figure 35: Determining the effect of NFE2L1 overexpression on α-synuclein accumulation in the striatum of Vehicle, LB and noLB-injected mice. Representative images and quantifications of untreated (-PK) and treated (+PK) slices immunostained with α-syn in Vehicle, LB and noLB-treated mice with NFE2L1 overexpression or stuffer. Comparisons were made using one-way ANOVA followed by Tukey's post-hoc analysis, n=9-10 per group. * $p<0.05$

Discussion

In this study, we have demonstrated the beneficial effects of overexpressing NFE2L1 in the LB mouse model of PD. LB-injected mice overexpressing NFE2L1 demonstrated a decrease in the loss of dopaminergic neurons of the SN and a decrease in PK-resistant α-syn species in both the SN and the striatum compared to LB-injected mice.

We observed that noLB-injected mice having received the NFE2L1 virus demonstrated a loss of dopaminergic fibers in the striatum compared to those that received the Stuffer virus. However, inconsistencies were seen as these noLB + NFE2L1 mice did not demonstrate a

decrease in TH-positive neurons in the SN or an increase in the accumulation of α-syn. This could indicate a specific toxicity when combining the virus with noLB fractions, but this remains to be studied in detail. This should be further tested with immunohistochemistry to assess the overexpression of NFE2L1 in the neurons of these mice.

In a first attempt, we have only assessed the implication of NFE2L1 overexpression on dopaminergic neurons and fibers, as well as α-syn accumulation. However, given the implication of NFE2L1 in many cellular mechanisms, it would be interesting to further investigate the specific effect of NFE2L1 in this model. This would be even more interesting if we could identify the reason for the differences observed in mice injected with noLB fractions compared to LB fractions. The overexpression of NFE2L1 could impact either proteasome clearance, oxidative stress, directly on dopaminergic neuron survival, or on a combination of these mechanisms.

Taken together, the investigation of overexpressing NFE2L1 in a PD model induced by the injection of LB fractions could be interesting for potential therapeutic strategies. As NFE2L1 is important for dopaminergic neuron survival as well as other cellular mechanisms, its pleiotropic effects could be that much more effective in halting PD progression.

Conclusions

Despite certain inconsistencies observed on the varying effects of NFE2L1 overexpression in LB and noLB-injected mice, NFE2L1 could be a very interesting therapeutic target for PD. These LB-injected mice represent a progressive model of PD, and the positive effects of NFE2L1 overexpression on dopaminergic neuron loss and α-syn accumulation are promising for future studies to develop on.

PROJECT 4: INHIBITION OF α-SYN ACCUMULATION USING ANLE138B IN A PD MOUSE MODEL

Targeting α-syn accumulation has been shown to be a valid therapeutic option to reduce neurodegeneration and α-syn accumulation (Teil *et al.*, 2020). For this targeting, several small molecules have been discovered by high-throughput drug screening or drug repositioning to treat synucleinopathies through an anti-aggregative strategy. Different drug mechanisms of action have been reported for this panel of small anti-aggregative molecules: either a direct interaction with α-syn aggregation pathway or an indirect direction, such as the modulation of chaperons to inhibit α-syn aggregation. Among these small molecules able to directly interact with pre-fibrillary structures, an oligomeric modulator was discovered through a systematic high-throughput screening: anle138b [3-(1,3-benzodioxol-5-yl)-5-(3-bromophenyl)-1H-pyrazole]. First tested *in vitro* on the prion scrappy protein and α-syn, anle138b demonstrated an ability to inhibit the formation of aggregates. Following *in vitro* testing, anle138b was tested in three mice models of PD, including rotenone or MPTP-intoxicated mice and A30P-α-syn transgenic mice. In these three animal models, anle138b blocked the formation of oligomers and aggregates of α-syn, as well as neuronal degeneration and disease progression (Wagner *et al.*, 2013). In the A30P-α-syn transgenic mice model, anle138b was administered after the appearance of symptoms but retained its ability to decrease the formation of oligomers, reduce motor impairments and prolong mouse survival (Levin *et al.*, 2014). In addition to its efficacy on α-syn in PD pathology, anle138b also showed its capacity to prevent neurodegeneration, motor impairments and α-syn aggregation in MSA mouse 3-nitropropionic acid toxin and transgenic models (Fellner *et al.*, 2016; Heras-Garvin *et al.*, 2019). Recently, using a new transgenic mouse model of PD, the MI2 mouse line, anle138b once more demonstrated that it could prevent dopaminergic neuronal loss, gait impairment, and large aggregate formation (Wegrzynowicz *et al.*, 2019). Given the success of all these studies, it is not surprising that clinical studies have started using anle138b in both PD and MSA patients (Lemos *et al.*, 2020).

Despite its proven effect in transgenic and neurotoxin models, no studies have used anle138b in patient-derived brain extract models of either PD or MSA. In this study, we wanted to determine not only the ability of anle138b to prevent neurodegeneration but also α-syn accumulation when administered concomitantly or with a delayed start compared to the

injection of LB in mice. For this, we delivered anle138b to mice through their diet either the day after their stereotaxic injections or with a postponed treatment.

Objective

This objective of this study was to assess the effect of Anle138b in a spreading model of PD and determine its anti-aggregative properties once the pathology had already begun to develop.

Experimental procedures

Diet

After stereotaxic injections, mice received either a classic diet or a modified diet with anle138b [3-(1,3-benzodioxol-5-yl)-5-(3-bromophenyl)-1H-pyrazole]. The food pellets contained 2g/kg of anle138b. Two treatment plans were designed, one where mice received anle138b diet the day after their stereotaxic injections, and the other where mice received the modified anle138b diet two months after their intracerebral injections (**Figure 36**). Hence, the first group received concomitant treatment and pathological induction, whereas the other group allowed a development of pathology before beginning the treatment. From the two different time points, mice were fed with the anle138b modified diet until the end of the experiment, four months after stereotaxic injections.

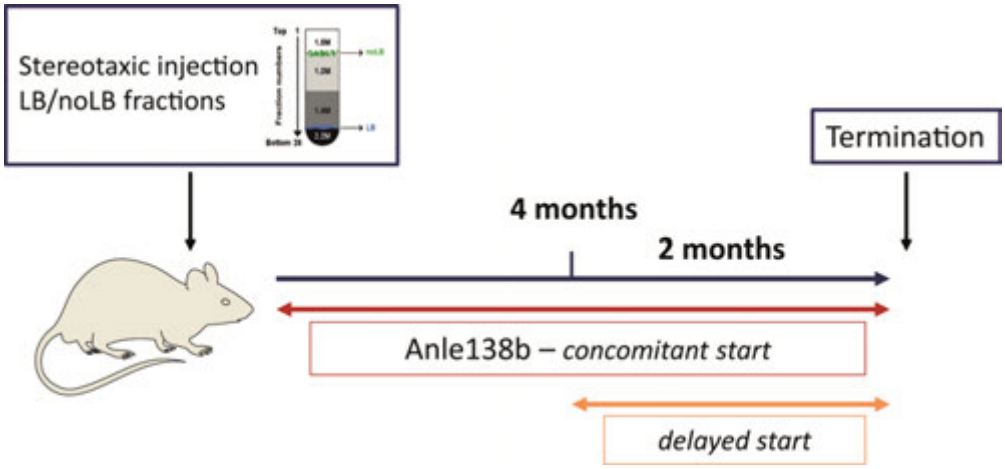

Figure 36: Schematic representation of the experimental timeline of the study. Mice were injected in the right substantia nigra with either LB or noLB fractions. During the 4 months of inoculation, mice received either a concomitant or delayed start treatment of anle138b in their diet. The delayed start treatment started after 2 months and lasted 2 months.

Results

Despite the abundance of studies using anle138b in many animal models of proteinopathy, little is known on its efficacy in models relying on the prion-like propagation of α-syn *in vivo*. Here, we wanted to assess the neuroprotective effects of anle138b on LB-injected models compared to controls. We thus used TH staining to determine the loss of dopaminergic neurons and fibers in both the SN and the striatum of mice treated or not with anle138b. In untreated LB-injected mice, we observed a decrease in the number of TH-positive cells in the SN compared to controls. However, in mice treated concomitantly with anle138b, the number of dopaminergic neurons was significantly higher than in untreated LB mice (**Figure 37**). In comparison, in mice that were treated with a delayed start, we observed that the number of TH-positive cells was once again lower than in control mice. This indicates that anle138b seems to be effective in protecting dopaminergic neurons when mice are treated concomitantly to the LB injection, however, this is not the case with delayed treatment.

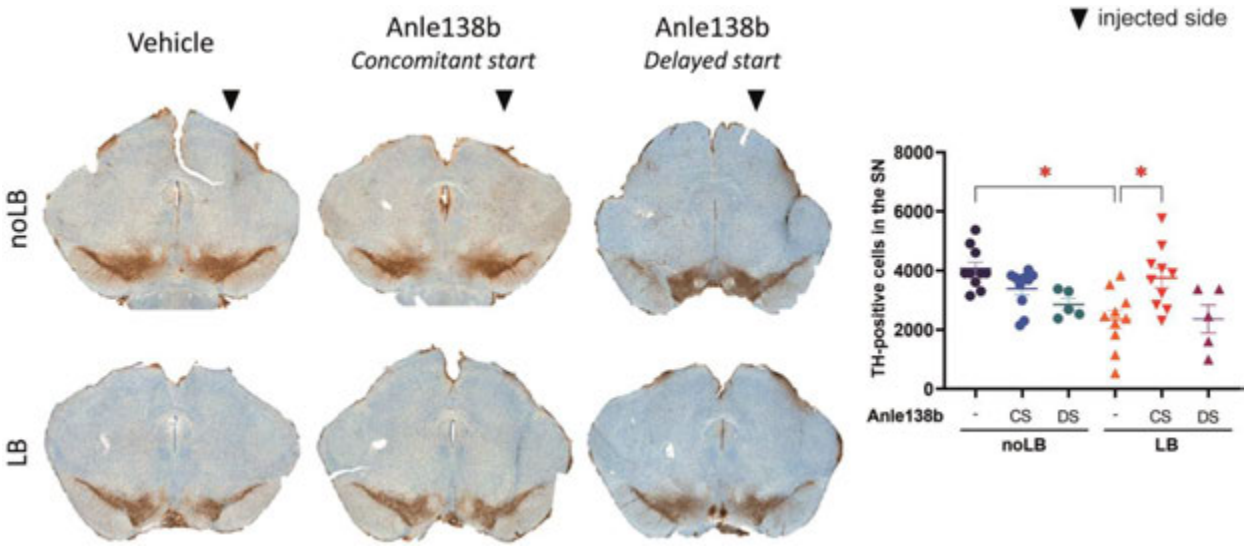

Figure 37: Assessing neurodegeneration in the substantia nigra of LB and noLB-injected mice treated with anle138b, either with a concomitant or delayed start. Illustrative images and quantifications of TH staining in the right SN in mice injected with LB or noLB fractions that received anle138b with a concomitant start (CS) or a delayed start (DS). Comparisons were made using one-way ANOVA followed by Tukey's post-hoc analysis, n=5-10 per group. * $p<0.05$

We then assessed the changes in striatal TH in treated and untreated mice to determine whether anle138b protected dopaminergic fibers. In LB-injected mice, the intensity of TH staining in the striatum was significantly decreased compared to control mice. This was not the case in LB-mice treated with anle138b both with concomitant start and delayed start (**Figure 38**). In fact, in untreated LB mice compared to delayed start treatment, there was a significant difference in the TH staining in the striatum. Taken together, these data indicate that dopaminergic neurons were affected by the change in treatment timing, but this was not the case for dopaminergic fibers.

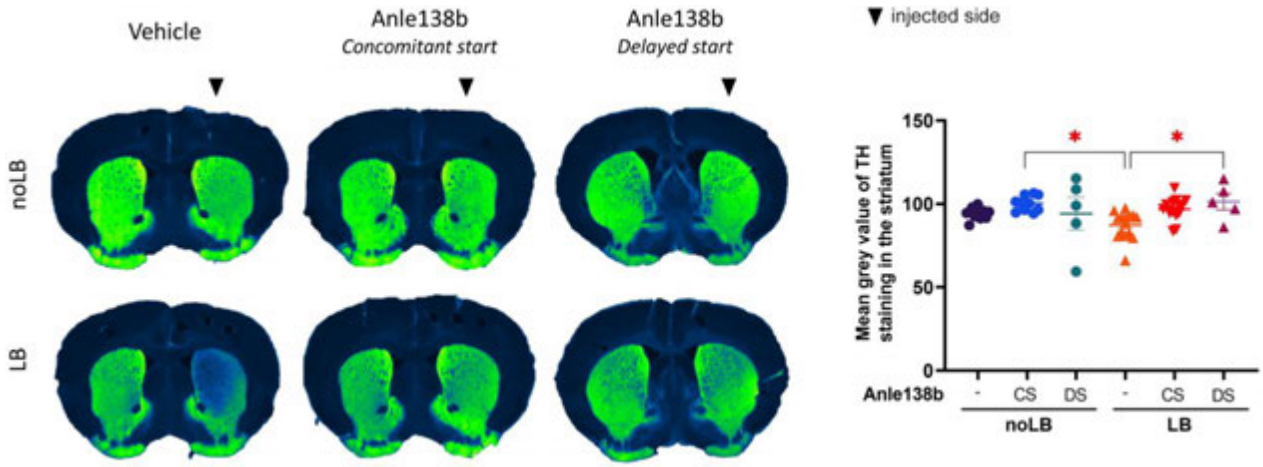

Figure 38: Assessing neurodegeneration in the striatum of LB and noLB-injected mice treated with anle138b, either with a concomitant or delayed start. Illustrative images and quantifications of mean grey value of TH staining in the striatum of mice injected with LB or noLB fractions that received anle138b with a concomitant start (CS) or a delayed start (DS). Comparisons were made using one-way ANOVA followed by Tukey's post-hoc analysis, n=5-10 per group. * $p<0.05$

To pursue in the assessment of the effect of anle138b, we wanted to examine the difference in α-syn accumulation. For this, we measured total α-syn staining in the SN and striatum of mice and also assessed PK-resistant α-syn. In the SN, we observed no significant changes between untreated noLB and LB injected mice. However, an increase in total α-syn staining was seen in mice having received a delayed start treatment, both with noLB and LB injections (**Figure 39**). The same increase in α-syn staining was observed even after PK treatment in these same groups. However, when comparing the ratios of ipsilateral and contralateral α-syn staining in PK-treated slices, there is a trend towards an increase in LB-injected mice compared to noLB mice. Altogether, minimal alterations in α-syn staining can be observed between the different treatments with anle138b.

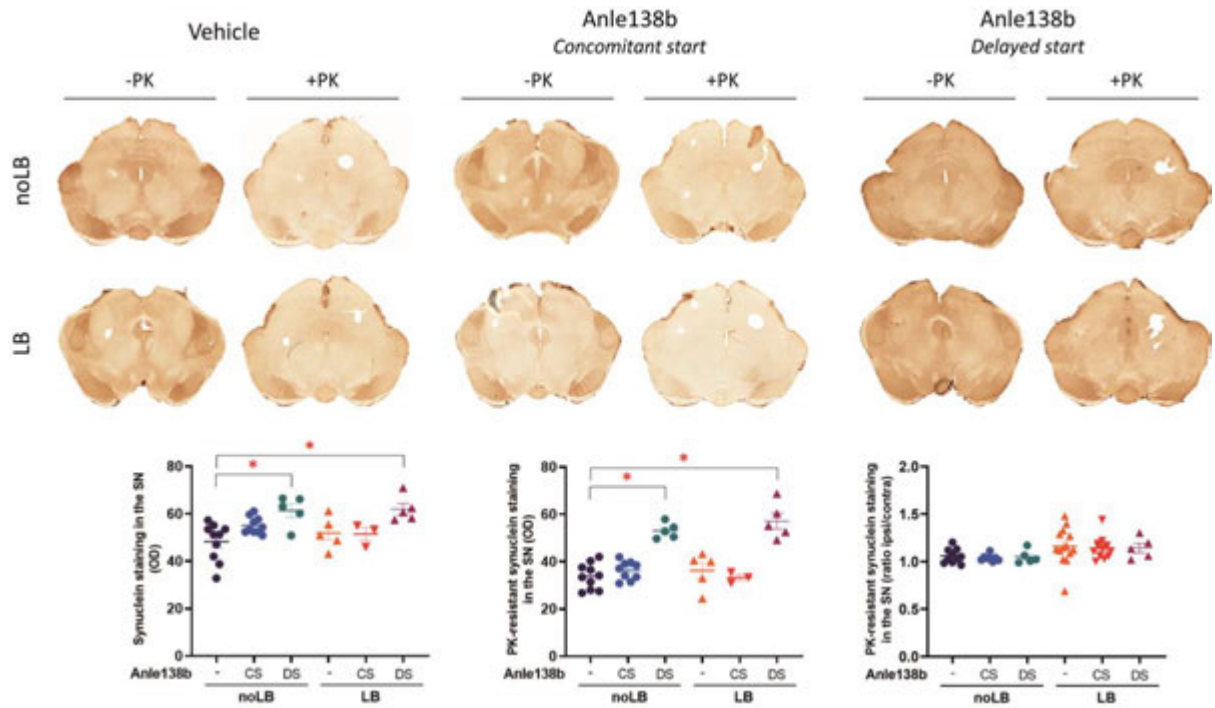

Figure 39: Determining the effect of anle138b concomitant or delayed treatment on α-synuclein accumulation in the substantia nigra of LB and noLB-injected mice. Representative images and quantifications of untreated (-PK) and treated (+PK) slices immunostained with α-syn in LB and noLB-treated mice with vehicle, anle138b concomitant start (CS) or delayed start (DS) treatments. Comparisons were made using one-way ANOVA followed by Tukey's post-hoc analysis, n=3-10 per group. * $p<0.05$

Finally, we also assessed the total α-syn levels and the PK-resistant α-syn stainings in the striatum (**Figure 40**). For total α-syn staining, there were variations between groups, but we observed that LB-injected mice treated concomitantly with anle138b had a trend to be decreased in total α-syn staining. With PK treatment, we observed a trend towards an increase in untreated LB-injected mice compared to controls. This trend was inversed in the case of LB-injected mice concomitantly treated with anle138b. This suggests that anle138b could decrease PK-resistant α-syn accumulation in the striatum of LB-injected mice compared to untreated LB mice. However, animals from the delayed start group, both noLB and LB, once again demonstrated an increase in PK resistance. Together, it seems that with a delayed start treatment of anle138b, there is no decrease in α-syn accumulation and PK resistance. In conclusion, with a concomitant treatment, the anti-aggregative properties of anle138b seem to be more efficient in LB-injected mice than with a delayed start treatment.

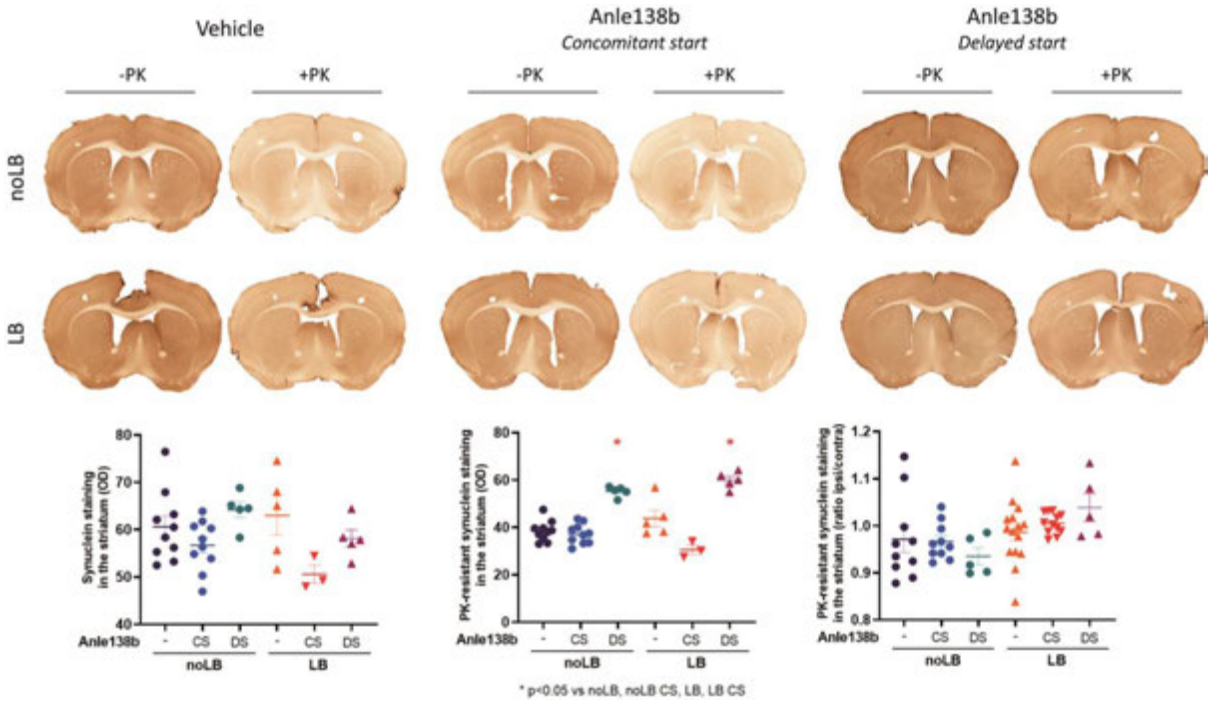

Figure 40: Determining the effect of anle138b concomitant or delayed treatment on α-synuclein accumulation in the striatum of LB and noLB-injected mice. Representative images and quantifications of untreated (-PK) and treated (+PK) slices immunostained with α-syn in LB and noLB-treated mice with vehicle, anle138b concomitant start (CS) or delayed start (DS) treatments. Comparisons were made using one-way ANOVA followed by Tukey's post-hoc analysis, n=3-10 per group. * $p<0.05$

Discussion

In this study, we challenged the neuroprotective effect of anle138b through two different experimental designs, i.e., administered in the diet of LB-injected mice for 4 or 2 months. LB-injected mice with concomitant anle138b treatment demonstrated a decrease in nigral dopaminergic neuron and fiber loss. These mice also demonstrated with a slight decrease in PK-resistant α-syn species compared to controls. Conversely, LB-injected mice with a delayed treatment had a dopaminergic lesion in the SN, which was nevertheless not translated by a loss in dopaminergic fiber in the striatum. However, when mice had a delayed treatment with LB and noLB fractions, we observed less effect of the anti-aggregative molecule on the progression of the pathology. Concerning α-syn accumulation, delayed treatment mice demonstrated an increase in α-syn staining and PK resistance compared to mice with concomitant treatments. Given these results, the delayed start treatment of 2 months seems to be insufficient to inhibit α-syn aggregation and dopaminergic neuron loss compared to a more prolonged treatment.

The alterations in α-syn staining and PK resistance in mice having received delayed treatments could be due to a different perfusion date or in the variability between cohorts. However, this could not be due to experimental differences in immunohistochemistry since all immunolabeling was done simultaneously to avoid possible biases. These inconsistencies could lead to the visible differences between groups which could lead to potential misinterpretations. Nevertheless, when observing dopaminergic neuron and fiber staining, the delayed or concomitant anle138b treatments do not have the same effect on neuronal and fiber survival in LB-injected mice.

Despite the anti-aggregative properties of anle138b and its positive effects with concomitant treatment and LB injections, this molecule seems to have a lower neuroprotective power when given after the beginning of pathological induction. This suggests that it might not be the therapeutic target that studies have indicated. In fact, with clinical treatments only being a possibility after diagnosis of PD, a potential therapy should be efficient to slow the progression of PD after pathology has been triggered. From what we have observed, this might not be the case for anle138b.

Conclusions

Anle138b has previously been shown to own neuroprotective effects in mouse models of PD when used concomitantly and with a delayed start (Levin *et al.*, 2014). However, in this spreading model of the disease, anle138b had a beneficial effect when used concomitantly to LB injections, but these effects were less significant when mice were treated with a delayed start. Taken together, anle138b could be a less attractive therapeutic target in the case of PD pathology.

DISCUSSION

The sum of my thesis work has been to study the mechanisms and potential therapeutic targets of synucleinopathies, emphasizing a translational approach led in the team "Physiopathology of Parkinsonian Symptoms" in the Institute of neurodegenerative diseases. The objective of this thesis work was to better understand the mechanisms implicated in these diseases, to find potential therapeutic strategies that allow us to translate these results from *in vivo* models to human patients. In this discussion, I will develop each aspect led during this thesis and their place in the current field of research, with both the novelties and questions that remain to be answered, before concluding on the entirety of this work. The two main aspects of my work were first to better understand the mechanisms implicated in α-syn, its propagation, and its ability to induce a new model of MSA, and secondly to identify new potential therapeutic strategies to reduce neurodegeneration and α-syn accumulation, via indirect or direct α-syn targeting. In the first project, I participated in an exploratory *in vivo* study aiming to decipher the effect of intrastriatal injection of patient-derived extracts from MSA patients. This study led to the development of a new mouse and non-human primate model of MSA, recapitulating an MSA-like pathology. In the second project, I focused on a therapeutic angle, where I challenged the same PD mouse model with multiple potential therapeutic strategies. The use of the same experimental model allowed for better comparison between these approaches and the determination of their efficacy *in vivo*. Together, these therapeutic strategies demonstrated a general ability to reduce neurodegeneration, with a variable profile alteration towards α-syn accumulation. Nonetheless, the last project opened the door to the potential problems we face in the field and questions to what the next steps should be in the future. Altogether, I chose to highlight in this manuscript the most important projects I participated in during this thesis and discuss their benefits and critical aspects in this last section.

Models of synucleinopathy

The different approaches used in this thesis implicate the use of animal models for synucleinopathies. First, the mechanistic approach to better understand MSA and its characteristics led us to develop a potential new model to study this synucleinopathy. Second, to test potential therapeutic targets, it is necessary to examine their benefits or drawbacks in an *in vivo* context. However, developing the correct models to study these two aspects remains one of the central challenges that are faced in the field.

With an increase in the study of synucleinopathies, the development of a multitude of models has escalated in the last few years. These models range from *in vitro* models, to rodents, to NHP, each with their own benefits and limitations. Though less close to humans both in anatomy and brain function, rodent models have been widely used for several reasons. The genetic variability between rodents used in laboratories is more controlled, combined with a quick reproduction and short lifespan are definite advantages for these animals. Nonetheless, they also present a brain anatomy that is quite different than ours, with little to no existence of the pre-frontal cortex, which plays an essential role in cognitive functions. In addition, for the study of age-related diseases, it is difficult to use rodents given their shorter lifespan, making it a challenge to create models associated with neurodegeneration. NHP, on the other hand, display a wide range of similarities with humans since they are genetically the closest species and have a long lifespan. NHP also present the advantage of having brains with the closest size, cell number, physiology, anatomy, and functions as humans. In addition to this, NHP produce neuromelanin, similarly to humans, in dopaminergic neurons of the SN (Vila, 2019). Studies have suggested that neuromelanin-positive dopaminergic neurons could be the first neurons to degenerate, making their presence in NHP that much more interesting. Once again, rodents do not have the ability to produce neuromelanin, confirming the need for NHP. Neuromelanin production has been induced in rat and mice models with the injection of AAV-tyrosinase, which has led to the development of models with dopaminergic neuron loss, cellular dysfunction and LB-like inclusions (Carballo-Carbajal *et al.*, 2019). This model, however, is once again difficult to compare to humans given the synthetic production of neuromelanin at high levels, which could itself be deleterious to rodents. NHP are also interesting due to their behavioral patterns, which are closer humans than those observed in rodents. However, NHP also present with drawbacks including their expense, controlled use in science, lifespan, and the time to develop diseases. They also present with inter-individual heterogeneity, similar to that seen in humans, but for scientific purposes, it makes studies difficult and there is usually a low number of individuals per group. This adds to the challenge of statistical relevance using NHP (Teil *et al.*, 2021).

For now, no one model has been shown to recapitulate synucleinopathies. Of course, certain aspects of these diseases are often demonstrated, such as the loss of dopaminergic neurons in the SN, some motor impairment, or an accumulation of α-syn species either in neurons or in oligodendrocytes. Nonetheless, no studies have been able to demonstrate all hallmarks of the disease, in particular the formation of LB or GCI *per se*. The presence of these inclusions

requires not only the overexpression of α-syn since classic overexpression models, via transgenic or viral vectors, do not suffice. It is highly possible that it is a combination of α-syn increase and spreading, cellular mechanism impairments, and dopaminergic vulnerability that each has a role to play in triggering the disease. In addition, there could be a plethora of factors that come into play that we do not yet know about. Thus, building models based solely on the acute overexpression of α-syn could be insufficient to model these diseases. This is complicated further by the fact that no two laboratories use the same models, or exact same techniques to induce the models. The variability between injections and quantities injected, but most importantly, in the nature of α-syn assemblies used for intracerebral injections are responsible for many differences between models. These differ in α-syn inoculum preparation (protein folding state(s), concentrations, composition, and purification protocol), α-syn inoculum purity (molecular size, sonication protocol, and storage), the concentration of α-syn injected, and the time post-injection. Together, these differences result in the presence of different anatomopathological features present *in vivo*. It is also important to note that no current consensus exists to identify the criteria of synucleinopathy in models in the field, making comparisons between models difficult. In fact, the readouts used by different laboratories are not always the same as certain teams focus more on aggregated α-syn, others on the presence of certain PTMs, others on the PK-resistance of α-syn. Most laboratories focus on one PTM: the phosphorylation at Ser129, but do not consider the other PTMs, including other phosphorylation sites or truncations of the protein. With such focus on one readout for PTMs, it is difficult to understand the implication of other PTMs and if they could be more adequate markers of pathological α-syn accumulation. Of course, this being said, we are also at fault for using these same markers in our studies, as they are what is recognized in the field as being the go-to pathological hallmarks.

The models presented in this manuscript rest on the injection of purified patient-derived fractions from either MSA or PD patients. Previously, the purification of LB from the midbrain of PD patients induced neurodegeneration and synucleinopathy in a large cohort of NHP (Bourdenx *et al.*, 2020). This was also confirmed when these same LB fractions were injected in wild-type mice in multiple studies (Bengoa-Vergniory *et al.*, 2020; Bourdenx *et al.*, 2020; Soria *et al.*, 2020). Given that the injection of LB fractions from PD patients induced a PD-like pathology, the question of the potential for GCI to induce a pathology similar to MSA was raised. Here, we aimed to resolve that question through the use of the same NHP species as used previously for LB injections, *Papio papio* baboons (Project 1).

With a purification protocol identical to that of the LB and noLB fractions, we extracted the GCI from the putamen of MSA-p patient brains. The site of injection and the age of injection of these NHP were also identical between studies. This allowed for protocols that were identical, with the exception of the material injected. These almost identical protocols were also used in the case of mice injected with LB or GCI fractions. However, much work remains to be done in this project, including the comparison of LB and GCI-injected monkeys to determine the effect of different inoculates at the same injection site. Concerning GCI-injected monkeys, an observation at earlier and later time points could give us an idea of the progression of the disease over time and help us understand the mechanisms behind this progression.

Given that these fractions come from patient brains, it could bring one to question the composition of the fractions injected and whether α-syn is the key player that induces neurodegeneration or not. Previous studies have demonstrated that these fractions are composed in large part by α-syn (90%), with the presence of other proteins present in LB, including Tau and Aβ. However, when LB fractions were double-immunodepleted of α-syn, they lost their potential to induce dopaminergic degeneration. These LB fractions were also injected in α-syn knockout mice and demonstrated no neurodegeneration either (Recasens *et al.*, 2014). Together, these combined results demonstrate the important role of both exogenous α-syn in brain fractions and endogenous α-syn in the injected species.

As seen in the introduction, the number of animal models for MSA is quite limited. In fact, only two types of potential models have been mainly developed for MSA: transgenic or recently viral-vector-based models (Lee *et al.*, 2019). These two models are based on a significant overexpression of the α-syn protein in oligodendrocytes specifically. These models have brought many insights into the development of MSA and the potential therapeutic targets that could be used to slow the progression of the disease. Certain studies have also aimed at investigating the effect of injecting MSA patient-derived extracts to understand whether this has the ability to induce an MSA-like pathology (Watts *et al.*, 2013; Prusiner *et al.*, 2015; Woerman *et al.*, 2020). These studies were done using TgM83 homozygous and hemizygous mice that were intracerebrally injected with brain extracts from MSA patients, leading to neurodegeneration and formation of α-syn positive inclusions after 3 months. Following this study, another study used injections of brain homogenates from sick M83 transgenic mice or human MSA patient extracts in TgM83 mice to demonstrate the transmissibility of α-syn species. This study showed a pathological accumulation in

transgenic mice that was higher when having received homogenates from sick M83 transgenic mice than from human MSA patients. These studies demonstrate the potential for patient brain extracts to induce MSA pathology and the significant discovery of the more potent effect of same species transmission. However, none of these results were observed in wild-type mice, which begs the question of a higher vulnerability of transgenic mice due to the overload of synuclein to these patient extracts compared to wild-type mice. In our hands, and with a different protocol for purification of MSA-patient brain extracts, we observed an effect in wild-type mice. This could potentially be explained by the potency of the inoculum, which in our case could be more pure due to the sucrose gradient purification compared to their more classic tissue homogenization.

In NHP, the studies on MSA have been extremely limited as well, and have been based solely on viral-vector-based approaches. The first potential indication of the effect of oligodendrocyte-specific overexpression of α-syn in NHP was by an AAV under the control of a ubiquitous promoter that infected oligodendrocytes specifically in the putamen (Mandel *et al.*, 2017). This pilot study was followed by a larger study that confirmed their initial observations of oligodendroglial α-syn accumulation, demyelination, and microglial activation after the injection of α-syn (Marmion *et al.*, 2020). Another study was based on the MBP-driven overexpression of α-syn in macaque, which did not lead to neurodegeneration or α-syn accumulation in the SN (Bassil *et al.*, 2017). Together, the development of MSA-like models in NHP has not been extensive. Our study allowed for another potential model for NHP to emerge amongst other studies, this time with the specificity of being a transmission-based model. Although we don't observe the formation of GCI that resemble those seen in human neuropathology, we do see prodromal signs of MSA pathology with an important loss of dopaminergic neurons and fibers in the SN and the striatum, respectively, along with significant demyelination and oligodendroglial loss.

One of the most puzzling aspects in synucleinopathies is the localization of α-syn aggregates either in neurons or oligodendrocytes and how these separate pathologies are triggered. With the observation that both LB and GCI fractions induce aspects associated with PD and MSA, respectively, the notion of pathological strains comes to mind. The difference in α-syn fibrillary structures in these two pathologies has started to be studied, but no consensus has yet to be reached. To resolve this problem, further work must be done to understand what structures are found in patient-derived fractions as well as oligomers. Few studies have done high-resolution structural characterizations, and the ones that have been carried out have

mainly used recombinant α-syn (Bousset *et al.*, 2013; Rodriguez *et al.*, 2015; Tuttle *et al.*, 2016). Currently, recent studies have used cryo-electron microscopy to better understand the structure of recombinant α-syn fibrils, but none have yet been done on patient-derived species (Guerrero-Ferreira *et al.*, 2018; Li *et al.*, 2018; Boyer *et al.*, 2019). One of the essential questions would be to determine the difference in pathological induction between these two different types of patient-derived fractions, with similar extraction and purification protocols, as well as a combination of cryo-electron microscopy or other high-resolution techniques. A comparison study between these induced pathologies could determine the potential existence of strains that exist in human patient brains, with short fibrils having been shown to be more toxic (Froula *et al.*, 2019; Lau *et al.*, 2020). This comparison should be made at multiple stages of the disease, to assess the modulations in α-syn species and their presence at different points during the progression.

Concerning MSA, the specific accumulation of α-syn in oligodendrocytes makes us wonder why and how much α-syn would be required to induce pathology in oligodendrocytes if not for the difference in conformation of α-syn aggregated structures. Another critical question that remains to be answered is where the aggregated α-syn in oligodendrocytes emerges from. In fact, oligodendrocytes have been shown to express very little α-syn, begging the question of how α-syn manages to accumulate in these cells (Miller *et al.*, 2005). Two contradictory theories are possible: one that relies on the increased expression of α-syn in oligodendrocytes, leading then to the formation of aggregates in these cells, the other relies on the transmission of pathological α-syn from other α-syn-expressing cells, such as neurons, to oligodendrocytes, thus leading to the formation of GCI (Asi *et al.*, 2014; Reyes *et al.*, 2014). Nonetheless, no convincing proof currently exists. To discern which hypothesis could be the correct one, we could consider trying more recent techniques such as RNAscope in patient brains to test the expression of oligodendroglial cells and α-syn without mentioning the single-cell transcriptomics approach (Chamling *et al.*, 2021). This question remains one of the central questions amongst synucleinopathies and answering it could indicate how α-syn is transmitted and how to halt the progression of this fatal disease.

Therapies based on metals

The implication of metals in synucleinopathies, and particularly in PD pathology, has been studied extensively. The most studied metal associated with PD has undoubtedly been iron.

Iron dyshomeostasis has been shown to be implicated in both the loss of dopaminergic neurons and α-syn accumulation. Given this implication, the use of chelators has been widely used in experimental studies to determine their potential to reduce iron accumulation and thus inhibit PD pathology (Carboni *et al.*, 2017). Studies have shown such promise that one specific iron chelator has made its way to clinical trials on PD patients to assess its effects (Devos *et al.*, 2020).

Compared to iron and copper, zinc has been studied very little but has nonetheless shown interest in the study of PD. Excess of zinc in the diet of rats has been shown to be able to induce a loss of dopaminergic neurons, the accumulation of α-syn, and responsiveness to L-Dopa (Kumar *et al.*, 2018). This ability to induce PD-like pathology implicated zinc directly in the development of pathology. With the implication of multiple metals in the progression of PD, a study was done to determine whether the concentrations of these heavy metals were altered in the SN of NHP having been injected with LB fractions. A synchrotron X-ray fluorescence-based analysis allowed for a precise measure of SN metal concentrations and showed that zinc levels were the only dysregulated metal concentrations of all metals measured. This increase in nigral zinc was showed to be a good predictor for neurodegeneration in an artificial network used in machine-learning (Bourdenx *et al.*, 2020). With this in mind, our study based on zinc modulation was a direct follow-up to this demonstration and aimed at further understanding these zinc variations.

The prion-like hypothesis and Braak staging cannot completely explain what we can observe in synucleinopathy patients. One other hypothesis relies on the potential cellular vulnerability of dopaminergic neurons, which makes these neurons more susceptible to degeneration (Surmeier *et al.*, 2017). Among the hypotheses that are discussed, one relies on the metabolism of dopamine via the production of neuromelanin in nigral dopaminergic neurons. Neuromelanin can stock heavy metals, particularly iron and zinc, which have been shown to be dysregulated in NHP injected with LB fractions (Zecca *et al.*, 1994; Bourdenx *et al.*, 2020). The dysregulation of metals in the SN, potentially in neuromelanin-positive neurons, could explain a part of the vulnerability observed in synucleinopathies.

To determine the effects of zinc, in particular in the reorganization of zinc within the cell, we wanted to use a metal ionophore. Clioquinol, a ionophore previously used as an antibiotic, had already been tested in several neurodegenerative diseases and demonstrated to decrease neurodegeneration in Alzheimer's, Parkinson's, and Huntington's disease models (Nguyen *et*

al., 2005; Lei *et al.*, 2015; Finkelstein *et al.*, 2016). However, clioquinol has the ability to bind several metals, predominantly zinc and copper, but has shown iron-binding properties as well. For this reason, it is important to estimate the changes in metals in the SN when using clioquinol to confirm the correct depletion in zinc concentrations in LB-injected mice (Project 2). In our study, this will be assessed using synchrotron X-ray fluorescence, for which the experiments have already been carried out. The analyses are ongoing and will allow us to determine whether there are any changes in the concentration of zinc or other heavy metals in the SN of clioquinol and zinc-supplemented diets.

Several studies have tried to understand the role of clioquinol within the cell and how it could have beneficial effects in neurodegeneration and even cancer. With the use of *in vivo* and *in vitro* experiments, multiple potential roles of clioquinol have emerged. One of the first identifications of the role of clioquinol was its role in autophagy. Through its zinc ionophore abilities, clioquinol is thought to be implicated in autophagy, in particular in cases of oxidative stress in cultured astrocytes (Park *et al.*, 2011). This molecule has demonstrated its ability to activate autophagy through the Akt/mTOR pathway in MPTP-lesioned monkeys (Shi *et al.*, 2020). Given this potential role in autophagy, we thought that the neuroprotective effects of clioquinol could be related to this ability in our PD mouse model. For this reason, we analyzed the effect of clioquinol and zinc-supplemented diets on our LB-injected mice. Results remained variable with a trend towards LC3-II accumulation and a significant increase of p62 in zinc-supplemented mice. Taking into account these results, we remained unconvinced that clioquinol was in fact, acting on autophagy clearance. However, the potential for clioquinol to act on autophagy could also be through the lysosome. Clioquinol has shown an ability to target zinc to the lysosome. Even though this lysosomal targeting has been shown to have the potential to activate autophagy, the dysfunction of lysosomes after LB-injection could be compensated by lysosomal zinc (Yu *et al.*, 2009). With this in mind, we wanted to look at the lysosomal function in LB-injected mice with clioquinol and zinc-treated mice. In LB-injected mice, we have already seen that lysosomal function is impaired by the localization of these organelles within the cell. When the lysosomes are functional, they are predominantly located around the nucleus, and their increased distance to the nucleus demonstrates their inactivity (Johnson *et al.*, 2016; Pu *et al.*, 2016). Our study demonstrated that when LB mice were treated with clioquinol, the number of lysosomes located close to the nucleus was increased compared to untreated LB-injected mice. This result determines one of the potential mechanisms of neuroprotection of clioquinol in our PD mouse model. Future

experiments should focus on the alterations in autophagy, potentially through the mTOR/Akt pathway, TFEB activation, and the verification of other zinc transporters or proteins that have been shown to be implicated in neurons (TRPML1, MT-3, TMEM163, and NLRP3). These changes could reflect how clioquinol is altering the zinc concentrations in complement to the results of our synchrotron X-ray fluorescence results.

Clioquinol has other potential targets in the synaptic transmission of zinc. The fact that clioquinol is a zinc ionophore indicates its ability to redistribute the zinc contained within the cell depending on cellular requirements (Ding and Lind, 2009). Zinc has demonstrated increased importance at the synapse and transmission has been implicated in correct brain function. With the decrease in ZnT3 expression in our LB-injected mice treated with clioquinol, we could hypothesize that this is related to the trafficking of cytosolic zinc towards synaptic vesicles. ZnT3 decrease could indicate a lower zinc concentration at the synapse, leading to potential neuroprotection. Combined with increasing lysosomal function, clioquinol could play a protective role in LB-injected mice through postsynaptic zinc signaling.

However, one of the potential issues that arise with the use of chelators or ionophores is the widespread effect of these molecules. In fact, these molecules are not specifically able to target one cell type or one organ. They can target most cells in the body, which makes them difficult to control and the dose at which they are used must be thinly adjusted. When used at concentrations that are too high, these metal-altering molecules can lead to deficits in metals, which could, in turn, lead to problematic consequences to the cell. Conversely, doses that are not high enough could be inefficient and defeat the purpose of their use. Therefore, the use of chelators must be extremely controlled before using on patients. Despite this challenging adjustment, chelators and ionophores remain very interesting to target metal dyshomeostasis in neurodegenerative diseases through their widespread approach and their ability to cross the blood-brain barrier.

α-Syn-based therapeutic strategies

With the rise of α-syn and its importance in synucleinopathies, therapies based on the direct or indirect targeting of α-syn have increased significantly. Multiple types of potential therapies have emerged between targeting α-syn synthesis, aggregation, clearance or

accumulation (Teil *et al.*, 2020). Concerning α-syn synthesis, most strategies rely on gene therapy with siRNA or miRNA, which have yet to be very successful, mostly given the difficulties of translating this technique in patients. However, silencing α-syn in the brain or the periphery completely could have serious complications given the physiological role of the protein. To decrease α-syn aggregation, many types of strategies have been tested between altering PTMs, using anti-oxidant strategies or small anti-aggregative molecules. These small anti-aggregation molecules have found interest in multiple studies and including clinical trials. Anti-oxidants such as coenzyme Q10 have also been found to be of interest and were also tested in three phases of clinical trials (Shults *et al.*, 2002; Yang *et al.*, 2009). In the case of PTMs, the difficulty lies in the contradictory observations of which PTMs are helpful or harmful, making the choice of targeting difficult. An indirect strategy to target α-syn has been predominantly by increasing clearance through autophagy or the proteasome. Given that these cellular mechanisms are impaired in an age-dependent manner and in synucleinopathies, these various strategies have proven to be interesting to enhance α-syn degradation. Several drug candidates to enhance these pathways have also led to clinical trials (Hebron *et al.*, 2013). Finally, one of the most studied potential strategies has been through immunotherapy with the development of antibodies targeting either oligomeric or aggregated forms of α-syn. Through all these various strategies, despite several having been moved to clinical trials, none have yet to be approved or make a significant difference in patients (Brys *et al.*, 2019; Schofield *et al.*, 2019; Shin *et al.*, 2019; Weihofen *et al.*, 2019; Lemos *et al.*, 2020). Given the difficulties faced with certain strategies, in particular gene-therapy-based targets, it seems that, with the right amount of resources, the strategies to focus on could be the use of anti-aggregation molecules, immunotherapies, and clearance-boosting approaches.

The first strategy we used involved a gene implicated in decreasing oxidative stress, increasing clearance, and promoting overall dopaminergic neuron survival. The NFE2L1 transcription factor has been shown to be decreased in PD, therefore indicating that increasing the expression of this transcription factor could be of interest in this pathology (Project 3). We know that NFE2L1 acts on promoting anti-oxidant response elements, which decrease the mitochondrial release of ROS. NFE2L1 is also implicated in promoting protein degradation via the proteasome (Villaescusa *et al.*, 2016; Taniguchi *et al.*, 2017). With these effects in mind, this project aimed at determining whether the increase in *Nfe2L1* could be neuroprotective in our LB-injected mice. We observed that NFE2L1 could in fact decrease the loss of dopaminergic neurons and trended to decrease the accumulation of PK-resistant α-

syn species. However, we have yet to go further in the examination of the exact mechanisms by which NFE2L1 was beneficial in LB-injected mice. For this, it will be necessary to determine whether oxidative stress was in fact decreased or the proteasome activity increased. Determining the mechanisms of action of NFE2L1 efficacy could be a pathway to finding targets that could be altered via molecules *in vivo*.

This study also brought unexpected results with the observation that in noLB-injected mice, NFE2L1 seemed to decrease the survival of dopaminergic fibers in the striatum without affecting the neurons in the SN. We suspect that this could be linked to the combined toxicity of the virus with noLB fractions. These noLB fractions are composed of small aggregates of α-syn and have previously been demonstrated to be non-toxic when injected in mice. However, the combination of these small aggregates with the virus could prove to add toxicity to these species when injected into mice.

The potential of NFE2L1 should also be assessed in another *in vivo* model, which is already a project underway. In fact, this project is in collaboration with the laboratory of Chris Rochet, whose team will inject the same batch of virus in an AAV-A53T-α-syn rat model to determine the efficacy of NFE2L1 in a viral-based model. The combination of the data between these two models should be able to give us a better notion of the ability of NFE2L1 to be a potential interesting therapeutic target. However, given the data that we have observed with anle138b, it would be necessary to test this in at least one other model in addition to the AAV-A53T-α-syn rat to have a better representation of the efficacy of this overexpression. We should also verify the benefits of increasing NFE2L1 expression with a delayed start compared to the injection of LB fractions. As seen with anle138b, the time at which the treatment is administered can influence the efficacy. This first demonstration of the therapeutic potential of NFE2L1 is important, but not sufficient to ensure that it could be a valid target to decrease dopaminergic loss and α-syn accumulation. Furthermore, viral-based therapies have yet to be well developed and require much adjustment. The modulation of NFE2L1 expression seems to be a difficult feat to achieve, at least for now.

Another strategy we used has involved the use of small anti-aggregative molecules, which includes anle138b (Project 4). Anle138b has shown to be efficient in multiple animal models to decrease neurodegeneration and α-syn accumulation, including transgenic and toxin models for both PD and MSA. Our study aimed at assessing the efficacy of anle138b in our LB-based model with a concomitant and a delayed-start treatment. The delayed start

treatment was meant to determine the efficacy of anle138b after the beginning of the installment of the disease compared to the concomitant start treatment, which was to determine the efficacy of anle138b to inhibit the development of the disease. The delayed start treatment resembles more closely what can be seen in clinical trials in early diagnosed PD patients. Of course, it is essential to note that our LB model is progressive and after 2 months, the disease onset is not as far along as what can be seen in patients. Nonetheless, the question of the anti-aggregative ability of anle138b in this model has yet to be determined. In these LB-injected mice, we observed that a concomitant treatment with anle138b could reduce nigral neurodegeneration and decrease slightly PK-resistant α-syn accumulation. On the other hand, with a delayed-start treatment, no alterations in dopaminergic cell loss were observed in the SN compared to untreated LB-injected mice, and there was even an increase in the levels of PK-resistant α-syn. These observations demonstrate the lack of efficacy of the delayed-start treatment compared to the concomitant treatment. One potential explanation to this lack of efficacy in the delayed start could be that the window of opportunity for anle138b to reduce α-syn aggregation has passed and the α-syn accumulation is already too important. Thus, the neurodegeneration induced by these patient-derived extracts could have already been initiated and there is no way of turning back. Concerning the concomitant start, the simultaneous anle138b treatment with the patient-derived extracts could inhibit the beginning of spreading of α-syn and neurodegeneration, explaining its beneficial effects.

When put into perspective with other studies that have been conducted using anle138b, the varying results in our study compared to others are important to note. In fact, most studies have shown, in different models, the potential for anle138b to decrease neurodegeneration and α-syn aggregation (Wagner *et al.*, 2013; Levin *et al.*, 2014; Fellner *et al.*, 2016; Heras-Garvin *et al.*, 2019; Wegrzynowicz *et al.*, 2019). The differences that we observe put forth the interest of studying one same molecule on different models of pathology. As previously stated, no two models recapitulate the same aspects of disease. Certain models have higher accumulation and pathological forms of α-syn, others focus more importantly on the neurodegenerative aspects combined with motor impairments. The catalog of models allows a comparison of potential therapeutic strategies on models based on different approaches and with varying neuropathological hallmarks. The testing of the same drug candidate in different models allows a better understanding of the potential benefits of the candidate. With this study, the lack of effect of anle138b in our model with a delayed start as compared to the concomitant start demonstrates perfectly the interest of different approaches. Previously,

delayed start administration of anle138b proved to be efficient in a transgenic PD model (Levin *et al.*, 2014). The discrepancies between our study and previous studies effectively demonstrate the interest in testing one same drug in multiple models.

Clinical trials are initiated based predominantly on the efficacy of certain candidates in pre-clinical animal models. Given that no one model recapitulates synucleinopathy in its entirety and most mechanisms implicated in disease onset have yet to be elucidated, it seems difficult to base a patient-based clinical trial on its effectiveness in one or two animal models. To start clinical trials that have potential, it seems increasingly important, with the facts previously discussed in this section, that we take a step back to determine the true potential of therapeutic approaches. For this, we should take into consideration the imperfect models that we use and test one candidate in multiple models based on a combination of different approaches including transgenic, viral-mediated, patient-derived fraction or toxin models. By challenging one molecule's efficiency in all these models, with an additional preference for delayed-start treatments, we could have a better indication of their pre-clinical potential.

Another challenge that we face with therapeutic approaches is the latency before testing these molecules in actual patients. Indeed, before diagnosis of synucleinopathies is even given, neuropathology has already progressed enough for motor symptoms to arise, and in the case of MSA this is not a definite diagnosis. With this late diagnosis, the effect of therapeutic strategies must be very efficient mostly in halting the already advanced progression of the disease. This also restricts the ability to stop early progression of the disease and inhibit the development of motor symptoms. Achieving this early diagnosis requires the development of correct biomarkers that could represent earlier stages of the disease. This quest for biomarkers has been of great interest in the field and a global effort. However, the discovery of one or several biomarkers has yet to be efficient to allow an early diagnosis, in particular given the heterogeneity between patients with PD, MSA or DLB.

We are at a turning point in the search for both understanding the mechanisms and the search for therapeutic strategies to slow the progression of synucleinopathies. In the next years, we should try and focus some of our energy on key points of mechanistic understanding of these diseases. For this mechanistic approach, the key lies potentially in using a combination of models and species, with viral-vector based model, PFF models, and patient-derived extracts. Future therapeutic strategies could require not just one but multiple different approaches to help in halting disease progression. We could imagine that these next therapies could involve

a combination of targets. For example, a combination of increased clearance through autophagy with an anti-aggregative molecule targeting α-syn could be a bridge to increase the neuroprotective effects of these strategies. Of course, this should involve a collaborative effort on the part of different teams to access more techniques and more animal models to put this in place.

CONCLUSION

In this thesis manuscript, we have discussed the central role of α-syn in the development of synucleinopathies as well as potential therapeutic approaches that target α-syn directly or indirectly. The first part of this thesis demonstrated the ability of α-syn containing MSA patient extract to induce a neuropathology spectrum similar to what we see in patients. This confirms the crucial importance of α-syn in the induction of this disease among the other synucleinopathies. This work was then continued by challenging our PD mouse model to various therapeutic approaches. Targeting both α-syn, directly and indirectly, demonstrated the importance of these two approaches throughout these projects.

Through my participation in these various projects, and through my participation in several review articles, I had the opportunity to better comprehend the gaps that exist in synucleinopathies. In fact, certain problems are faced throughout the field, but particular attention must be paid to the different models that are used, as well as how we approach the development of therapeutic strategies. This work has highlighted the importance of developing the correct models to therefore better assess future therapeutic approaches. The role played by α-syn is clearly seen in most studies in this field of research, but also throughout this manuscript. The similarities in α-syn dysregulation in synucleinopathies could lead to certain molecules that would be efficient for multiple synucleinopathies. Therefore, developing strategies that could target α-syn directly and indirectly through the modulation of cellular mechanisms impaired in synucleinopathies could be a gateway to finding efficient strategies. This approach has already been observed in a recent clinical trial assessing the impact of one same antibody on both PD and MSA (Meissner *et al.*, 2020; Volc *et al.*, 2020). However, given the complexity of these synucleinopathies, it seems unlikely that just one agent could be developed that could have diverse effects and bring to a halt the progression of the disease. As is the case for certain viruses or in cancer treatments, the potential solution would be a combinatory approach that would target several aspects that are affected in these diseases. As also demonstrated in this manuscript, one of the central problems we face is the late diagnosis of patients, which highlights the need to develop the correct biomarkers for earlier treatment. Although much work remains to be achieved at different levels in parallel, we should continue to better understand the role that is played by α-syn by itself and the community is beginning to understand the need for common ground to be found so that we should reinvent ourselves and take steps forward in the treatment of synucleinopathies.

DIDACTIC ARTICLES

cells

MDPI

Review

Autophagy in Synucleinopathy: The Overwhelmed and Defective Machinery

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Received: 28 May 2019; Accepted: 8 June 2019; Published: 9 June 2019

Abstract: Alpha-synuclein positive-intracytoplasmic inclusions are the common denominators of the synucleinopathies present as Lewy bodies in Parkinson's disease, dementia with Lewy bodies, or glial cytoplasmic inclusions in multiple system atrophy. These neurodegenerative diseases also exhibit cellular dyshomeostasis, such as autophagy impairment. Several decades of research have questioned the potential link between the autophagy machinery and alpha-synuclein protein toxicity in synucleinopathy and neurodegenerative processes. Here, we aimed to discuss the active participation of autophagy impairment in alpha-synuclein accumulation and propagation, as well as alpha-synuclein-independent neurodegenerative processes in the field of synucleinopathy. Therapeutic approaches targeting the restoration of autophagy have started to emerge as relevant strategies to reverse pathological features in synucleinopathies.

Keywords: Parkinson's disease; multiple system atrophy; synucleinopathy; α-synuclein; macroautophagy; chaperone-mediated autophagy; mitophagy; neurodegeneration

1. Introduction

Synucleinopathies are neurodegenerative diseases characterized by the presence of alpha-synuclein (α-syn)-positive intracytoplasmic inclusions in the central nervous system (CNS), such as Parkinson's disease (PD), multiple system atrophy (MSA), and dementia with Lewy bodies (DLB). PD is the most prevalent synucleinopathy worldwide, with 1–5% of the population over the age of 60 developing this disease [1]. PD is clinically characterized by a triad of motor symptoms (resting tremor, rigidity, and akinesia) caused by dopaminergic striatal depletion due to the loss of dopaminergic neurons in the substantia nigra (SN). The major neuropathological hallmark of PD is the presence of α-syn-positive intracytoplasmic inclusions called Lewy Bodies (LB), which accumulate in neurons [2,3]. DLB is the second most prevalent synucleinopathy, clinically characterized by parkinsonian syndrome associated with cognitive deficits. DLB patients exhibit neurodegeneration in the SN, as well as LB deposition in the brain, but DLB differs from PD by the presence of amyloid plaques in cortical areas [2]. MSA is a rarer synucleinopathy, affecting 1–9/100,000 persons worldwide, divided into two clinical phenotypes: the MSA-parkinsonian with levodopa non-responsiveness parkinsonian syndrome due to neurodegeneration in the nigro-striatal pathway, and the MSA-cerebellar with gait, speech, and limb ataxia, as well as oculomotor dysfunction, due to neurodegeneration in the olivo-pontocerebellar system. As part of synucleinopathy, MSA patients' brains exhibit α-syn-positive intracytoplasmic inclusions, which accumulate mainly in the oligodendrocytes, called glial cytoplasmic inclusions (GCI) [2].

Although synucleinopathy regroups heterogenous clinical disorders, the neuropathological common denominator is the accumulation of the 140-amino acid protein, α-syn, in different brain regions of PD, DLB, and MSA patients. The α-Syn accumulation leads to the formation of misfolded α-syn species that aggregate in the cytoplasm, forming LB and GCI. Although the trigger phenomenon remains to be elucidated, three cellular factors have been suggested to play a role in α-syn aggregation: (i) increased expression of the *SNCA* gene encoding α-syn, (ii) post-translational modifications or mutations favoring α-syn aggregation, and (iii) impaired α-syn protein degradation. The α-Syn degradation is ensured by both the ubiquitin-proteasome system [4] and by the autophagy-lysosomal pathways (ALP) [5] protein degradation machinery. The signals responsible for targeting α-syn to either degradation pathway remain not fully understood.

ALP was shown to be implicated in the degradation of monomeric, small, soluble oligomeric species, as well as aggregated forms of the α-syn protein. ALP is a cellular proteolytic system which allows the degradation of long-lived proteins, protein aggregates, and abnormal organelles through both macroautophagy (MA) and chaperone-mediated autophagy (CMA) processes [6]. MA degrades cellular waste after the fusion of the autophagosomes carrying the material with the lysosome containing the enzymatic material [6]. CMA is a selective pathway that degrades proteins after recognition of pentapeptide (KFERQ-like motif) sequence by the cytosolic chaperone heat-shock cognate 70kDa protein (Hsc70) and delivery to the lysosome [6]. Here, we review clinical and experimental evidence focusing on PD and MSA, suggesting that defects in ALP machinery actively participate in α-syn accumulation in synucleinopathies and neurodegenerative pathological process.

2. Clinical Evidence of Autophagy Implication in Synucleinopathies

2.1. Genetic Evidence of Autophagic Involvement in Familial PD Cases

Genetic studies have been conducted for PD identifying familial forms of the disease representing approximately10% of the PD cases with hereditary parkinsonism and earlier onset of the disease.

Among PD-linked genes, the first autosomal dominant mutation identified for PD was localized in the *PARK1/SNCA* gene encoding for the α-syn protein. This point mutation constitutes the A53T substitution in the *SNCA* gene, identified in Italian and Greek families [7]. In 1998, Kruger and collaborators identified the A30P point mutation in a German family [8] and the E46K point mutation was described in a Spanish family in 2004 by Zarranz and colleagues [9]. All these three *SNCA* point mutations were associated with α-syn accumulation and PD development, and were suggested to participate in autophagy impairment [10–12]. The autosomal dominant mutations in the *PARK8* gene encoding the leucine rich repeat kinase 2 protein (LRRK2) were reported to be the most known genetic cause of familial PD cases [13]. As LRRK2 is composed of multi-domain proteins, mutations in *PARK8* gene induce neurodegeneration by alteration in multiple cellular pathways, including the autophagy machinery [14]. The G2019S point mutation is the most common *PARK8* mutation, accounting for 5% of familial cases worldwide, and up to 42% of familial cases from North African Arab patients [15]. This mutation affected the kinase domain of the protein leading to impaired autophagy degradation. In 2006, two autosomal recessive mutations in the *PARK9* gene encoding for a lysosomal ATPase cation transporter (ATP13A2) in a Chilean family with early-onset Parkinson disorder were reported [16]. The ATP13A2 protein physiologically colocalizes with the lysosomal-associated membrane protein 2 (LAMP-2). The PD heterozygous *PARK9* mutations lead to an unstable truncated ATP13A2 protein, which is abnormally retained at the endoplasmic reticulum (ER) before its degradation [17]. Regarding genes involved in the CMA process, an autosomal recessive mutation in the *DNAJC6* gene, encoding DnaJ heat shock protein (Hsp) family member C6, was initially found in two members of a family in Palestine and was associated with fast-progressing parkinsonian syndrome [18]. Subsequent follow-up studies reported other *DNAJC6* mutations in families with very early-onset parkinsonism [18]. More recently, several groups also reported heterozygous mutations in the DnaJ heat shock protein family member C13 (*DNAJC13*) gene leading to pathological features resembling idiopathic PD [19].

Related to mitophagy-linked genes, *PARK2* gene encoding the E3-ubiquitin ligase protein Parkin was the second identified PD-linked gene with a large spectrum of autosomal recessive mutations observed in different familial cases [20–25]. Most of the *PARK2* mutations are deletions affecting the ubiquitin-like domain of the protein, disturbing the stabilization of other cellular proteins. The second mitophagy-linked gene constituted by *PARK6* gene mutations encoded the phosphatase and tensin homolog-induced putative kinase 1 (PINK1) protein is found in 1–9% of familial PD cases [26–28]. The majority of *PARK6* mutations affected the kinase domain of the protein, leading to a loss-of-function of the protein, physiologically involved in eliminating damaged mitochondria. Finally, autosomal recessive mutations in *PARK7* gene encoding the DJ-1 protein are rarer and lead to its reduced antioxidant activity [29,30].

Besides PD-linked genes, genome-wide association studies pointed to some polymorphisms in genes that are associated with increased risk of developing PD. Among them, the *GBA* gene encoding the lysosomal enzyme β-glucocerebrosidase (GCase) and involved in glycolipid metabolism is a well-validated PD-associated risk factor [31]. *GBA* mutations cause an inherited autosomal recessive lysosomal storage disorder, known as Gaucher disease (GD), characterized by a loss-of function of the GCase protein leading to accumulation of glucocerebroside in different organs, such as the liver, the blood, the spleen, the lungs, and the nervous system. Interestingly, 8–14% of PD patients carried mutations in their genome, suggesting an increased risk of developing PD [32]. Similarly, patients with GD carrying heterozygous *GBA* mutations exhibited higher risks of developing PD [33]. Strikingly, a recent study discovered a significant burden of new susceptible loci, likely damaging lysosomal storage disorder gene variants in association with PD risk, reinforcing the importance of lysosomal mechanisms in PD pathogenesis [34].

As no genetic forms or polymorphisms have been reported in the literature so far for MSA, no genetic evidence regarding this other synucleinopathy can be reviewed here. Nevertheless, genetic studies on PD already indicate the potential role of autophagic processes into PD pathogenesis.

2.2. Post-Mortem Evidence of Autophagic Involvement in Synucleinopathies

Post-mortem studies have provided numerous pieces of evidence of autophagy impairment in pathologically affected areas of the brains of MSA and PD patients compared to age-matched controls. In 1997, Anglade and collaborators already observed an impairment in the autophagic process, noticing a drastic decrease of autophagic vacuoles in the nigral dopaminergic cells on PD post-mortem brains [35]. The most striking example highlighting an ALP impairment associated with α-syn accumulation in synucleinopathies is the presence of the microtubule-associated protein 1A/1B-light chain 3 (LC3), an autophagosomal marker, within the LB of PD brains and within the GCI of MSA brains. In 2010, different groups showed that LC3 colocalized with LB and Lewy Neurites (LN) in the SN of PD patient brains [36], with an estimation of up to 40% of cortical LB [37] and up to 80% of nigral LB [38] being positive for LC3 in PD patients. Post-mortem studies on DLB patients—another synucleinopathy showing α-syn positive inclusions in cortical and cognitive areas—further corroborated this data, demonstrating co-immunostaining of LC3 with LB in the hippocampus [39] or in the temporal cortex [40]. Co-staining of LC3 and phosphorylated forms of α-syn in GCI were also observed in the oligodendrocytes of MSA brain patients [41], with up to 84% of colocalization found in pons tissue [42]. Taken together, these results suggest the accumulation and defective clearance of autophagosomes in synucleinopathies. In PD cases, Dehay and coworkers also observed that the transmembrane lysosomal transporter ATP13A2 is a component of LB in the SN of PD cases, as evidenced by 90% of ATP13A2 immunoreactivity in LB-positive dopaminergic neurons of the SN [43]. The autophagic regulator protein Beclin-1 was shown to be caspase-cleaved and increased in PD brains [44]. Finally, a more systemic autophagy impairment occurs in synucleinopathy, as both MA and CMA activity are decreased in cultured peripheral blood mononuclear cells of PD patients [45].

At a cellular level, alterations in the ALP could be explained by a decreased expression of the ALP transcription factor LIM homeobox transcription factor 1 beta (LMX1B) observed in melanized

dopaminergic neurons of post-mortem PD brains [46]. Among lysosomal components, the amount of the lysosomal-associated membrane protein 1 (LAMP-1) [47] and the ATP13A2 protein [43,48] are reduced in PD patients in comparison with age-matched controls, suggesting a defect in the MA process in PD pathogenesis. Corroborating this data, Dehay and colleagues described an impairment in the lysosomal-mediated clearance of autophagic vacuoles with an accumulation of autolysosomes containing non-degraded material in fibroblasts derived from PD patients harboring ATP13A2 loss-of-function mutations [43]. Pontocerebellar fibers of MSA patients showed increased staining of LAMP-1 and LAMP-2 with abnormal random distribution into the cytoplasm compared to control tissues, suggesting an MA impairment that is also involved in MSA pathogenesis [49]. Tanji and colleagues further demonstrated a decreased expression of the *GABARAP* gene encoding for the Gamma-aminotubyric acid receptor-associated protein and the *GABARAPL2* gene encoding for the Golgi-associated ATPase enhancer of 16KDa (GATE-16), which both play important roles in autophagosome formation associated with impaired maturation of the proteins in the cerebellum of MSA patients [41]. Finally, Compagnoni and colleagues demonstrated an autophagic activation with LC3-II accumulation in the cytoplasm of iPSCs derived from MSA patient skin fibroblasts associated with defect in lysosomal clearance [50], confirming impairment on the MA pathway.

Regarding the CMA pathway, Alvarez and colleagues demonstrated decreased protein levels only for the isoform type A of LAMP-2 (LAMP-2A) rate-limited protein for CMA process in the SN and the amygdala of PD patients, which is associated with decreased Hsp70 protein levels [36]. Corroborating this data, Murphy et al. (2015) showed that decreased levels of LAMP-2A and Hsc70 were associated with α-syn accumulation at the early stages of PD [51], and α-Syn protein was also shown to be colocalized with Hsc70 in the oligodendrocytes from MSA brains [52], confirming an impairment in the CMA machinery.

Finally, alterations in lysosomal enzymatic activities, involved in both MA and CMA processes, have been also described in post-mortem brains of patients suffering from synucleinopathies. The lysosomal GCase enzyme activity was shown to be decreased in the cerebellum, the amygdala, the putamen, and the SN of PD brains from *GBA* mutation carriers [53]. Regarding sporadic cases, its activity is reduced in the cerebellum and in the SN by 24% and 33%, respectively [53], and in the caudate nucleus [54]. Its decreased activity was found to correlate with an increased α-syn amount at the early stages of the disease [55]. GCase activity was also demonstrated to be decreased in the cerebrospinal fluid (CSF) of PD patients compared to controls [56,57], as well as a decrease in α-amminosidase [50,56] and α-fucosidase [58] activity and increase in β-hexosaminidase [57] and β-galacosidase [58] activity. Reduced activity of lysosomal hydrolase Cathepsin D (CathD) was observed in the SN of PD patients [47] or in ATP13A2 mutant fibroblasts derived from PD patients associated with defective processing of cathepsins [43]. In MSA cases, alterations in hydrolases were also observed with increased levels of CathD found in the pontocerebellar fibers [49] and in the white matter of the cerebellum [41].

In conclusion, post-mortem analysis of PD and MSA patients pointed out the ALP, macroautophagy, as well as CMA as be part of α-syn accumulation and neurodegenerative processes in synucleinopathies.

3. Dual-loop Between Autophagy and α-Syn Pathogenesis

Multiple pieces of evidence suggested that defective ALP processes could participate in α-syn accumulation, aggregation, and propagation into neuronal cells, thus confirming the role of deficient autophagy machinery in synucleinopathy.

3.1. ALP Processes Participate in α-Syn Accumulation and Aggregation

As suggested by genetic and post-mortem studies, ALP machinery is impaired in synucleinopathies and seems to be involved in α-syn accumulation. Of interest, multiple studies have provided evidence that ALP participates in α-syn degradation, in addition to the ubiquitin proteasome system.

In an α-syn transgenic mouse model, Klucken and collaborators observed the colocalization of α-syn positive inclusions with autophagic markers, such as LAMP-2 and LC3, as observed in patients' brains [59]. In fibroblasts derived from PD patients harboring a loss-of-function mutation in the *PARK9* gene, an accumulation of α-syn protein was observed in parallel with the accumulation of lysosomes and decreased proteolytic activity [43,60]. Knocking-down ATP13A2 expression in primary cortical neurons also enhanced accumulation of endogenous α-syn protein in addition to lysosomal degradation impairment, which can be reversed in part by the expression of wild-type (WT) ATP13A2 in ATP13A2 knockdown cells [60], confirming that α-syn is degraded by the lysosomes. Tsunemi and collaborators showed that *PARK9* deficiency leads to defective lysosomal exocytosis through impaired lysosomal Ca^{2+} levels contributing to intracellular α-syn accumulation [61]. This study suggested that ATP13A2 regulated neuronal lysosomal exocytosis, which modulates α-syn intracellular levels [61]. Recently, Sato and collaborators generated a Tyrosine-Hydroxylase cell-specific Atg7 conditional knock-out (KO) mouse model that presented α-syn accumulation and LB-like inclusions (positive for α-syn, p62, and ubiquitin) after autophagy impairment [62]. Thus, α-syn is degraded by ALP processes and several studies aimed to determine the contribution of MA and CMA pathways in α-syn degradation.

In PC12 cells that overexpress WT or mutated forms of α-syn (A30P, A53T), Webb and colleagues demonstrated that the induction of MA using the mTOR inhibitor rapamycin leads to an increased clearance of both WT and mutated forms of α-syn [63]. Conversely, increased levels of intracellular α-syn were observed after inhibition of the autophagosomal formation using the PI3K inhibitor 3-methyladenine (3-MA) or inhibition of the fusion of the autophagosomes with the lysosomes with BafilomycinA1 (Baf) [63,64]. In neuronal cells overexpressing WT α-syn, de novo α-syn aggregates colocalize with lysosomal markers CathD and LC3 [65]. Overexpression of Beclin-1 in this cellular model and in a transgenic α-syn mouse model leads to a decrease of α-syn aggregates but not monomeric forms, confirming an autophagic role in α-syn aggregate clearance [64,65]. On the contrary, Beclin-1 or Atg5 silencing in A53T-α-syn expressing neuroblastoma M17 cells induced a significant increase of α-syn oligomers and aggregates [66]. Altogether, these results suggest that the MA system is involved in clearance of α-syn oligomers or aggregates.

Interestingly, another study showed that clearance of human α-syn-aggregates induced by rotenone exposure to COS-7 cells is decreased after lysosomal inhibition using Baf treatment, but not after macroautophagic inhibition using 3-MA treatment [64]. Likewise, Cuervo and colleagues also demonstrated that rat ventral midbrain dopaminergic neurons exposed to 3-MA did not inhibit α-syn degradation, whereas a significant increase of α-syn half-life was observed after exposure to the lysosomal proteolysis inhibitor ammonium chloride [67]. These observations suggested that even if lysosomes are clearly involved in α-syn degradation, a non-macroautophagic pathway also took part in α-syn aggregates clearance [64]. Interestingly, the α-syn protein sequence contains the CMA-recognition motif VKKDQ [68]. Mutations in the CMA recognition motif of the α-syn protein sequence dramatically decreased its translocation to the lysosomal lumen [67]. In the same line of evidence, knocking-down the rate-limited CMA receptor LAMP-2A in rat embryonic cortical neurons increased detergent-soluble and -insoluble α-syn levels [69]. In vivo LAMP-2A silencing in dopaminergic cells in a rat model also induced autophagic defects with accumulation of autophagic vacuoles surrounded by α-syn punctates, which were positive for ubiquitin [70]. Altogether, this emphasized that CMA could be the non-macroautophagic pathway contributing also to α-syn degradation.

Additionally, the expression of micro RNA (miRNA) targeting LAMP-2A or Hsc70 CMA proteins in SHSY5Y cells also led to α-syn accumulation after defects in CMA [71,72]. Interestingly, these miRNAs, such as miR106a, miR320a, and miR301 (for Hsc70), or miR224 and miR373 (for LAMP-2A) levels are upregulated in the SN of PD patients, suggesting a CMA-mediated α-syn clearance defect in pathological conditions that could be involved in their accumulation and aggregation. Further confirming these results, addition of the chaperone protein DJ-1 to the α-syn solution inhibited the production of α-syn protofibrils, whereas the addition of L166P parkinsonism mutated DJ-1 failed to

inhibit the generation of α-syn protofibrils [73]. Knocking-out DJ-1 chaperone protein in SHSY5Y cells or in the parkinsonian MPTP mouse model induced increased levels of α-syn oligomers associated with decreased Hsc70 levels [74]. This suggested that DJ-1 deficiency enhanced the accumulation and aggregation of α-syn through a decrease of CMA-mediated degradation. Regarding other PD-linked proteins, loss-of-function mutations in the *GBA1* gene increased α-syn oligomeric accumulation, as demonstrated in vitro [75–77]. Likewise, decreased GCase activity after β-epoxide treatment in vitro [78] or in a GD rodent model [76,77,79] enhanced pathological α-syn accumulation and aggregation. Altogether, these data further highlighted the contribution of CMA impairment in pathological α-syn accumulation and aggregation. Altogether, these data further highlighted the contribution of CMA impairment in pathological α-syn accumulation and aggregation.

A30P and A53T mutated forms of α-syn seemed to be specifically degraded by the MA process, as they were poorly internalized to the lysosomal lumen despite their high affinity to the LAMP-2A receptor [67]. This induced a compensatory toxic gain-of-function activation of the MA process to counteract the CMA blockade induced by these mutated forms [67,69], which was not sufficient to maintain α-syn at physiological levels.

Altogether, these data suggest that α-syn is a macroautophagic and a CMA substrate (Figure 1a). Impairment of ALP in pathological conditions participates in α-syn accumulation and aggregation, as observed in synucleinopathies (Figure 1b).

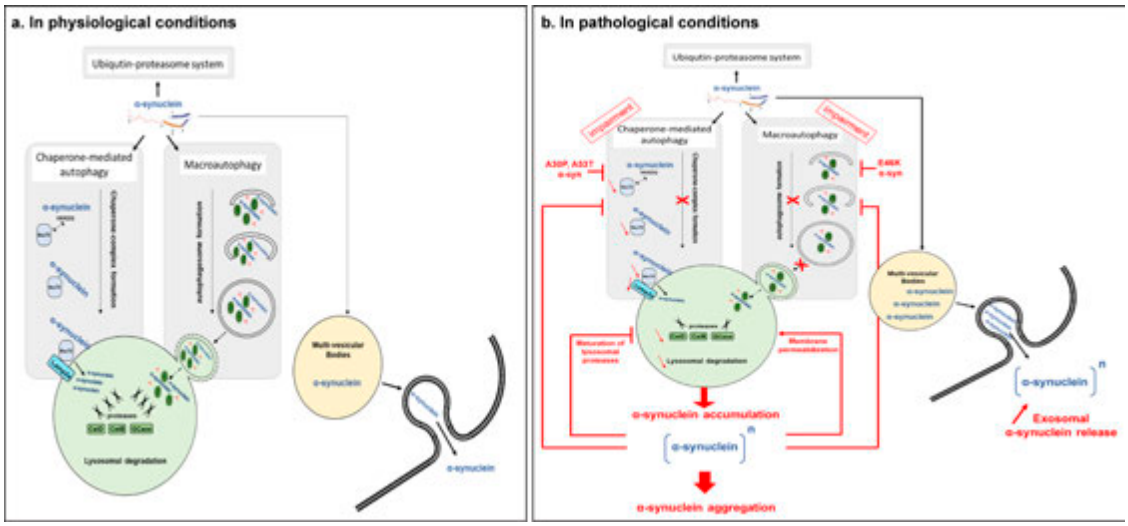

Figure 1. Dual loop between autophagy lysosomal pathway and α-synuclein. (**a**) In physiological conditions, autophagy machinery participates in α-synuclein degradation through both macroautophagy and chaperone-mediated autophagy. The intracellular α-synuclein levels are maintained at basal conditions and exosomal α-synuclein release is low. (**b**) In pathological conditions, autophagy impairment leads to decreased α-synuclein clearance. Intracellular α-synuclein accumulates in the cells and participates in the α-synuclein aggregative process. To compensate for this defect, the cell attempts to eliminate α-synuclein through the exosomal pathway, inducing increased released α-synuclein amounts and cell-to-cell propagation of the protein. Reciprocally, accumulated pathological α-synuclein induces cell toxicity mediated in part by autophagy impairment, diving the cell into an endless cycle of dyshomeostasis. Hsc70: cytosolic chaperone heat-shock cognate 70kDa protein; CatD: Cathepsin D; CatB: Cathepsin B; GCase: β-glucocerebrosidase.

3.2. ALP Pathways Participate in α-Syn Propagation

The prion-like hypothesis of α-syn in synucleinopathies suggests that it is able to propagate by cell-to-cell transfer into the CNS, as described in graft post-mortem studies [80–83] or in different experimental models [81,84,85]. Among the possible routes of propagation, membrane-bound vesicles of endocytic origin called exosomes were suggested to carry α-syn from one donor cell toward a recipient one [86]. Interestingly, α-syn protein was found to be increasingly associated with vesicle fractions from human CSF of LBD and PD patients compared to control subjects [87]. Isolation of exosomes from WT α-syn overexpressing SHSY5Y conditioned medium confirmed this association between α-syn and exosomes [88]. Incubation of naïve SHSY5Y cells to these α-syn-positive-exosomes showed the transmission of α-syn to the recipient cells [88,89]. The integrity of the

from human CSF of LBD and PD patients compared to control subjects [87]. Isolation of exosomes from WT α-syn overexpressing SHSY5Y conditioned medium confirmed this association between α-syn and exosomes [88]. Incubation of naïve SHSY5Y cells to these α-syn-positive-exosomes showed the transmission of α-syn to the recipient cells [88,89]. The integrity of the exosomal compartment is essential for α-syn transmission, as its membrane disruption prevents the α-syn transmission to the donor cells [88,89]. Several studies confirmed this exosomal-mediated cell-to-cell α-syn transmission in vitro using different sets of co-culture techniques [89,90].

Interestingly, inhibition of autophagy using chemical treatments or Atg7-KO cells led to a significantly increased α-syn release in the conditioned media, and particularly in the exosomal fractions associated with increased levels of α-syn in recipient cells [88–90]. In contrast, activation of autophagy using rapamycin induced significant lower levels of α-syn exosomal release [88–90] and cell-to-cell transfer of α-syn in vitro. Systemic treatment of α-syn transgenic mice with Baf increased α-syn levels in the CSF, as well as neuronal death and neuroinflammation [91]. Likewise, injection of CSF-derived exosomes from LBD patients into mice brains increased the occurrence of α-syn-positive inclusion bodies in neurons, suggesting that the delivery of exosomal α-syn induces synucleinopathy [87]. These data suggest that autophagy impairment caused increased α-syn secretion and enhanced cell-to-cell transmission of α-syn via release of non-degraded α-syn-containing exosomes.

Further confirming this hypothesis, Tsunemi and collaborators demonstrated that fibroblasts from patients carrying *PARK9* mutation produced higher amounts of multivesicular endosomes with sparse intraluminal vesicles, which represent exosome precursors, suggesting that the ATP13A2 protein may control the exosomal process [92]. Using ATP13A2 overexpression in neuroblastoma H4 cells and primary neuronal cultures, they showed that α-syn levels are decreased in association with a higher production of exosomes, confirming that ATP13A2 plays a critical role in α-syn transport mediated by exosomes [92]. Kong and collaborators suggested that ATP13A2 mediates exosomal α-syn transmission by controlling zinc homeostasis in these vesicles [93]. Finally, *GBA* KO SHSY5Y cells accumulated intracellular α-syn and increased α-syn extracellular release, which was taken up by recipient naïve cells [94]. This phenomenon was reversed by overexpression of WT *GBA* in KO cells. Transplantation of *GBA* KO cells into the hippocampus of human α-syn transgenic mice showed that grafted cells were more sensitive to host-derived α-syn transfer [94]. Thus, reduced GCase activity by *GBA* mutation or in pathological conditions enhances the cell-to-cell transmission of α-syn.

Altogether, these studies demonstrated that autophagic impairment present in synucleinopathies could enhance α-syn cell-to-cell transmission by increasing the amount of non-degraded delivered exosomes to the extracellular media, thus aggravating the α-syn spreading into the CNS (Figure 1b).

3.3. α-Syn Induces Alterations on ALP Processes

Several studies also reported a detrimental role of the protein α-syn on ALP, further contributing to the pathology through an endless cycle of cellular alterations. PC12 cells overexpressing WT or mutated A53T α-syn protein showed abnormal morphology with accumulation of aberrant vesicular structures associated with diminished lysosomal activity, suggesting an impairment of the autophagy machinery in correlation with α-syn levels [95].

WT α-syn overexpression induced autophagy inhibition through impairment in autophagosome formation [96–98]. One possible mechanism relates to a decrease in α-syn-induced Rab1 activity, which, in turn, perturbs the localization of the Atg9 protein involved in autophagosome formation at the early stage of the biogenesis [97]. Song and collaborators suggested that α-syn-mediated autophagy inhibition was caused by the interaction between α-syn and the autophagic stimuli trigger HMGB1, associated with decreased Beclin-1 protein levels. This prevented the physiological cytosolic interaction between Beclin-1 and HMGB1 essential to activate autophagy [96]. Moreover, dopamine-modified α-syn showed inhibitory effects on the CMA activity by: (i) binding the LAMP-2A protein at the lysosomal membrane, (ii) forming oligomeric complexes at the lysosomal membrane, and (iii) being poorly translocated to the lysosomal lumen, thus blocking the degradation of other CMA cellular

substrates [98,99]. Finally, when α-syn transfers from cell to cell, autophagic impairment was also triggered in the recipient cell with an accumulation of enlarged deficient lysosomes, further contributing to the expansion of the pathology [100].

In parallel to WT α-syn effects on autophagy, pathologically mutant forms of α-syn induce alteration in ALP processes, per se. Overexpression of mutated A53T α-syn decreases the autophagy activity in PC12 cells. A53T α-syn lacking the CMA recognition motif expressed in the same cells did not alter autophagy activity, suggesting that the mutant A53T α-syn induced alterations only in the CMA-mediated degradation process [98]. In this situation, the MA pathway was upregulated to compensate for the CMA deficit but resulted in detrimental conditions that were partly responsible for the observed cell death. Disruption of CMA-mediated degradation induced by A53T α-syn also led to abnormal accumulation of the myocyte enhancer factor 2 (MEF2D) in the cytosol. This compromised its binding to DNA and its consequent role in gene regulation of multiple cellular functions, inducing cellular dyshomeostasis [101]. The mutant E46K α-syn induced impaired autophagy through inhibition of autophagosome formation in an mTOR-independent pathway [102]. It was suggested that E46K α-syn reduced the phosphorylation of Bcl2 protein through upstream JNK1 inactivation, provoking the sequestration and the loss-of-function of Beclin-1 in the Bcl2/Beclin-1 complex [102]. The mutant A30P α-syn was suggested to inhibit autophagy by increasing the translocation of the repressive autophagic transcription factor ZKSCAN3 to the nucleus in a JNK-dependent manner [11].

In α-syn preformed fibrils-treated HEK293T cells, phosphorylated α-syn (P-α-syn) colocalized with autophagosomal markers p62 and LC3II, but not with lysosomal marker LAMP-1, suggesting that α-syn inclusions are associated with autophagy machinery at the early stages [103]. Moreover, activation or inhibition of lysosomal activity did not alter the levels of P-α-syn, suggesting that matured α-syn aggregates were not efficiently degraded by the lysosomes. These cells also exhibit an impairment in autophagy with defective autophagosome maturation and delivery to the lysosomes. This work suggested that once α-syn aggregates are matured and the pathology is initiated, autophagy is no longer effective and is dysregulated by α-syn [103]. Detrimental effects of α-syn aggregates also occur through interaction of these aggregates with membrane lipids, inducing lysosomal membrane permeabilization and consequent impairment in autophagy [104,105].

In the lysosomal lumen, α-syn alters lysosomal hydrolases activity, such as Cathepsin B, GCase, β-galactosidase, or β-hexosamidase, in midbrain neurons generated from iPSC cell line derived from healthy subjects transfected with WT α-syn or from idiopathic PD patients [77,106]. Strikingly, the levels of total cellular hydrolases were not altered, suggesting a problem in maturation and trafficking of the enzymes rather than a biosynthesis alteration. This idea was confirmed by experiments showing that α-syn modified the location of the key-regulator of ER-Golgi trafficking, Rab1, leading to disruption of the hydrolase trafficking at the Golgi compartment [106]. Interestingly, interaction of α-syn with GCase at its active site in the lysosomal compartment [107], as well as decreased GCase lysosomal activity in presence of WT or A53T α-syn due to alteration in protein maturation [77], further strengthened the risk-association of *GBA* mutations between PD and GD.

Finally, α-syn also dysregulates specific autophagy pathways, such as mitophagy. Choubey and colleagues showed that cortical neurons transfected with mutant A53T α-syn increased levels of healthy mitochondria colocalizing with autophagosomes, and were associated with decreased ATP levels that provoked bioenergetic deficits for the neuron [12]. In this situation, the physiological role of Parkin, which is to mark the mitochondria for their lysosomal addressing, is completely altered [12].

Altogether, these data suggested that the α-syn protein alters the ALP machinery through various deleterious pathways, thus participating in cellular dyshomeostasis and neuronal death, but also exacerbating the pathological synucleinopathy by a dual-loop effect (Figure 1b).

4. Role of Autophagy Impairment in Neurodegeneration

Since autophagy plays a role in the accumulation of α-syn, and in turn in the formation of a α-syn aggregates, its contribution in neurodegeneration must be questioned. Autophagic genes,

such as *Atg5* and *Atg7*, have been implicated in neurodegeneration. Both *Atg7* or *Atg5* conditionally KO mice displayed neuronal cell death, presence of ubiquitin-positive inclusions, and behavioral deficits [108–110], suggesting that autophagy impairment is implicated in neurodegeneration. In synucleinopathies, autophagy dysfunction has been observed through different mechanisms within the cell from the lysosomal stress, to mitophagy defect (a mitochondrial-centered autophagy), and finally in cellular trafficking disturbances that lead to neuronal loss.

4.1. Lysosomal Dysfunction

Neurons require a tight regulation and recycling of proteins involved, for the most part, in neurotransmission, making lysosomal efficacy that much more important for neuronal function and survival [111]. Correct completion of autophagy results in efficient cargo degradation via lysosomal digestion. Issues with lysosomal stress have been shown to affect the elimination of cargo.

Suitable autophagosomal degradation is based on the fusion of the autophagosomes with the lysosomes to be able to correctly degrade their content. Specific lysosomal proteins have been involved in the formation of the lysosome, such as LAMP-1 and LAMP-2. In LAMP-1 and LAMP-2 double-mutant mice, early embryonic death from E14.5 to E16.5 was observed with accumulation of autophagic vacuoles containing non-degraded material [112]. The same cellular disruptions were observed in fibroblast cell lines derived from these double mutant embryos, but lysosomal enzyme activity and protein degradation rates remained unchanged. These results suggest the important role of both LAMP proteins in lysosome formation that goes beyond structural maintenance of lysosomes.

Lysosomal destruction of the cargo requires a specific acidic environment to activate the hydrolases and proteases to, in turn, digest cargo. Neurotoxic agents such as MPP+ lead to the alkalinization of the lysosomal compartment associated with neurodegeneration [113]. In genetic PD models, such as ATP13A2 KO mice, not only does the loss of this protein cause a lysosomal alkalinization associated with a reduction of lysosomal proteolysis, but also triggers cell death [43]. In addition, when restoring this protein in dopaminergic cell lines, the reestablishment of lysosomal acidification and function leads to a decrease in cell death. Supplementary studies using ATP13A2-null mice were conducted and confirmed that ATP13A2 deficits were enough to induce neurodegeneration. Despite overexpressing α-syn in these mice, no pathology alterations were observed, indicating that ATP13A2 deficits act independently from α-syn [114].

Mutations in the LRRK2-encoding gene also indicated an impairment in the lysosomal pH and cathepsin activity [115]. LRRK2 G2019S mutations diminish lysosomal capacities and induce an enlargement of these organelles. These mutations also lead to an increased expression of ATP13A2, suggesting a link between these two lysosomal proteins associated with PD. The pathogenic mechanism originating from LRRK2 mutations remains unclear in PD pathogenesis. As LRRK2 KO mice present a little neurodegeneration in the brain [116], double knockouts of both LRRK proteins (LRRK1 and LRRK2) have been generated to elucidate the potential compensation of these proteins [117]. These double-mutant mice showed early mortality as well as dopaminergic cell loss accompanied by autophagy dysfunction, highlighting the importance of this protein in the regulation of ALP and neurodegeneration.

In line with acidification of the lysosomes, deficiencies in lysosomal proteases and hydrolases have been observed in PD models. First, mutations in the lysosomal protease CathD in *C. elegans*, as well as in mice, were shown to have a deleterious impact on autophagy and to cause the formation of α-syn aggregates and neurodegeneration in the CNS [118]. In addition, mutations in the *GBA1* gene have demonstrated parkinsonian symptoms linked to neurodegeneration [119]. In the case of MSA pathology, after differentiation of iPSC-derived dopaminergic neurons from MSA patients, impairment of autophagy and particularly of lysosomal enzymes were also observed [50].

The MPTP mouse model recapitulates some of the major features of PD, such as modifications in mitochondrial structure and function, reactive oxygen species (ROS) production, and activation of apoptotic pathways, which induce dopaminergic cell death. In this model, a pathogenic lysosomal

depletion occurs prior to an autophagosomal accumulation contributing to PD-related dopaminergic neurodegeneration [38].

Taken together, these studies emphasized the occurrence of lysosomal defects in in vitro and in vivo PD models as a key player in the initiation of neurodegeneration (Figure 2a).

4.2. Mitophagy Defects

Main cellular functions of the cell also reside in mitochondria, as they are the key organelles in the cell for the production of energy [120]. Defects in mitochondrial functions have been associated with genetic forms of synucleinopathies, particularly in PD with protein deficiencies, such as PINK1 and Parkin [121–124]. In the cell, degradation of mitochondria via the autophagic pathway, known as mitophagy, is essential for the elimination of defective mitochondria. When mitochondria are defective, there is recruitment of PINK1 to the outer membrane, where it accumulates and in turn phosphorylates Parkin, leading to its activation. The activation of Parkin induces the recruitment of a polyubiquitin chain. This polyubiquitin chain is then phosphorylated by PINK1, leading to the recruitment of autophagy receptors, activating the induction of autophagy and the clearance of the defective mitochondria [125].

In PD, this mitochondrial clearance does not function properly. Studies using deletions in genes of Parkin and PINK1 were first described in *Drosophila melanogaster* [121–123]. Given the central role of these two proteins in mitophagy, it is important to understand the contribution of this specific autophagic pathway in the induction of neurodegeneration. In Parkin-deleted adult mice, Stevens and colleagues observed declines in mitochondrial mass as well as age-dependent loss of dopaminergic neurons [126]. Recently, Sliter and collaborators showed that Parkin and PINK1 defects induced an inflammatory response that can play a role in neurodegeneration [127]. Nevertheless, the exact contribution of Parkin or PINK1 defective mitophagy in neurodegeneration need further exploration [128]. DJ-1, a PD-linked protein associated with familial cases, seems to also play a role in mitophagy, as it is able to rescue the phenotype of PINK1 mutants in Drosophila, but not in Parkin mutants. This suggests that DJ-1 could act between PINK1 and Parkin [129]. Interestingly, DJ-1 overexpression increased α-syn clearance and decreased neuronal death [130]. In DJ-1-deficient cells, increased levels of oxidative stress via glutathione inhibition were responsible for an increased sensitivity of cells to ROS, acting on the correct functions of the cell and its viability [131]. Loss of DJ-1 has also been shown to disrupt CMA, in addition to inducing mitochondrial defects [132]. Finally, Li and colleagues observed that *GBA* heterozygous mutations impair mitophagy by altering the mitochondrial priming to the lysosomes, indicating that *GBA* mutations not only affect ALP, but also mitochondrial recycling [133].

Thus, impairment of the mitophagy process seems to contribute to oxidative stress conditions, ultimately leading to neurodegeneration (Figure 2b).

4.3. Vesicular Trafficking Defects

Finally, trafficking defects have been observed in α-syn-related pathologies. Trafficking of endosomes and autophagosomes is essential in the autophagic pathway as it allows the transfer of the cargo to the ALP. VPS35 is implicated in endosomal trafficking. Mutations in VPS35 have been involved in late-onset PD [134]. In VPS35 PD-causing mutations, localization of the WASH complex within the endosomes was reduced and autophagy defects associated with cell death occurred [135]. WASH complex is involved in binding with retromers and inducing the formation of F-actin patches to guide cargo for transport. This complex has been shown to be involved in the induction of autophagy, particularly in the autophagosome formation step [135]. The VPS35 protein seemed to be essential in the regulation of the WASH complex to maintain cargo trafficking. Parkin has also emerged as a key player in endosomal trafficking. Williams and colleagues showed a novel interaction between Parkin and VPS35, in which Parkin mediated the ubiquitination of VPS35 [136]. VPS35 ubiquitination acts

directly on WASH-dependent cargo sorting and alters endosomal trafficking, thus affecting correct autophagic function, and finally playing a role in neurodegeneration.

The interaction between the two proteins Beclin-1 and Vps34, implicated in cargo trafficking, is the launching point to initiate autophagy. Huang and colleagues showed that HMGB1 protein played an indirect role on autophagy impairment via the malformation of the Beclin1-Vps34 complex in rotenone-treated cells, which inhibits autophagosome formation [137]. HMGB1 overexpression and rotenone exposure had the same effects on the cells—they induce cellular shrinkage and decrease cell viability.

As membrane components, lipid and cholesterol metabolism have also been shown to be players in vesicle integrity and trafficking. Cholesterol has been implicated specifically in autophagosome trafficking. Accumulation of cholesterol observed in *GBA* N370S mutant fibroblasts leads to an accumulation of multilamellar bodies and a disruption of autophagy [138], increasing their susceptibility to apoptosis. In *GBA1*-deficient cells, an impairment of secretory autophagy can also be seen [139]. Lysosomal membrane ceramide species are produced by the GCase enzyme and then are converted into other lipids, such as fatty acids, by acid ceramidase [140]. Kim and colleagues overexpressed acid ceramidase in WT cells, which induced the accumulation of autophagic markers (p62, LC3, Rab7), as well as cell death [139]. Inhibiting acid ceramidase in *GBA1*-deficient cells led to a partial rescue of the lipid abnormalities, as well as decreased neurodegenerative markers [139].

Trafficking defects compromise autophagosomes delivery to the lysosomal compartment, thus inhibiting correct protein turnover, finally leading to neurodegenerative processes (Figure 2c).

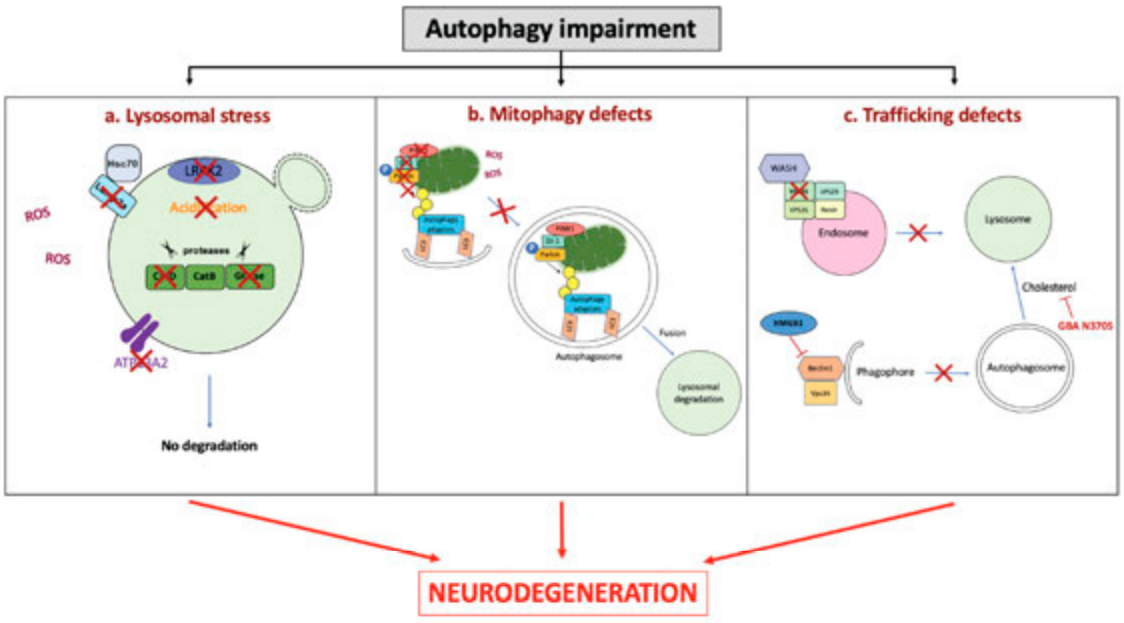

Figure 2. Autophagy impairment induces neurodegeneration. Autophagy can be impaired at different levels, namely: (**a**) Lysosomal stress; (**b**) mitophagy defects; (**c**) vesicular trafficking defects. (**a**) Lysosomal stress, characterized by either lysosomal alkalinization, lysosomal structural modifications, or defective lysosomal proteases, blocks the correct degradation of the cargo. (**b**) Mitophagy relies on actors such as PINK1, DJ-1, and Parkin, which allow a correct priming of defective mitochondria to be addressed to autophagy. Alterations in these proteins induce decreased mitophagy. (**c**) Vesicular trafficking is the starting point for the autophagosome formation though the recruitment of the WASH complex. Mutations in VPS35 inhibits the WASH complex, and thus autophagosome formation. HMGB1 overexpression inhibits the Beclin1-Vps34 binding, and thus the phagophore formation. Mutations in *GBA* genes can also modify cholesterol, impairing autophagosome trafficking. GCase: β-glucocerebrosidase; CatD: Cathepsin D; ROS: reactive oxygen species.

5. Autophagy Recovery as a Therapeutic Target

We have reported here that ALP impairment plays a pivotal role in α-syn accumulation and release, as well as in neurodegenerative processes. Many studies have highlighted the importance of increasing autophagy activity or restoring its functionality as a therapeutic strategy in the field of synucleinopathies [141].

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5.1. mTOR-Dependent Targeting

ALP is regulated upstream by the mTOR protein, in which the inhibition activates autophagy. Molecules such as rapamycin increase mTOR inhibition, and thus increase the autophagy levels within the cell. This molecule has been tested in multiple models of PD, including, but not limited to, rotenone-exposed cells and MPP+-treated cells, MPTP mouse model, and α-syn transgenic rodents [40,142–144]. All these models have shown beneficial effects of rapamycin administration in both neurotoxin-induced PD models and α-syn overexpressing models. Specifically, there has been shown to be a reduction in dopaminergic cell death, decreased levels of phosphorylated α-syn, and reduced mitochondrial dysfunction. To target the mTOR pathway directly, some studies have also targeted the downstream mTOR target, transcription factor EB (TFEB). The mTOR inhibition activates the transport of TFEB to the nucleus, in which it binds DNA to regulate ALP gene expression [145]. Attempts at activating TFEB have been done through molecules such as FDA-approved 2-hydropropyl-ß-cyclodextrin, which are capable of increasing autophagy levels, as well as α-syn clearance in neuroglioma cell lines [146]. Gene therapy using adeno-associated viral mediated TFEB overexpression in α-syn-overexpressing rat conferred a protective effect on rat midbrain neurons, associated with an increased clearance of pathologic α-syn through autophagic recovery [147]. Natural compounds such as pomegranate extracts enhanced TFEB activity, reversing the effects of α-syn mediated neurodegeneration in neuroblastoma cells through induction of both mitophagy and autophagy [148].

Neuroinflammation also contributes to neurodegeneration in synucleinopathies, but its role in triggering autophagy defects has not yet been completely elucidated. Nonetheless, innate immunity receptors, such as Toll-like receptors (TLR), have been suggested to play a role in neurodegeneration, as well as neuroinflammation, through autophagic dysregulation dependent on the mTOR pathway [149]. In fact, TLR2 expression has been shown to be increased in PD and LBD patients, as well as models of these diseases [150]. Activating TLR2 expression in vitro induced an accumulation of α-syn aggregates and increased neurotoxicity through autophagic dysregulation. On the other hand, knockdown of TLR2 in a mouse model of PD allowed an improvement in α-syn-induced pathology and alleviated motor deficits, suggesting that TLR2 can represent a novel interesting therapeutic target.

5.2. mTOR-Independent Targeting

Other molecules aimed to target mTOR-independent pathways through various mechanisms. A pioneer molecule for this strategy is the invertebrate disaccharide trehalose, which targets SLC2A transporters and induces an AMPK-dependent increase of autophagy. Trehalose has been tested in multiple models of PD, ranging from drug-induced pathology to α-syn overexpressing in vitro and in vivo models [100,151,152]. Using this molecule, there has been shown to be an increase in α-syn clearance, as well as a reduction in cell loss and neuroinflammation. Chronic treatments of natural caffeine molecules on α-syn fibrils-injected mice decreased α-syn positive inclusions, cellular apoptosis, microglial activation, and neuronal loss, all mediated by an increase in autophagy [153]. Lithium constitutes another strategy to enhance autophagy in an mTOR-independent pathway in different models via the inhibition of inositol phosphatase [154,155]. Lithium treatment was shown to increase the autophagy marker LC3 and to decrease the dopaminergic cell loss in the SN of MPTP mice [155]. Additionally, Hou and colleagues observed mitochondrial protection,

in addition to autophagy recovery, increasing cell viability after treatment of rotenone-exposed SH-SY5Y neuroblastoma cells with lithium [154]. Nonetheless, lithium is a molecule that is primarily used to treat bipolar disorders, indicating that possible unwanted side effects could emerge from the use of this molecule. Other molecules, such as calcium channel blockers, potassic ATP channel blockers (Minoxidil), and Gi signaling activators (Clonidine), have also been shown to be effective in inducing autophagy and clearing protein aggregates through regulation of cAMP levels [156]. Finally, inhibition of the autophagy blocker c-Abl using Nilotinib has been tested in PD patients who showed an increased clearance of α-syn in biological fluids associated with reduced symptomatology during clinical trials [157,158].

To target lysosomal activity directly, the use of acidic nanoparticles has been tested for their potential to restore lysosomal pH [113,159,160]. Bourdenx and colleagues tested the effect of nanoparticles in MPP+-treated cells, ATP13A2 mutant fibroblasts, and MPTP-treated mice. In all the mentioned models, the nanoparticles had the capacity to restore lysosomal pH, lysosomal function, and finally inhibit dopaminergic cell death in mice brains [113]. To mediate the lysosomal disruption caused by a loss-of-function of ATP13A2, strategies involving metal homeostasis have been used as therapeutic strategies. Tsunemi and coworkers recently observed that loss of ATP13A2 function in fibroblasts induced a Ca^{2+} dyshomeostasis in the lysosomes, in turn leading to exosomal disruptions and autophagic impairment [61]. TRPML1, the principal receptor responsible for Ca^{2+} import in lysosomes, was used as a target to modulate Ca^{2+} levels and restore correct function of this organelle. Other studies have also used strategies to target metals such as zinc, as well as cAMP to restore lysosomal acidification [161].

Knowing the importance of autophagy-related genetic mutations and associated protein loss-of-function in PD pathology, restoration of protein activity implicated in synucleinopathies have also demonstrated encouraging beneficial effects. Targeting GCase activity using ambroxol in *GBA1* mutant fibroblasts [162,163] restored lysosomal function and reduced oxidative stress. Preclinical trials using ambroxol are currently being conducted on PD dementia patients to observe the possible beneficial effects of this molecule on GCase activity and PD pathology [164]. Given the implication of Beclin-1 in inducing autophagy, targeting this protein to increase autophagic clearance has been tested in different models. Using activators of Beclin-1 in multiple PD models has been shown to increase α-syn clearance, decreasing cellular toxicity [65,165,166]. Despite the interaction of Beclin-1 with proteins able to induce apoptosis, such as Bcl2, the activation or overexpression of Beclin-1 has not seemed to increase the amount of cell death. Possibilities of targeting CMA are more limiting than targeting macroautophagy. Nonetheless, downstream factors of the CMA pathway could be an appealing alternative to macroautophagy. CMA targeting has principally been through the lysosomal marker LAMP-2A and its chaperone Hsc70. Overexpression of LAMP-2A has been induced in cellular models of PD using SH-SY5Y cells, in primary neuron cultures, as well as in rats [167]. In these models, an increase in the expression of the lysosomal protein LAMP-2A increased CMA activity, thus decreasing α-syn accumulation, allowing neuroprotection [167]. In addition to overexpressing LAMP-2A, retinoic acid receptors have been shown to be inhibitors of CMA. This inhibition decreased oxidative stress and protein toxicity, proving the interest of increasing the CMA pathway [168]. Mitochondrial oxidative stress also plays an important role in autophagy impairment in synucleinopathies. The antioxidant properties of the DJ-1 protein have been previously reported, as it could decrease oxidative stress and restore autophagy [169–171]. Increasing DJ-1 expression in astrocytes co-cultured with neurons proved to have beneficial effects against rotenone-induced cell oxidation [169]. This was also shown in vivo using a rotenone-treated rat model, where DJ-1 was specifically increased in astrocytes [172]. This DJ-1 overexpression allowed an inhibition of dopaminergic cell loss, a decrease in neuroinflammation, as well as an inhibition of CMA deficits. These studies indicate DJ-1 as an interesting genetic target because it acts at multiple levels of synucleinopathies: α-syn interaction, mitophagy activation, and oxidative stress reduction.

Pupyshev and colleagues, in an attempt to test the synergistic effect of mTOR-dependent and -independent targeting, combined the use of trehalose and rapamycin in a MPTP mouse model of PD [173]. By combining both molecules, this study showed an even more pronounced improvement in both the number of protected dopaminergic neurons and motor function recovery.

Knowing the crucial role that autophagy plays in the maintenance of cellular homeostasis, reestablishing its proper function in synucleinopathies was proven to be efficient in clearance of pathological α-syn aggregates and neuroprotection. However, this strategy could be detrimental to the well-being of neurons, as increasing the ALP could also target other proteins' elimination. Nevertheless, autophagy remains a promising and relevant target that could be at the source of possible therapies for synucleinopathies, as well as other protein accumulation-based neurodegenerative diseases.

6. Conclusions

Here, we summarized distinct pieces of evidence suggesting that autophagy is involved in synucleinopathy. The presence of autophagy alterations was first provided by genetic and post-mortem studies on brains of PD and MSA patients. Since then, experimental studies attempted to demonstrate the role of autophagy in the pathology of synucleinopathy. First, α-syn is mainly degraded by both macroautophagy and chaperone-mediated autophagy. Thus, autophagy defects induce intracellular α-syn accumulation, participating in its aggregative state towards the formation of α-syn-positive intracytoplasmic inclusions. In addition to this aggregative-prone phenomenon, autophagy defects also increase the α-syn secretion by the non-autophagic exosomal pathway, leading to increased cell-to-cell transmission of the protein, and thus the propagation of the α-syn-linked pathology in different brain regions of the CNS. On the other hand, autophagy defects also cause detriment effects in cellular homeostasis: (i) lysosomal impairment through structural or functional defects leads to accumulation of non-degraded products and increased production of ROS; (ii) decreased mitophagy leads to neuronal bioenergetic imbalance; and (iii) defective cargo trafficking impairs the addressing of vesicles to lysosomal clearance. All these detrimental cellular conditions lead to neurodegeneration. Finally, increased evidence demonstrated that inducing the autophagy pathways by natural or chemical compounds, as well as genetic approaches, has become a relevant therapeutic approach to counteract the deleterious effects of autophagy impairment in synucleinopathy.

Funding: This work was supported by Fondation de France Grant number 00066525, a France Parkinson Grant and an IDEX Emergence Grant number OPE-2018-410 (B.D.). M.L.A and M.T. are recipients of a MSER fellowship (France). The LABEX Brain, the University of Bordeaux, and the Centre National de la Recherche Scientifique provided infrastructural support.

Acknowledgments: We apologize to the authors of several high-quality scientific articles that contributed significantly to the development of the field, which could not be cited due to space limits.

Conflicts of Interest: The authors declared no conflict of interest.

Abbreviations

α-syn	α-synuclein
ALP	Autophagy-lysosomal pathway
Baf	BafilomycinA1
CathD	Cathepsin D
CMA	Chaperone-mediated autophagy
CSF	Cerebrospinal fluid
DLB	Dementia with Lewy bodies
ER	Endoplasmic reticulum
GABARAP	Gamma-aminobutyric acid receptor-associated protein
GATE-16	Golgi-associated ATPase enhancer of 16kDa
GCase	β-glucocerebrosidase
GCI	Glial cytoplasmic inclusions

GD	Gaucher's Disease
Hsc70	Heat-shock cognate 70kDa
Hsp	Heat shock protein
KO	Knock-out
LAMP-1	Lysosomal-associated membrane protein 1
LAMP-2	Lysosomal-associated membrane protein 2
LB	Lewy Bodies
LC3	Microtubule-associated protein 1A/1B-light chain 3
LMX1B	LIM homeobox transcription factor 1 beta
LN	Lewy neurites
MA	Macroautophagy
MEF2D	Myocyte enhancer factor 2
miRNA	micro RNA
MSA	Multiple system atrophy
P-α-syn	Phosphorylated α-synuclein
PD	Parkinson's Disease
PINK1	Phosphate and tensin homolog-induced putative kinase 1
ROS	Reactive oxygen species
SN	Substantia nigra
TFEB	Transcription factor EB
TLR	Toll-like receptor
WT	Wild-type
3-MA	3-methyladenine

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Review

Targeting α-Synuclein for PD Therapeutics: A Pursuit on All Fronts

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Received: 29 January 2020; Accepted: 29 February 2020; Published: 3 March 2020

Abstract: Parkinson's Disease (PD) is characterized both by the loss of dopaminergic neurons in the substantia nigra and the presence of cytoplasmic inclusions called Lewy Bodies. These Lewy Bodies contain the aggregated α-synuclein (α-syn) protein, which has been shown to be able to propagate from cell to cell and throughout different regions in the brain. Due to its central role in the pathology and the lack of a curative treatment for PD, an increasing number of studies have aimed at targeting this protein for therapeutics. Here, we reviewed and discussed the many different approaches that have been studied to inhibit α-syn accumulation via direct and indirect targeting. These analyses have led to the generation of multiple clinical trials that are either completed or currently active. These clinical trials and the current preclinical studies must still face obstacles ahead, but give hope of finding a therapy for PD with time.

Keywords: Parkinson's disease; α-synuclein; neurodegeneration; therapy; aggregation

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease, affecting about 1–5% of the population over the age of 60 [1]. Clinically, PD is associated with motor impairments including bradykinesia, akinesia, rigidity, resting tremor and gait disturbance [2]. These symptoms are, in major part, due to the progressive loss of dopaminergic neurons in the substantia nigra (SN). In addition to this loss of dopaminergic neurons, another hallmark of PD is the presence of intraneuronal cytoplasmic inclusions, named Lewy Bodies (LB) [3], composed of the misfolded, aggregated α-synuclein (α-syn) protein, but also of other proteins and organelles [4,5]. Genetic and post-mortem evidence of PD patients undoubtedly highlight the key role of α-syn in PD pathology [6]. α-syn has also been shown to aggregate in other diseases, grouped under the term synucleinopathies. Targeting neuronal accumulation of α-syn is thus appealing to potentially halt or delay the progression of PD and other synucleinopathies.

In this review, we aimed to highlight α-syn properties that make this protein an appealing therapeutic target exclusively in the PD context. We then tried to expose the current therapeutic strategies that have been tested to target α-syn to inhibit its accumulation, aggregation or toxicity. We focused this review on discussing strategies that target specifically α-syn in preclinical and clinical studies.

2. α-syn: A Relevant Therapeutic Target for PD

2.1. α-syn Aggregation and Its Deleterious Impacts on Cellular Homeostasis

α-syn is a 14 kDa natively unfolded protein of 140 amino acids, encoded by the *SNCA* gene. This protein is highly abundant in presynaptic compartments, where it can associate with vesicles via its possible binding to membranous phospholipids [7]. It is composed of three domains: an N-terminal α-helix domain allowing lipid binding, a central non-amyloid-β component (NAC) domain responsible for its amyloid aggregation, and an unstructured flexible C-terminal domain. The protein has been shown to form a native tetramer in physiological conditions [8], but these findings are still controversial in the field [9]. In 1997, it was discovered that early-onset familial forms of PD were associated with a mutation in the *SNCA* gene [6]. That same year, α-syn was identified as the major component of LB presented by affected neurons of PD subjects [4]. These two studies shed light on this protein as a possible major actor in PD pathogenesis.

In pathological conditions, misfolded and aggregated forms of α-syn accumulate in LB. The NAC domain of α-syn confers a high propensity for the protein to misfold and to form β-sheet rich amyloid assemblies, also termed fibrils [10]. α-syn assembly into amyloid fibrils is dynamic and the existence of intermediate oligomeric species has extensively been studied. These oligomers provoked more seeding and neurotoxic effects than larger fibrillar assemblies, inferring that they might in fact be the real pathogenic species [11,12]. Deciphering the pathological relevance and exact role of these assemblies in the pathogenesis of PD is very challenging due to their reduced size, massive heterogeneity and transient nature. Recently, studies have aimed at identifying the different α-syn strains between synucleinopathies that could explain their divergent pathologies and clinical manifestations. It has been shown by several teams that there appears to be a specific aggregated form of α-syn that induces PD pathology [13–15].

α-syn aggregation can be considered as a stochastic event, which would increase with age and/or cell stress conditions, forming initial seed nuclei that would escape cellular clearance due to perturbed proteostasis. Increased α-syn expression and point mutations have been extensively shown to promote aggregation [16]. External factors such as viral [17] or bacterial [18] infections, and cell stress due to pesticide or toxin exposure [19,20] can also trigger the aggregation of the protein. Perturbed calcium homeostasis, mitochondrial failure, oxidative stress, and neuroinflammation are implicated in the initiation of the accumulation of α-syn as well [21–23].

The accumulation of intracellular aggregated α-syn shows pleiotropic pathological effects on the cell such as synaptic vesicle impairments, mitochondrial dysfunction, oxidative and endoplasmic reticulum (ER) stress, or dysfunction in the clearance pathways [24] which in turn contribute to neurodegeneration. The physiological interaction of α-syn with vesicles [25] and its role in vesicle trafficking via interactions with the SNARE complex may explain the disruption of this complex and of synaptic vesicle motility by α-syn oligomers associated with increased dopamine (DA) release [26,27]. A synaptic loss-of-function of normal α-syn, which could be triggered by its abnormal accumulation, has been shown to induce perturbations in dopamine release [28,29]. Conversely, increased levels in oxidized dopamine also impact the formation of oligomeric α-syn species and lysosomal activity [30,31]. In addition, misfolded α-syn aggregates can associate with ER membrane and cause a morphologic dysfunction, perturbations in ER chaperone levels, resulting in increased reactive oxygen species (ROS) levels, or calcium leakage to the cytosol [32]. The transport of proteins from the Golgi apparatus to the ER can also be affected by α-syn aggregates, increasing the ER stress and leading to a lack of function of protein production and quality control [33]. Mitochondrial swelling and depolarization combined with an accelerated release of cytochrome c and calcium dyshomeostasis have also been observed in vitro due to α-syn oligomers [34]. α-syn aggregates could also lead to an increase in oxidative stress by disturbing mitochondrial respiration via inhibition of mitochondrial complex I subunits [35], increasing the production of ROS by NADPH oxidase [36] or failure in antioxidant proteins [37]. These pathological assemblies are also capable of interacting with membrane lipids, thus

inducing lysosomal membrane permeabilization and compromising autophagic function [38]. Lastly, it has been shown that aggregated α-syn can activate microglial inflammation, in turn participating in the induction of cell death [39]. Altogether, these various toxic effects of aggregated α-syn contribute to the loss of cellular homeostasis, therefore contributing to neurodegeneration.

In parallel, disruptions in cellular functions could also have a crucial role in α-syn toxicity towards neurons in PD. A dysfunction in α-syn clearance can lead to the abnormal accumulation of the protein, which in turn can disrupt autophagic-lysosomal and ubiquitin-proteasomal functions, entering in a vicious circle [40]. In addition, metal dyshomeostasis within the cell has been shown to impact α-syn by itself and its aggregation, via the metal binding sites present on α-syn [41–43].

Altogether, these findings reveal the tight links between α-syn aggregation and cellular dysfunctions in PD pathogenesis, but do not decrypt which is the trigger and which is the consequence.

2.2. The Prion-Like Hypothesis as a Model of α-syn Pathogenicity

The prion-like hypothesis of α-syn was raised in 2003 when six different neuropathological stages of PD were classified based on the spreading of α-syn deposits throughout affected brain areas [44]. Given that the successively affected regions are interconnected, they hypothesized the prion-like propagation of PD: the Lewy pathology would appear at the brainstem or olfactory bulb, for still unidentified reasons, and expand spatiotemporally to the neuronal connectome. Even if still under debate, this hypothesis was further strengthened in 2008 with the discovery of the development of Lewy pathology in fetal neurons grafted in the brains of PD patients more than a decade before their death [45–47], highlighting a potential cell-to-cell propagation of pathological α-syn.

Evidence showing that α-syn can transfer from a donor cell to a recipient cell was demonstrated in vitro using neuroblastoma cells [48] and in vivo when the transfer of α-syn to grafts in 6-OHDA-lesioned rats was observed [49], confirming the seminal findings of α-syn transfer from host to graft. These processes have since then been intensively described, with the demonstration that α-syn assemblies can be taken-up by neurons [48,50,51] via binding to the cell surface [52], or interaction with protein partners [53–55]. Once inside the neuron, aggregated α-syn has been shown to be directed to the lysosomal compartment [50] and can be transported along the axon [56,57]. The intracellular aggregates can, in the end, be transmitted to neighboring cells via several non-exclusive possible routes: exported extracellularly [56,58], in exosomes [59], or simply released after the affected neuron death.

This prion-like propagation of aggregated α-syn was further demonstrated following intrastriatal inoculation of recombinant α-syn pre-formed fibrils (PFF) in WT mice. This PFF injection induced the formation of α-syn-positive cytoplasmic inclusions in neurons of the striatum as well as interconnected regions such as the cortex, associated with a loss of dopaminergic neurons in the SN and motor dysfunctions [60], while a similar injection of soluble α-syn had no effect [61]. PFF also induced a progressive synucleinopathy after intracerebral injection into susceptible transgenic mice [62–64], and in non-human primate models [65]. In 2014, LB-enriched fractions purified from PD brains containing pathological aggregated α-syn were injected unilaterally in the SN and striatum of WT mice and non-human primates. This resulted in the induction of a progressive accumulation of pathological and aggregated forms of α-syn in nigral neurons and interconnected brain regions, leading to nigrostriatal neurodegeneration [66]. These pathogenic effects were abolished when injections were performed into mice lacking α-syn expression, or when inoculates were experimentally deprived of α-syn. The propagation of α-syn has also been demonstrated from the gut to the brain by injections of PFFs in the gut, which did not occur in α-syn knockout mice [67].

In addition to its ability to aggregate and propagate, α-syn has shown the capacity to seed the formation of new aggregates. The exposure of cells to exogenous α-syn fibrils has been shown to induce the accumulation of aggregated endogenous α-syn in many independent studies [68,69]. In these studies, fluorescently labelled α-syn exogenous fibrils were found to colocalize with newly formed endogenous α-syn aggregates [70]. The exposed cells developed a synucleinopathy resembling the one found in PD affected neurons, with the formation of cytosolic inclusions and Lewy Neurites (LN),

containing pathological phosphorylated α-syn at the serine residue position 129 (pS129). These findings show that exogenous aggregates of the protein are able to recruit endogenous α-syn monomers and serve as templates in their seeded aggregation.

Altogether, these models have shed light on the capacity of α-syn to form aggregates that can be transferred from an affected cell to a naive one and induce the recruitment and seeded aggregation of the endogenous protein, leading to the expansion of the pathology. These events, recapitulated under the term "prion-like propagation", highlight the implication of α-syn and its aggregation in the pathogenesis of PD.

All the above-mentioned findings and models undoubtedly show the central implication of α-syn in the expansion of PD pathology, leading to the disruptions and impairments of physiological cellular processes, giving rise to a progressive neurodegenerative disorder. There are currently no curative therapies for PD. In line with the pathogenic mechanisms linked to α-syn mentioned above, global effort has been focused on: (1) reducing α-syn expression and synthesis, (2) inhibiting its aggregation or reducing its accumulation through post-translational modifications, immunotherapy, and anti-aggregative molecules, (3) enhancing the degradation of α-syn as possible therapeutic strategies for PD, (4) modulating oxidative stress via anti-oxidative molecules or metal alteration.

3. Reducing α-syn Synthesis

The discovery of seven missense mutations, A53T, A30P, E46K, H50Q, G51D, A53V and A53E [6,71–77], as well as duplication and triplication [78,79] of the *SNCA* gene encoding for α-syn as a cause of several PD familial cases undoubtedly rises the interest of the field towards *SNCA* gene targeting. Moreover, the association between α-syn expression levels and the severity of the pathology [80,81] in sporadic and familial cases, strongly reinforced the link of *SNCA* gene expression to the pathophysiology of PD. Silencing *SNCA* or normalizing its levels of expression has thus been deeply explored as an appealing genetic-based therapeutic approach for PD.

The discovery of RNA interference (RNAi) paved the way to reduce *SNCA* expression level in vitro and in vivo. RNAi is a conserved process by which the double-stranded RNA targets a specific sequence of mRNA resulting either in the degradation of the targeted mRNA by the RISC complex when using short hairpin (sh)RNA, or in its translational inhibition when using small interfering (si)RNA [82,83]. Both RNAi strategies have been used in the last decades in an attempt to reduce α-syn levels through *SNCA* silencing, aiming to delay or prevent dopaminergic cell loss. In 2006, it was first shown that a lentiviral-mediated delivery of shRNA targeting human *SNCA* was efficient to silence *SNCA* expression in vitro and in rat brains when it was co-expressed with the human *SNCA* gene [84]. That same year, successful down-regulation of *SNCA* gene expression in vivo occurred using an Adeno-Associated Virus (AAV)-based approach. They were able to prevent nigral dopaminergic cell loss by injecting hammerhead ribozyme-mediated inhibition of α-syn into MPP^+-treated adult rats [85]. Another delivery method demonstrated that stabilized naked siRNA against *SNCA* gene infused into mice hippocampi was resistant to endo- and exonuclease activity and led to efficient knockdown of *SNCA* locally (up to 70%) that lasted for up to three weeks post-infusion [86]. In addition to RNAi strategies, two microRNA (miR) targeting the 3'-untranslated region of *SNCA* have been identified as potential therapeutic targets to downregulate *SNCA* transcription. miR-7 was demonstrated to repress post-transcriptional *SNCA* gene expression in neurons and to contribute to protection against oxidative stress [87]. One year later, miR-7 and miR-153 overexpression reduced endogenous expression of α-syn in vitro and expression of these microRNA mirrored the *SNCA* expression in tissues, making them interesting for genetic-based approaches [88].

Despite the benefits obtained by such genetic-based therapies to reduce α-syn expression, the first demonstration of neurotoxicity induced by α-syn downregulation in WT rats raised long-lasting adverse effects [89]. In this study, they showed that intranigral unilateral injection of AAV carrying shRNA against *SNCA* in WT rats led to a rapid and progressive dopaminergic lesion in the nigrostriatal pathway, associated with pronounced amphetamine-induced behavioral asymmetry which correlated

with the level of reduction of *SNCA* expression [89]. In 2012, these results were confirmed using an AAV delivery of shRNA against human *SNCA* into the dopaminergic neurons of the SN of rats ectopically overexpressing human α-syn [90]. They showed that human α-syn knockdown to undetectable levels aggravated the dopaminergic cell loss presented by this model despite behavioral motor improvement [90]. The same team tested a miR-embedded shRNA against human *SNCA* in vitro and in the same model of α-syn overexpressing rats [91,92]. They showed that the miR-30-embedded shRNA silencing vector was effective to reduce α-syn expression level in the rat SN and the striatum, and to prevent dopaminergic cell loss and motor impairment at two months post-injection [92]. Nevertheless, this new genetic tool also induced negative effects such as toxicity on dopaminergic fibers in the striatum, reduction of tyrosine hydroxylase global expression and neuroinflammation in the SN [92]. Finally, in 2016, three months after injection of shRNA against *SNCA* in four St. Kitts green monkeys, a loss of dopaminergic cells and fibers in a titer-dependent manner occurred [93]. These results highlighted a critical window of *SNCA* expression which is required to maintain dopaminergic cell viability. To directly test the effect of *SNCA* silencing on endogenous α-syn, the neurotoxicity induced by injection of shRNA targeting rat *SNCA* in the SN of adult rats was assessed. They showed that the nigrostriatal cell loss observed one-month post-injection was specifically due to the silencing of endogenous *SNCA* as no cell loss occurred in *SNCA* knockout mice. Loss of endogenous α-syn was toxic specifically for dopaminergic neurons as they described the initiation of both the innate and adaptive immune systems induced by *SNCA* silencing that finally contributed to a neuroinflammatory cascade and dopaminergic cell death [94]. They thus confirmed a critical threshold of endogenous *SNCA* silencing within nigral neurons above which neurotoxicity and neuronal death were certain. However, another study recently demonstrated that long-term reduction by 90% of α-syn expression using AAV-mediated delivery of shRNA against *SNCA* in rats during one year did not induce neurodegeneration in WT rats, observing only a slight decrease in tyrosine hydroxylase due to non-specific toxicities that were attributable to cellular transduction [95]. Altogether, most of these studies have shown the potential detrimental effects of complete α-syn knockdown, but these results remain controversial given the latest studies.

Even though the physiological role of α-syn is not yet completely understood, some evidence has pointed to synaptic functions [26], which could explain such adverse effects after long-lasting downregulation of *SNCA* gene. Genetic-based therapies required refining to efficiently reduce *SNCA* at physiological levels and to protect dopaminergic cells without neurotoxic side-effects. In 2010, this issue was highlighted when by partially suppressing α-syn expression using siRNA targeting specifically the squirrel monkey transcript [96]. In this first successful primate study, they produced a 21-base pair siRNA duplex targeting squirrel monkey *SNCA* transcript that was infused for four weeks unilaterally in the left SN of three monkeys. They demonstrated that naked si*SNCA* infusion induced a significant partial reduction of α-syn expression by 40–50% in nigrostriatal dopaminergic neurons [96]. Despite this α-syn reduction, they did not observe any dopaminergic cell loss, phenotypic tyrosine hydroxylase alteration, neuroinflammation or significant neurochemical dysfunction in the striatum (dopamine, DOPAC and HVA levels were unchanged). They thus confirmed the absence of deleterious adverse effects with partial α-syn silencing induced by naked siRNA infusion in an animal model highly pertinent to humans, but they did not assess the efficacy of this strategy on the reduction of *SNCA* in PD neuropathology. Furthermore, α-syn knockdown using shRNA in human neuroblastoma SHSY5Y cells enhanced cell survival and resisted methamphetamine-induced neurotoxicity, reinforcing the interest of silencing α-syn expression as an effective therapy for neuroprotection in PD pathology [97]. To revive the interest of the field in genetic-based therapies, a Japanese group developed siRNA against human *SNCA* that reduced the expression of α-syn by half in PD patient's fibroblasts carrying *SNCA* triplications, normalizing α-syn levels. Using *Drosophila melanogaster* as a PD model, they developed transgenic lines that co-expressed human α-syn and a siRNA directed against *SNCA* and showed that motor dysfunctions of flies were improved depending on α-syn reduction levels. They thus confirmed that moderate α-syn silencing, normalizing *SNCA* expression levels, was efficient to improve

motor deficits in a simple model of PD [98]. A short-term reduction by 35% of α-syn expression by AAV-mediated delivery of shRNA targeting endogenous rat *SNCA* transcript was shown sufficient to prevent motor deficit and dopaminergic cell and fiber loss presented by rotenone-exposed rats [99]. This study also showed that even if dopamine release was slightly affected by this *SNCA* reduction, motor function or nigral dopaminergic integrity were not affected in WT rats. They thus brought the first proof of concept that partial reduction of α-syn levels could be effective in slowing down neuropathology in a rodent PD model. Altogether, these studies demonstrated that partial reduction of *SNCA* could be beneficial for PD, without the neurotoxicity observe with total ablation of *SNCA*.

Another strategy employed to reduce pathologic α-syn levels was based on the principle of allele-specific RNAi effectors. In familial cases, mutated forms of α-syn are responsible for the development of the pathology. Changes of a single nucleotide in the 21–23 nucleotides that composed an RNAi effector can abrogate the silencing ability of an RNA trigger. Allele-specific RNAi silencing can thus eliminate transcripts of mutated forms of *SNCA* without affecting WT transcripts. In 2011, a study succeeded in developing shRNA that discriminated between A30P mutated and WT *SNCA* transcripts in vitro [100]. This study highlighted the interest of targeting mutant-specific silencing of *SNCA* as genetic-based therapy in the case of hereditary forms of the pathology.

Despite emerging efforts to refine strategies aiming at silencing *SNCA* in vivo, this approach has not yet been developed enough to pursue clinical studies using *SNCA*-genetic-based therapies. Indeed, limitations of RNAi strategies slow down the application to humans, such as the need: (i) to use vectors to express certain RNAi, (ii) to directly inject into the brain to target local *SNCA* and (iii) to repeat this protocol chronically to enable sustained expression of genetic tools. The first major challenging point of such strategy for clinical application is thus to find safe and efficient vectors to cross the blood brain barrier (BBB) and deliver the genetic tools in a sustained way into the brain after a systemic administration. To overcome this limitation, modified exosomes were developed that expressed a brain-targeting peptide, the rabies virus glycoprotein peptide (RVG), and loaded with siRNA against *SNCA* gene in order to specifically target α-syn silencing to the brain [101]. After intravenous administration of RVG-modified exosomes containing si*SNCA*, they showed a significant decrease of α-syn mRNA in the midbrain, the striatum and the cortex of transgenic Tg13 mice model, with no toxicity observed [101]. More recently, RVG-decorated liposomes were used to deliver siRNA for α-syn silencing into mice primary hippocampal neurons as a first validated step towards in vivo systemic therapy [102]. To optimize viral vector penetration into the brain, a non-invasive magnetic resonance-guided focused ultrasound was tested combined with systemic injection of microbubbles to locally and transiently increase the BBB penetrance [103]. Using this non-invasive methodology, they succeeded in silencing human *SNCA* by 60% in four mice brain regions (hippocampus, SN, olfactory bulb and dorsal motor nucleus) after systemic delivery of AAV-sh*SNCA* vectors through the tail vein of transgenic mice overexpressing human *SNCA*, with no deleterious side effects [103]. Another group proposed the use of intranasal delivery—a semi-permeable BBB region—of antisense oligonucleotide against *SNCA* conjugated with indatraline, a triple monoamine transporter blocker with differential affinities, to specifically target monoamine neurons involved in PD pathology [104]. They obtained a time-dependent knockdown of α-syn specifically in serotoninergic and dopaminergic neurons of the SN, the ventral tegmental area, the putamen, the caudate and the locus coeruleus [104]. Finally, a non-viral vector approach was developed based on the systemic injection of a small peptide derived from the envelope protein of the rabies virus (C2–9r) complexed with siRNA targeting α-syn in MPTP-mice [105]. This showed a sustainable neuronal specific knockdown of α-syn by 60–90% associated with dopaminergic neuroprotection and motor improvement. Recently, the same group developed another peptide derived from the apoB protein in order to carry the si*SNCA* and showed equivalent efficient neuronal specific silencing of α-syn and neuroprotection after intraperitoneal injection in human-*SNCA*-overexpressed transgenic mice [106]. Even though no clinical trials exist yet, reduction of *SNCA* expression and delivery of these gene-based therapies are being developed and could be promising for PD therapeutics.

Despite all these technical advances and appealing strategies to silence efficiently and with refinement the *SNCA* gene in the brain, genetic-based therapies targeting α-syn silencing reflected the complexity of modifying gene expression in the brain, and notably in the field of PD pathology. Interestingly, the recent discovery of ZSCAN21 as a transcriptional factor for *SNCA* [107] and its upstream regulation with TRIM17 and TRIM41 [108] could be an alternative route to modulate *SNCA* gene expression. In the same line of evidence, a recent paper reported the role of β2-adrenoreceptor agonists on *SNCA* gene expression and regulation showing that β2-adrenoreceptor agonists decreased *SNCA* expression in a dose-dependent manner through Histone 3 Lysine 27 deacetylation in WT mice SN [109]. Future strategies to silence partially *SNCA* gene expression may be focused on drug candidates that may act on these new pathways in order to pave the way of genetic-based approaches targeting α-syn silencing with high translational impact for PD. Nonetheless, these genetic-based strategies still require refinement to perturb pathological forms of α-syn all the while maintaining the physiological levels of α-syn.

4. Targeting α-syn Post-Translational Modifications

α-syn has been shown to undergo several post-translational modifications (PTM), of which numerous are associated with the pathological forms of the proteins. These modifications, such as phosphorylation, truncation, acylation, ubiquitination, or glycation can impact pathological α-syn aggregation, or oligomer formation, with many different mechanisms, and represent possible molecular targets in the development of disease-modifying therapeutic approaches.

4.1. Phosphorylation

Phosphorylation of α-syn has been the most studied PTM of the protein since the finding in 2002 that α-syn is phosphorylated at residue S129 in LB [110]. Importantly, non-fibrillar forms of α-syn extracted from tissues of PD patients can also be phosphorylated at this site [111]. pS129 α-syn has since emerged as a major hallmark of the disease, as well as other synucleinopathies. Other serine and tyrosine residues of the protein can be phosphorylated, such as S87, Y125, Y133 and Y136 [112–114]. The exact roles of the latter in PD pathogenesis are still to be determined, as many independent laboratories have shown it to be either neuroprotective or neurotoxic. Multiple studies have shown that pS129 α-syn promotes its fibrillization in vitro [110], or accelerates the formation of inclusions and exacerbates its toxicity in cell models [115]. Other studies have shown a neuroprotective effect of this PTM, as the phosphorylation deficient mutant S129A was found to be more toxic in different animal models compared to WT α-syn [116,117]. Paradoxically, this mutant was shown to induce the formation of large inclusions, rather than—possibly more toxic—small oligomers ending with a protective effect [118,119].

In an attempt to modulate the phosphorylation of α-syn as a therapeutic approach, two molecular players in this pathway have been targeted: kinases and phosphatases. Numerous kinases phosphorylate α-syn, especially at S129, such as casein kinases [112], G protein-coupled receptor kinases [113,120], or Polo-like kinases (PLK) [121]. PLK2 was shown to phosphorylate α-syn more efficiently than PLK1 and PLK3, and this PTM was largely reduced in PLK2-/- transgenic mice [117,121]. In transfected cells expressing WT α-syn and treated with recombinant PFF, PLK2 phosphorylated α-syn under its monomeric, or aggregated forms, allowing the modulation of its phosphorylation status at different stages [122]. In this model, PLK2-induced pS129 α-syn had no effect on the aggregation of the protein, but could mediate its clearance through the autophagy-lysosomal pathway [123]. The modulation of PLK2-mediated phosphorylation was obtained by its inhibition with selective and brain-permeable chemical compound BI2536 [121,122]. In WT mice and rats, treatment with BI2536 inhibited pS129 α-syn [121,124], and could be delivered locally by nanoparticles [125] but the exact consequences on this inhibition on the pathology of PD are still to be decrypted. However, as kinases phosphorylating α-syn have ubiquitous distribution and possibly compensatory effects, the development of safe and efficient brain-penetrant compounds selectively modulating this PTM is extremely challenging.

To modulate α-syn phosphorylation, phosphatases may represent another possible therapeutic target. Despite the interest in its phosphorylation, few studies have targeted phosphatases selectively dephosphorylating α-syn. Protein phosphatase 2A (PP2A) has been shown to dephosphorylate pS129 α-syn [126], although these results remain controversial [127,128]. Recently, the antidiabetic drug metformin significantly reduced the levels of pS129, through activation of PP2A, in cells and in vivo [129,130]. In MPTP-exposed mice, activation of PP2A by metformin presented neuroprotective effects, with restored dopamine depletion and behavioral impairments [130]. This phosphatase is ubiquitous and its specificity is determined by regulatory B subunits [126]. Assembly of pS129-dephosphorylating PP2A is regulated by reversible methylation, representing a possible control mechanism [131,132]. Thus, the use of activators to increase its S129 phosphatase activity represent an interesting therapeutic approach for PD. This has been achieved in mice brains with the use of a coffee component, eicosanoyl-5-hydroxytryptamide (EHT), which, by inhibiting demethylation of PP2A, enhanced its phosphatase activity toward α-syn [126]. In a human α-syn transgenic mouse model, using an EHT-enhanced diet, S129 phosphorylation was decreased, with concomitant reduction in α-syn aggregation and improvement of neuronal integrity leading to improved motor performance. Equivalent results were obtained on a similar WT α-syn transgenic mouse model, showing a synergistic effect of caffeine and EHT in enhancing PP2A activation [133]. These mechanisms of enhancing PP2A dephosphorylation of α-syn appear as promising therapeutic approaches, as they are safe and can be specific to α-syn through an allosteric activation, therefore avoiding the deleterious effects towards off-target proteins.

4.2. Truncation

Numerous studies have reported the presence of different truncated forms of α-syn in LB, and about 15% of LB-contained α-syn is truncated [134–136]. The generation of C-terminally truncated forms of the protein has been demonstrated in vivo [137] and was shown repeatedly to promote fibril assembly and to enhance full-length α-syn propensity to aggregate [138–141]. C-terminal truncation of α-syn is required for its cellular processing and can be found in control brains [137], but truncated α-syn is present in higher amounts and within higher molecular-weight species in PD brains [140]. The underlying mechanisms of α-syn proteolytic cleavage are not yet understood, but several α-syn-cleaving proteases have been identified, and represent interesting potential targets for modulating its aggregation.

Calpain I is a calcium-dependent neutral protease, activated by increased intracellular calcium levels, therefore prone to cleave α-syn in presynaptic terminals where it is predominantly localized. In vitro, calpain I cleaved α-syn after amino acid 57, and within the NAC domain, and the former cleavage was impossible on A53T mutant α-syn [142]. Under fibrillar forms, α-syn is cleaved at amino acids 117 and 122, generating C-terminal fragments that retain their fibrillar structure and induce the co-aggregation of the full-length protein. Calpain I-mediated cleavage of soluble monomeric α-syn inhibited its fibrillization, while processing of the fibrillar form of the protein by calpain I promoted its further aggregation [143]. In PD, calpain expression levels and activity were elevated in the brain, and led to synaptic dysfunction and neuronal death by contribution to the formation of toxic oligomers [144]. This study also revealed that calpain-cleaved α-syn was found in nigral LB and LN, colocalizing with activated calpain. Recently, fibrillar α-syn C-terminal truncation mediated by calpains was shown to participate in regulating seeding, fibrillization, and LB formation and maturation in vitro [145]. The inhibition of α-syn cleavage by calpain could thus represent an interesting therapeutic option. The effects of calpastatin, a natural calpain-specific inhibitor, were demonstrated in vivo, in double transgenic mouse models either overexpressing both human mutated A30P α-syn and human calpastatin showing reduced calpain activity, or overexpressing A30P on a calpastatin-deficient background associated with increased calpain activity [146]. In these animal models, calpastatin-induced reduction of calpain activity led to decreased α-syn aggregation, while the loss of calpastatin led to increased truncation of α-syn. Furthermore, overexpression of calpastatin ameliorated the neuropathology observed in A30P mutant mice. In a recent study, the effects of

systemically administered low molecular weight calpain inhibitors, Neurodur and Gabadur, on α-syn were demonstrated in vivo. In transgenic mice overexpressing human WT α-syn, increased calpain activity and α-syn cleavage resulted in its aggregation and toxicity [147]. Treatments with both calpain inhibitors resulted in the reduction in α-syn deposits, with a reduction in its C-terminal cleavage, and improvement in neurodegeneration and activity performance of these mice.

Caspase I was also shown to truncate α-syn in vitro, which generated highly aggregation-prone species. This truncation-induced aggregation was toxic to neuronal cultures, and the specific inhibition of caspase I by the chemical compound VX-765 improved neuron survival [148]. In addition, neurosin, a serine protease predominantly expressed in the central nervous system, previously detected in LB, inhibited α-syn fibrillization in vitro and was involved in its degradation [149] through α-syn cleavage within the NAC region [150], therefore inhibiting its aggregation. Lastly, similarly to calpain and neurosin, α-syn C-terminal cleavage has been shown to be mediated by cathepsin D [151] or matrix metalloproteinase 3—which were also detected in LB—[152,153], but their respective roles in truncating α-syn intracellularly in vivo remain unclear, rendering their inhibition challenging in therapeutic development for PD. Taken together, inhibiting truncation of α-syn has been shown to prevent fibrillation and aggregation, making it an interesting potential therapeutic target.

4.3. O-GlcNAcylation

The *O*-linked β-*N*-acetyl glucosamine (*O*-GlcNAc) modification is a dynamic glycosylation in which the uncharged acetylated hexosamine sugar N-acetylglucosamine (GlcNAc) is added and removed to the serine or threonine residues of proteins by intracellular enzymes *O*-GlcNAc transferase and O-GlcNAcase respectively [154]. α-syn has been shown to be O-GlcNAcylated at nine different threonine residues in proteomics experiments from mouse and human tissues [155–157]. Most of these modifications are located in the NAC region, with the T72 O-GlcNAcylation appearing as particularly important. Indeed, the addition of a single O-GlcNAcylated residue at T72 completely inhibited the oligomerization and fibrillization of the full-length unmodified protein, without affecting its ability to bind membranes, and prevented the toxicity of α-syn in the treatment of neuronal cultures [158]. S87 O-GlcNAcylation was also shown to decrease the aggregation of the protein [159]. Both T72 and S87 O-GlcNAcylation inhibit the calpain-mediated cleavage of α-syn, associated with its aggregation [160]. It was shown recently, through the synthesis of six site-specific O-GlcNAcylated α-syn variants, that O-GlcNAcylation in general inhibited the aggregation of the protein, and could also alter the structure of its aggregated forms [161]. In this study, several of the O-GlcNAcylations prevented the toxicity of extracellular α-syn fibrils in a primary neuron culture PD model. Additionally, O-GlcNAcylation inhibited the aggregation of an aggressive α-syn mutant. Interestingly, MK-8719, a CNS penetrant O-GlcNAcase inhibitor towards tauopathies, has been advanced to phase 1 clinical trial [162]. This molecule could also represent a promising anti-aggregative therapeutic target towards α-syn in PD.

4.4. Other PTMs: Ubiquitination, SUMOylation, Acetylation, Glycation and Nitration

As many other cellular inclusions associated with neurodegenerative disorders, ubiquitylated proteins are components found in LB [163]. α-syn itself in LB is mainly mono-, di-, and tri-ubiquitinated [164–166]. A recent study showed that ubiquitin-specific protease 13 (USP13) can regulate α-syn clearance [167]. This de-ubiquitinase was found to be upregulated in post-mortem PD brains. Furthermore, knocking down USP13 resulted in increased α-syn ubiquitination and clearance in WT mice, prevented neuronal death and improved motor performance in α-syn lentiviral or transgenic PD mouse models. The major issue with targeting these ubiquitinating ligases resides in the lack of specificity of these pathways, and on the opposite effects of ubiquitination on α-syn aggregates [168–175].

α-syn has also been shown to be SUMOylated at lysine residues by PIAS2. An increase in PIAS2 expression along with SUMOylated α-syn were detected in PD brains, and PIAS2 was even detected in LB within nigral neurons. SUMOylation by PIAS2 directly promoted the aggregation of

α-syn, and impaired its ubiquitination by the above cited ligases, preventing its degradation [176]. Importantly, the SUMOylation of α-syn was also shown in other studies to inhibit its aggregation and toxicity [177,178]. Thus, SUMOylation could be an interesting target but, given the inconsistencies observed, more research must be done on this PTM.

Acetylation of α-syn has also been demonstrated at several Lysine residues, predominantly in the N-terminus (K6 and K10). This acetylation at the N-terminus stabilized the helical structure of α-syn, increasing its binding to the membrane. In addition, acetylated α-syn led to a lower propensity to aggregate than non-acetylated α-syn, but also to a different fibril polymorphism [179]. Sirtuin 2 (Sirt2), a deacetylase, has been implicated in α-syn mediated toxicity and aggregation in *Drosophila melanogaster* [180]. More recently, α-syn acetylation at K6 and K10 modulated not only aggregation but also autophagy in vitro. In this same study, Sirt2 knockout mice injected with either AAV-α-syn or MPTP showed less dopaminergic cell loss [181]. Altogether, these studies elucidate the potential therapeutic effects of Sirt2 targeting to decrease α-syn aggregation.

Finally, glycation of α-syn was first shown to be implicated in its toxicity through DJ-1, which has both glyoxalase and deglycase activities [182]. The increased glycation on α-syn Lysine residues through the use of methylglyoxal (MGO) induced an increase in α-syn toxicity and aggregation in vitro and in vivo. MGO was shown to amplify α-syn oligomer production and interfere with the N-terminal structure of the protein. Using anti-MGO molecules such as aminoguanidine and tenisetam, this same study demonstrated a better α-syn clearance and lower aggregation in *Drosophila melanogaster* [183]. Glycation inhibition via MGO scavengers constitute potential novel possibilities to lower α-syn oligomer formation.

Lastly, nitration of all four tyrosine residues of α-syn have been described [184–186]. But this PTM has shown very diverse effects on the aggregation of the protein [186–188] and therefore is not yet a viable therapeutic target in the treatment of PD.

In summary, targeting α-syn PTM in the development of treatments for PD can represent an interesting therapeutic approach. However, we have to bear in mind that the exact roles of these protein modifications in the pathogenesis of the disease are not yet fully unraveled. In addition, the enzymes that must be targeted to modify these PTM do not act specifically on α-syn, so they could be deleterious for the function of other proteins. Modifying these enzymes must thus be precise to only modify α-syn PTM.

5. Immunotherapy

Immunotherapy relies on the boost of the immune system as a potential therapy. In the case of PD, it relies on the immune system being directed towards the elimination of α-syn aggregates. There exist different types of immunizations: passive, active and alternative.

5.1. Passive Immunization

Passive immunization relies on the use of antibodies directed towards α-syn protein sequence to protect against neurodegeneration and reduce α-syn accumulation and propagation. This strategy presents many unknowns, including the fact that antibodies must be able to recognize α-syn despite its cytoplasmic localization and pass the BBB. Nonetheless, the propagation and abnormal localization of α-syn could make passive immunization relevant as a therapeutic strategy. Two main regions have been used to target α-syn: the C-terminal and the N-terminal regions of this protein.

The 9E4 antibody was one of the first antibodies tested in transgenic mice overexpressing human WT α-syn that showed beneficial effects [189]. After six months of 9E4 injections, the antibody had indeed entered the CNS and localized in lysosomes. These mice showed an improvement in their motor and learning deficits, as well as a reduction of α-syn accumulation in synapses and axons of cortical and hippocampal neurons [189]. Following this positive study, this murine antibody was derived to create the first antibody used for human clinical trials under the name PRX002. Combined, two phase 1 clinical trials showed promising results in the safety and tolerance of PRX002 in healthy

volunteers and PD patients, and its potential to eliminate free α-syn [190,191]. Currently, phase 2 trials for this antibody are underway (NCT03100149). Targeting the C-terminus of α-syn using antibodies was also tested using α-syn transgenic mice. Weekly administrations of the antibody named 274 revealed its beneficial effects on α-syn clearance. The interaction of 274 antibody with α-syn formed complexes which were then internalized by microglia via Fcγ receptors. Injection in vivo of 274 decreased cell-to-cell transfer of α-syn, reduced accumulation of α-syn, and increased α-syn uptake and clearance by microglial cells [192]. The third group of antibodies targeting the α-syn C-terminus, 1H7, 5C1 and 5D12, were tested in transgenic mice overexpressing WT human α-syn. 1H7 and 5C1 antibodies were able to reduce accumulation of cortical and striatal α-syn, neurodegeneration and behavioral alterations in these mice. The authors determined that these antibodies were able to block α-syn truncation by calpain I, inhibiting propagation and accumulation of α-syn, and thus its neurotoxicity [147]. Following these results, a combined model of transgenic mice overexpressing WT α-syn and lentiviral α-syn unilateral injection was used to assess the effect of immunization with the 1H7 antibody. Injections of 1H7 reduced α-syn-induced neurodegeneration, motor impairment, as well as axonal accumulation and transport to the contralateral hemisphere after the lentiviral injection [193]. Recently, a fourth antibody targeting the C-terminus of α-syn has been proven to sequester extracellular α-syn and to decrease spreading in vitro and in vivo: MEDI1341. MEDI1341 was capable of blocking α-syn accumulation and its trans-axonal spreading in hippocampi of mice unilaterally injected with human α-syn [194]. MEDI1341 is currently in a single-ascending dose phase 1 human clinical trial in healthy volunteers to determine its safety and tolerability (NCT03272165).

Concerning antibodies targeting the N-terminus, α-syn monoclonal antibodies were tested in PFF-injected mice to determine whether immunization could block PFF entry, cell-to-cell propagation and neurodegeneration. Syn303 was capable of recognizing and blocking misfolded α-syn in vitro, and was then tested in PFF-injected mice. Syn303 treatment reduced and delayed the spread of α-syn pathology, improved motor performances, and inhibited dopaminergic cell loss compared to non-treated PFF mice [195]. In another study targeting the human N-terminus of α-syn, intraperitoneal injection of the AB1 antibody in an AAV-WT α-syn rat PD model was able to protect dopaminergic cells, but was not completely able to protect against the motor deficits. In addition, AB1 reduced levels of α-syn and microglial activation in the SN [196]. Recently, another human-derived antibody (BIIB054) targeting the N-terminus was capable of binding aggregated forms of α-syn. This study showed the ability of this antibody to bind to pathologic α-syn in different mice models (PFF-injected and AAV-A53T human α-syn mice) as well as in post-mortem PD human brains. In mice injected with PFFs, treatment using BIIB054 was able to reduce the loss of dopamine transporter DAT in the striatum, decrease α-syn spreading, and alleviate motor impairments [197]. This antibody has already been implicated in a phase 1 clinical trial to test its safety and tolerance in healthy and PD participants. Single-dose intravenous injections up to 130 mg/kg were tested and relatively well tolerated in healthy participants, with similar adverse effects for all doses up to 45 mg/kg. PD patients then received a single dose up to 45 mg/kg, which was well tolerated, and displayed pharmacokinetic profiles that were similar to healthy volunteers [198]. This encouraging phase 1 data supports the start of the phase 2 trial of BIIB054 (NCT03318523).

In addition to antibodies targeting the specific sequence of α-syn, certain antibodies are being developed to specifically target oligomeric or fibrillary pathogenic forms of α-syn [199–202]. The mAb47 antibody was shown to target specifically α-syn protofibrils in human A30P α-syn transgenic mice. Administrations of mAb47 proved to specifically reduce the amount of protofibrils, without affecting monomeric or fibrillary α-syn, and decreased the motor deficits present in this model [201]. Based on these results, the BioArctic company has developed an antibody targeting human α-syn derived from mAb47, BAN0805, which is currently being tested in phase 1 clinical trials (NCT04127695). Finally, five antibodies specific to oligomeric and fibrillary α-syn were developed and used to immunize mice weekly for over 3 months. Immunization with Syn-F1, Syn-O1 and Syn-O4 decreased accumulation of α-syn oligomers, reduced PK-resistant α-syn aggregates, prevented neurodegeneration

in the hippocampus, and improved behavior of WT α-syn overexpressing mice. Additionally, only Syn-O4 was able to reduce the activation of microglia [202]. Taken together, the last decade has seen the rise of passive immunization as a potential lead in the treatment of PD. Multiple promising phase 1 and 2 clinical trials are currently underway and many preclinical studies could give rise to other trials as well.

5.2. Active Immunization

Active immunization or vaccination relies on the activation of the immune system to generate antibodies targeting a specific antigen. In the case of PD, the goal is to target α-syn to potentially reduce its aggregation and propagation in patients in the long-term.

In 2005, a first study aimed at using active immunization in WT α-syn transgenic mice by injecting mice with purified full-length recombinant α-syn expressed in *E. Coli*. After immunization, human α-syn antibodies generated in mice were able to decrease the accumulation of α-syn in neurons and neurodegeneration in synapses. Given the colocalization of α-syn with the lysosomal marker cathepsin D, the immunized mice could activate degradation pathways and thus eliminate α-syn more efficiently [203]. Following this study, another vaccination approach fused α-syn epitopes (α-syn$_{85–99}$, α-syn$_{109–126}$, α-syn$_{126–140}$) with P30, a T-helper epitope from tetanus toxin, to avoid the activation of harmful T-helper responses after vaccination. These three α-syn epitopes fused with P30 were injected in WT mice, which induced a strong antibody response. The antibodies produced by this response were in turn able to recognize LB and LN in post-mortem brain samples [204].

Other studies have tried active immunizations using small peptides, or Affitopes. Affitopes are short peptides with a sequence mimicking the native epitope, which are capable of eliciting a B-cell response without activating T-cell responses. These peptides have already been tested in AD successfully and have reached clinical trials. AFF1 was used to target the C-terminus of α-syn in WT α-syn transgenic mice. Immunization with AFF1 promoted the clearance of aggregated α-syn without affecting murine monomers or total α-syn. In addition, AFF1 was able to ameliorate motor functions, reduce neurodegeneration, decrease inflammation of microglia and astroglia, and promote secretion of anti-inflammatory cytokines [205]. Following these positive results of AFF1, Affitopes PD01 and PD03 were developed for human clinical trials. In 2012, the first phase 1 clinical trial for PD01 including early-stage PD patients and healthy volunteers demonstrated low toxicity for both subcutaneously injected doses that were tested. This was followed by a boost immunization in participants, which was also well tolerated and participants are currently being observed over time. First immunizations with PD01 induced an immune response against the peptide and against the targeted α-syn epitope, with generation of α-syn antibodies. The boost immunization then showed an increase in reactivity in the immune response [206–208].

Recently, combining cellular and humoral immunization using a glucan particle (GP) vaccine containing both an α-syn antigen and rapamycin has also been tested in WT α-syn transgenic mice. This GP vaccine induced a strong immune response with activation of T-regulatory cells in the CNS as well as a strong α-syn antibody production. This in turn reduced α-syn accumulation, neuron loss, and microglial activation, which could not be observed without the combined immunization [209].

The pursuit of active immunization in the case of PD has been growing quickly in the last 15 years, in particular due to its large-scale implications if successful. Nonetheless, vaccination remains difficult due to the fact that the antibodies generated must find their way to the CNS in large enough quantities to have a substantial effect.

5.3. Alternative Immunizations

Other than passive and active immunizations, alternative immunizations have arisen where α-syn is not targeted directly by antibodies. Dendritic cell (DC)-based vaccination relies on the use of antigen-sensitized dendritic cells as vehicles for immunization. A53T α-syn transgenic mice were immunized with different α-syn peptide-sensitized DC vaccines over 17 months to assess their effect

on α-syn antibody production and α-syn clearance. Results indicated that peptide-sensitized DC vaccines were able to induce a humoral response and generate α-syn antibodies. They were also able to decrease the levels of pro-inflammatory cytokines and partially protect from locomotor defects [210].

Aptamers are short, single-stranded DNA or RNA molecules that have been used as an alternative to antibodies as they are not immunogenic or toxic and, given the fact that they are composed of nucleic acids, they are extremely stable in the organism. Recently, aptamers targeting α-syn have been developed to inhibit α-syn aggregation and rescue cellular functions associated with its accumulation [211]. After successful tests in vitro, two aptamers were tested via RVG-exosome delivery in PFF-injected mice. After injections of the aptamer-containing exosomes, mice showed a decrease in dopaminergic cell loss and in α-syn accumulation in the midbrain. In addition, motor symptoms were reduced, but no changes were observed in microglial inflammation [212]. This was the first study that successfully delivered aptamers to the CNS and reduced the accumulation of α-syn.

Immune Toll-like Receptor-2 (TLR2) has previously been implicated in synucleinopathy in multiple capacities: astrogliosis, cell-to-cell transfer of α-syn, and autophagy-mediated clearance [213,214]. In this respect, TLR2 appears to be a potentially interesting target to decrease α-syn accumulation and propagation, via its roles in autophagic clearance and cell-to-cell transfer. To target TLR2, TLR2-depleting antibody (T2.5) was used in WT α-syn transgenic mice. After treatment, mice showed a decrease in α-syn aggregation, astrogliosis and microgliosis, and neuronal loss [215]. This suggests a potential alternative immunization by targeting α-syn accumulation and propagation indirectly.

Finally, intrabodies are small 14–30 kDa proteins that are derived from antibody fragments and are engineered to act intracellularly. Intracellular antibodies, unlike classic antibodies, are composed only of the Fv variable regions which determine the specificity of the antibody. Different intrabody structures exist: single-chain Fv intrabodies, with both the variable heavy and light chain, single domain intrabodies, or camelid nanobodies, with small heavy-chain-only fragments [216]. Multiple studies aiming to target α-syn via intrabodies have reported efficiency in vitro and with computational modeling [217,218]. Recently, VH14 and NbSyn87 intrabodies, targeting the NAC region and C-terminus of α-syn respectively, have been shown to interfere with α-syn aggregation in vitro [219,220]. VH14 and NbSyn87 combined with the PEST motif that targets the ubiquitin-proteasome system, have additionally been shown to block α-syn accumulation and attenuate proteasomal stress [221,222]. VH14*PEST proved to be more successful in targeting α-syn accumulation and showed very little neuroinflammation, demonstrating its potential for future potential therapies.

To conclude, immunotherapy targeting α-syn has been extensively studied using passive, active or alternative immunizations. Independently of the type of immunization, it appears that it could be feasible to reduce the amount of both intracellular and extracellular α-syn. Additionally, self-made antibodies could also inhibit the cell-to-cell propagation of α-syn. This could have extremely beneficial effects in avoiding spreading of α-syn, and thus neurodegeneration and neuroinflammation. In addition, complete immunization would require several injections over a long period of time, but the procedure is relatively non-invasive for patients. Nonetheless, it is important to note the disadvantages that accompany immunization. Little is also known on the effect of producing such large amounts of soluble α-syn on the native and pathologic forms of the protein, nor the localization of the protein. To successfully target α-syn, these immunizations rely on targeting a specific type of oligomer or aggregate, which would restrict the immunization and omit other forms of oligomers/fibrils. Also, LB contain different forms of α-syn, modified by PTM and truncated forms, which could also reduce the efficiency of these α-syn antibodies. Furthermore, we have yet to determine the immune reaction that could occur in the long-term against these α-syn antibodies and repeated injections. Certain strategies such as nanobodies and Affitopes attempt to avoid this immune activation, but the reaction to other strategies remains to be seen. Finally, despite extensive studies in animal models, a method to measure the penetrance and efficacy of α-syn-targeting treatments has yet to be discovered. Measuring the CSF and serum levels of α-syn have not yet been completely refined nor been proved to correlate with brain

α-syn levels [223]. Without this measure in vivo, it seems difficult to determine the actual efficiency of the treatment.

6. Anti-Aggregative Small Molecules

Several small molecules have been discovered or developed by drug screening or drug repositioning to treat PD in the goal to provide an anti-aggregative strategy. Several mechanisms of action have been reported: direct interaction with α-syn by small molecules or indirect modulation of chaperones to inhibit α-syn aggregation.

6.1. Molecules Directly Interacting with α-syn

Among the existing anti-aggregative small molecules, some have the ability to directly interact with α-syn and have been explored for their ability to slow accumulation of α-syn, making them potential therapeutic targets for PD.

Certain small molecules have been developed to specifically target Lysine residues of α-syn. Molecular tweezers inhibit key interactions in the self-assembly of amyloidogenic protein by binding to positively charged amino acid residues and disrupting both hydrophobic and electrostatic interactions [224,225]. The molecular tweezer, termed CLR01, was demonstrated as an inhibitor of aggregation of α-syn into fibrils in cell culture and zebra fish embryo. It could also disaggregate de novo fibrils by binding specifically with the lysine residue of α-syn via the hydrophobic and electrostatic forces [226]. CLR01 was shown to bind multiple lysine residues and potentially modified the kinetically controlled aggregation process [227]. More recently, continuous intracerebroventricular administration of CLR01 in WT α-syn transgenic mice improved motor dysfunction and caused a significant decrease of soluble α-syn in the striatum [228]. In 2005, DA and its analogs were also showed to inhibit α-syn fibrillization. More specifically, DA oxidation products, termed quinones, interact with lysine residues of α-syn, leading to the inhibition of α-syn fibrilization [229]. Moreover, in vitro fibrilization assays were performed and revealed that five selected dopamine analogs affected the aggregation process [230]. Recently, developing an assay to screen compounds with α-syn modulating properties, dopamine agonists D-519 and D-520 were discovered and showed to modulate aggregation and toxicity of α-syn. They notably showed its neuroprotective effect against the toxicity caused by α-syn in a *Drosophila melanogaster* model of synucleinopathy [231]. Analyzing α-syn species by biochemical approaches, another small molecule, an *ortho*-iminoquinone (IQ) was shown to reduce amyloid aggregation by reacting with lysine residues. IQ also reacted with free amines within the amyloid fibrils preventing their dissociation and seeding capacity [232].

Another group of small molecules preferentially bind to the C-terminal region of α-syn. Among them, the protein endosulfine-alpha (ENSA), a member of the cAMP-regulated phosphoprotein family, has been reported to interact specifically with membrane-bound α-syn [233,234]. The interaction between ENSA and α-syn led to an inhibition of membrane-induced α-syn aggregation and ENSA overexpression decreased α-syn neurotoxicity in neuronal cultures [235]. In the same line of evidence, the isoquinoline derivative Fasudil is the first small molecule Rho-associated protein kinase (ROCK) inhibitor developed for clinical use in humans. Using cell culture and cell free assay, the anti-aggregative potential of Fasudil was revealed, through its effects mediated by ROCK-inhibition, binding to tyrosine residues Y133 and Y136 in the C-terminal region of α-syn. Furthermore, long term treatment improved motor and cognitive functions and significantly reduced the α-syn pathology in the midbrain in the A53T α-syn transgenic mouse model [236]. Finally, despite its lack of metabolic stability and low oral bioavailability, previous studies demonstrated promising beneficial effects of NPT100-18A, an α-syn misfolding inhibitor, in vitro and in α-syn overexpressing transgenic mice [237]. NPT200-11, an optimized compound, with pharmacokinetic properties suitable for clinical evaluations, maintained robust beneficial actions in α-syn-based animal models. NPT200-11 was shown to interact with a domain in the C-terminal region of α-syn, thus reducing α-syn pathology, neurodegeneration and CNS inflammation and improving behavior impairment in transgenic mice that overexpressed WT α-syn or

α-syn linked to GFP [238]. A phase 1 clinical trial for this molecule testing its safety and tolerability has already been completed, and another phase 1 clinical trial to test its safety in PD patients is planned (NCT02606682).

Finally, based on a systematic high-throughput screening campaign combined with medicinal chemistry optimization, the oligomer modulator anle138b [3-(1,3-benzodioxol-5-yl)-5-(3bromophenyl)-1H -pyrazole] was developed. In vitro and in two different PD mice models, anle138b blocked the formation of pathological aggregates of α-syn targeting specifically oligomeric forms. In both rotenone-exposed and A30P α-syn transgenic mice, it strongly inhibited oligomer accumulation, neuronal degeneration [239], and disease progression even if the treatment started after disease onset [240]. Recently, anle138b rescued the dopamine deficit and reduced the density of α-syn aggregates associated with an increase in dispersed monomeric and small assemblies in transgenic mice brains [241]. This promising molecule for PD therapy, with high bioavailability and low toxicity, is currently in phase 1 of a clinical trial started at the end of 2019 (NCT04208152).

6.2. Heat Shock Protein Modulators

Heat shock proteins (Hsp) are molecular chaperones that assist in proper conformational binding of proteins, including α-syn [242]. It was shown that α-syn co-immunoprecipitated with Hsp90 and Hsp70 [243] and that Hsp modulators were protective against α-syn-induced toxicity, and could prevent its aggregation. In protein quality control processes, Hsp90 and Hsp70 had opposing effects on target protein stability. Hsp90 stabilized the proteins and inhibited their ubiquitination, whereas Hsp70, along with its co-chaperone Hsp40, was required for the degradation of many proteins promoting ubiquitination and proteasomal degradation dependent of CHIP, a component of LB in the human brain [168]. Modulating proteostasis by inhibiting Hsp90 function or by promoting Hsp70 function enhanced the degradation of the critical aggregating proteins and could be used for the treatment of PD against α-syn toxicity [244].

The first compound that was investigated in PD models was Geldanamycin, a naturally occurring antibiotic of the Ansamycin family. Geldanamycin effectively prevented α-syn aggregation by increasing its clearance, leading to a reduced toxicity in yeast model of PD, expressing WT or A53T α-syn [245]. A *Drosophila melanogaster* model of α-syn toxicity confirmed the decrease of α-syn oligomerization associated with neuroprotective effects of Geldanamycin [246–248]. This protective effect was also showed in the MPTP mouse model of PD [249]. Mechanistically, Geldanamycin protected cells against extracellular α-syn-induced neurotoxicity by preventing re-secretion of α-syn [250]. Similarly to Geldanamycin, 17-AAG (Tanespimycin) attenuated α-syn toxicity, prevented oligomerization and facilitated α-syn clearance in cultured cells [251,252]. Despite encouraging results, the use of Geldanamycin or 17-AAG has been limited because of their poor solubility and BBB permeability. On the contrary, synthetic small-molecule inhibitors of Hsp90 such as SNX-2112 and its derivatives displayed good pharmacokinetic characteristics. Using the bioluminescent complementation assay, a decrease in both high-molecular weight and monomeric α-syn was showed, as well as a reduction of α-syn oligomerization in cell culture models treated with SNX compounds. Even if most derivatives inhibited α-syn oligomerization, the four compounds SNX-3113, SNX-3723, SNX-8891 and SNX-0723 were found most potent in this study [251]. The site of interaction between Hsp90 and α-syn affected regions that are responsible for vesicle binding and amyloid fibril assembly. Those processes are perturbed in an ATP-dependent manner. Indeed, in the absence of ATP, Hsp90 inhibited α-syn fibril formation and promoted α-syn oligomer accumulation whereas, in the presence of ATP and Hsp90, fibril formation was favored [253].

Overexpression of Hsp70 or Hsp40 reduced or prevented the formation of high molecular weight forms of α-syn in cellular PD models [254–256]. By inducing Hsp70, Geldanamycin effectively prevented α-syn aggregation in cell culture model of PD [257]. The chemical induction of Hsp70 by Carbenoxolone (CBX), a glycyrrhizic acid derivative, decreased α-syn aggregation and prevented α-syn-induced cytotoxicity in cell cultures [258]. Similarly, overexpression of CHIP inhibited α-syn

inclusion formation and reduced α-syn protein levels, [168] increasing ubiquitination of α-syn both in vitro and in solution [259]. Hsp70 was also shown to inhibit α-syn toxicity in a *Drosophila melanogaster* and transgenic mouse model of PD [247,254]. Moreover, AAV-Hsp70 overexpression into dopaminergic neurons significantly protected the nigrostriatal pathway in MPTP mouse model [260]. Several studies suggested that Hsp70 interacted with prefibrillar α-syn species and interactions between the Hsp70 substrate binding domain and the α-syn core hydrophobic region was sufficient for assembly inhibition [261–263]. Cooperating with Hsp70 and Hsp40, Hsp104 reduced the formation of phosphorylated inclusions and prevented α-syn-induced neurodegeneration in a rat PD model [264]. More recently, the efficacy of Hsp110 in preventing or reducing α-syn aggregation was demonstrated in cell cultures and in double-transgenic α-syn/Hsp110 mice [265]. Development of activators promoting Hsp104 or Hsp110 functions could be interesting for the treatment of PD.

Several small molecules, by direct interaction with α-syn or indirect chaperone modulation, are able to reduce α-syn aggregation and neurodegeneration in vitro and in vivo using different animal PD models. As described, binding to lysine residues or C-terminal domain of α-syn, some small molecules can prevent and/or inhibit de novo fibrils. For some molecules, this beneficial effect is associated with neuroprotection and motor improvement in preclinical studies. Even if no clinical proof exists yet, these promising results encourage the development of small molecules for PD therapy. Hsp modulators could also be good candidates for future treatments of PD.

7. Increasing Clearance of α-syn

Two cellular pathways are involved in α-syn clearance trying to maintain its physiological protein levels: the ubiquitin-proteasome system (UPS) [266] and the autophagy-lysosomal pathway (ALP) [267–269]. UPS is involved in short-lived, damaged and misfolded protein degradation through a step of ubiquitination followed by a proteolysis, involving the action of multiple proteases. ALP is a complex process in charge of long-lived and aggregated protein degradation, as well as clearance of damaged organelles through multiple pathways including the non-selective macroautophagy (MA) and the selective chaperone-mediated autophagy (CMA). MA degrades cellular waste through the fusion of the autophagosomes carrying the material into the lysosomes containing the enzymatic material, whereas CMA degrades the proteins after the specific recognition of a pentapeptide KFERQ-like motif by the cytosolic chaperone heat-shock cognate 70kDa (Hsc70) and delivery of the targeted protein to the lysosomes through the Lysosomal-Associated Membrane Protein 2A (LAMP-2A). Alterations in both ALP pathways involved in α-syn clearance have been implicated in PD pathology by both genetic and post-mortem studies [40]. Therapeutic strategies aiming to increase the α-syn degradation through activation of these clearance pathways have thus been deeply explored in order to reestablish physiological levels of the protein and prevent from its accumulation and propagation in a PD context.

7.1. Activation of the UPS

The discovery of ubiquitin as a component of LB [266] as well as decreased proteasome activity measured in the SN of PD patients [270] already highlighted a detrimental alteration of the UPS contributing to PD neuropathology. In vitro, UPS inhibition was shown to increase the amount of ubiquitin-positive α-syn aggregates, confirming the significance of the proteasome system deficiency in α-syn accumulation [271,272]. It has also been shown that α-syn aggregates inhibited the catalytic 26S proteasome subunit through its direct interaction with the regulatory 19S proteasome subunit [273,274], pushing the cell into a vicious circle of detrimental factors leading to α-syn accumulation.

Various strategies have been investigated in order to increase UPS activity, but only few studies demonstrated the benefits directly achieved by an enhancement of the UPS-mediated α-syn degradation. A first study proposed to deliver additional free ubiquitin molecules to the cell in order to facilitate the labeling of the targeted protein and thus the overall proteasomal activity in a fly model of PD [275]. They showed that co-transfection of WT or K48-ubiquitin into neuronal cells of flies provided neuroprotection against α-syn-induced toxicity. In 2014, a plant extract from the *Rhodiola rosea L.*,

called Salidroside, was shown to protect MPP^+-treated PC12 cells and MPTP-treated mice by reducing expression of proteins involved in the UPS pathway regulation [276]. The group demonstrated that administration of Salidroside induced dopaminergic neuroprotection, associated with decreased levels of α-syn protein in MPTP-treated mice [276]. They confirmed the relevance of using Salidroside in a second study. They showed in vitro that transfection with WT or A30P α-syn vectors, after Salidroside treatment for 24h, increased clearance of α-syn by 30%, associated with a 60–70% increase of the 20S proteasome activity [277]. Then, they administrated the molecule to 6-OHDA-treated SHSY5Y cells and showed increased cell viability and decreased levels of phosphorylated α-syn through UPS-dependent activation [277]. Similarly, a suitable screening assay found the PD163916 compound, which activated proteasomal activity through the inhibition of the p38aMAPK pathway [278]. They showed that PD163916 treatment in primary mouse neurons increased the proteasome activity and decreased α-syn levels, suggesting an increased clearance of α-syn through the UPS activation [278]. More recently, the T-006 compound properties, a derivative of tetramethylpyrazine, were explored for its potential therapeutic activities to enhance proteasomal α-syn clearance [279]. In an inducible PC12/A53T α-syn cell model, they demonstrated a dose-dependent decrease of soluble and insoluble α-syn levels upon T-006 treatment. T-006 was showed to activate the chymotrypsin-like UPS through LMP7 protein expression upregulation by activation of the PKA/Akt/mTOR/P70S6K pathway [279]. They confirmed the significance of the use of T-006 in vivo providing evidence that T-006 prevented from neurodegeneration and motor deficits and induced decreased α-syn levels in A53T α-syn transgenic mice [279]. Finally, as previously mentioned, neuroscientists used two nanobodies fused with a proteasome-targeting proline, aspartate or glutamate, serine, and threonine (PEST) motif targeting either the NAC domain or the C-terminal of the α-syn protein. They first demonstrated that treatment of WT-α-syn-overexpressed ST14A cells upon PEST-nanobodies treatment enhanced turnover of the α-syn protein through, at least in part, proteasomal activation [221]. They later showed that these two PEST-nanobodies reduced the levels of phosphorylated and aggregated α-syn in rats overexpressing the WT-α-syn in the SN [222]. PEST mediated clearance of α-syn also led to dopaminergic neuroprotection when the NAC-targeting nanobody was used in those animals [222].

Enhancing UPS seems efficient to prevent α-syn accumulation in PD neuropathology. Strategies aiming at targeting the UPS are still at their early stages and still need to be developed further. Nevertheless, it is important to note that distinct roles of UPS and ALP have been described in vivo. While UPS degrades α-syn in physiological conditions, ALP process could be in charge of degrading elevated intracellular α-syn levels in more advanced pathological conditions [280]. Enhancing α-syn degradation through ALP activation, by both CMA and MA, seemed thus compulsory in a PD context.

7.2. Activation of the CMA

α-syn is a substrate of the CMA as its protein sequence contains a KFERQ-like motif (VKKDQ) [281]. It has been demonstrated that CMA contributes to α-syn degradation in different cell types in vitro [268,269]. Through the same line of evidence, mutation in the CMA-recognition motif of α-syn sequence [268] or knocking-down the rate-limited LAMP-2A CMA receptor [282] led to accumulation of α-syn in vitro. CMA process has been suggested to be altered in PD brains as LAMP-2A and Hsc70 CMA protein levels are significantly reduced in the SN and the amygdala of PD patients [283]. Interestingly, this decrease was shown to be associated with α-syn accumulation at early stages of PD [284]. Reciprocally, CMA is also a target of α-syn toxicity as it has been demonstrated that A30P and A53T mutated α-syn [268], as well as dopamine-modified forms of α-syn [285,286], inhibited the CMA activity through their high affinity to the LAMP-2A receptor, associated with poor internalization and degradation into the lysosomal compartment. Some studies also pointed out a detrimental role of WT α-syn overexpression on CMA activity [286,287]. Therapeutic strategies aiming to increase α-syn clearance through CMA activation have thus been investigated to normalize the protein level and to prevent from its neuronal accumulation. In order to boost α-syn clearance through CMA, the rate-limited LAMP-2A receptor was overexpressed in the nigral dopaminergic neurons of WT-α-syn-overexpressed

rats. This was a non-toxic, efficient strategy to enhance CMA-mediated pathological and aggregated α-syn clearance, associated with dopaminergic neuroprotective effects [288]. Another strategy to modulate LAMP-2A levels is based on the use of retinoic acid derivatives. In 2013, targeting the retinoic acid receptor by chemical retinoic derivatives led to a specific CMA activation through LAMP-2A transcriptional upregulation and could be relevant therapeutic molecules to enhance CMA-mediated α-syn clearance [289]. Finally, miR-based therapies were also considered to enhance CMA activity. Interestingly, post-mortem studies revealed that increased LAMP-2A and Hsc70-targeted miR levels were associated with decreased LAMP-2A and Hsc70 protein levels, which correlated with the severity of LB pathology in the SN and the amygdala of PD patients [290]. A more recent study notably demonstrated that miR-21 upregulation led to decreased LAMP-2A transcription and protein levels and contributed to increased levels of α-syn protein in MPP^+-treated SHSY5Y cells and MPTP-treated mice [291]. This miR-21 expression was shown to be upregulated in the SN of PD patients [292]. Geniposide, an iridroid glucoside extracted from the fruit of *Gardenia jasminoides*, completely reversed the miR-21-induced effects, and decreased the α-syn levels through LAMP-2A upregulation in MPTP mice model [291].

7.3. Activation of MA and Lysosomal Function

Multiple evidence also suggested that the MA process is involved in the degradation of WT, mutated and aggregated forms of α-syn in different cellular models [267–269]. MA was shown to be recruited when the UPS activity was altered and intracellular levels of α-syn were elevated in α-syn-transgenic mice brains [280]. In PD conditions, alteration of MA has been observed with accumulation of the Microtubule-associated protein 1A/1B-light chain 3 (LC3) which colocalized with LB in the nigral dopaminergic neurons of PD patients [283,293]. This observation suggested a defective clearance of the autophagosomes by lysosomes. In the same line of evidence, decreased levels of lysosomal proteins such as the Lysosomal-Associated Protein 1 (LAMP1) and the ATPase Cation Transporting 13A2 (ATP13A2) have been observed in PD brains [294–296], confirming a deficit in lysosomal activity. Moreover, decreased enzymatic activity of the lysosomal protein β-glucocerebrosidase (GCase), a well-known genetic risk factor for PD, has been measured in the SN [297,298] of sporadic PD patients, and correlated with the accumulation of α-syn [299]. The role of MA deficits in α-syn accumulation have been directly demonstrated in rodents, notably with the conditional knock-out of the autophagic related gene 7 in dopaminergic neurons of mice which led to the accumulation of α-syn [300]. Inversely, α-syn has been shown to directly alter the MA function either by inhibiting autophagosome formation through the Rab1 pathway [286,301,302], by inhibiting HMGB1 and Beclin1 complex formation, which is essential for the autophagic activation [302], or by inducing lysosomal permeabilization [38,303]. Thus, multiple therapeutic strategies aimed to increase the MA activity in order to enhance α-syn clearance and to reverse the α-syn toxicity on the MA machinery. Although multiple molecules have been investigated as activators of the MA process, we will only present here the strategies which directly linked MA activation to α-syn clearance as therapeutic approaches.

As the upstream autophagic actor mTOR (mechanistic Target Of Rapamycin) negatively regulates MA, multiple approaches first aimed to activate MA through mTOR-dependent strategies. Rapamycin, an FDA-approved antibiotic, is an allosteric inhibitor of mTOR and was deeply studied as a MA enhancer in the context of PD. Rapamycin has been shown to increase α-syn clearance of WT and mutated forms in cellular models [267] and in α-syn transgenic mice [304] through activation of the MA. Rapamycin treatment also prevented neurodegeneration and motor deficits in human A53T α-syn transgenic mice through MA reestablishment, although they failed to prove any effect on human α-syn levels in their model [305]. However, another team succeeded in demonstrating beneficial effects in WT α-syn overexpressed rat model with decreased behavioral impairment and dopaminergic cell loss associated with reduced α-syn levels induced by an FDA-approved derivate of rapamycin, CCI-779 (temsirolimus) [306]. Nonetheless, the use of Rapamycin as a drug candidate for PD was limited due to

its lack of specificity, its action on immunosuppression, and its poor solubility and stability in aqueous solutions [307]. mTOR-dependent MA induction can also be achieved through AMPK protein activation, an upstream effector of the mTOR pathway, that directly inhibits the mTOR protein. In this context, Resveratrol, a natural phytoestrogen found in grapes and red wine, was shown to directly increase MA activity through AMPK activation. Resveratrol treatment in MPTP-injected mice enhanced α-syn degradation through MA activation that finally attenuated neurodegeneration and motor deficits [308,309]. It was suggested that Resveratrol activated MA through the induction of the LC3 deacetylation by NAD^+-dependent deacetylase SIRT1 activation in an AMPK-dependent manner [310]. mTOR signaling also regulates the action of the downstream transcription factor EB (TFEB) which emerged as the master gene regulator of autophagy machinery [311,312]. TFEB regulates the expression of autophagic genes through the Coordinated Lysosomal Expression and Regulation (CLEAR) signaling network, enhancing both lysosomal biogenesis and autophagy [313–315]. TFEB overexpression prevented motor deficits and induced dopaminergic neuroprotection associated with enhancement of α-syn clearance through MA activation in human WT and A53T α-syn overexpressing rats [306,316]. Similarly, TFEB overexpression in MPTP-intoxicated mice induced autophagosomal formation and lysosomal activity in addition to neurotrophic effects [317]. Chemical activation of TFEB using FDA-approved 2-hydroxypropyl-β-cyclodextrin (HPβCD) molecule also induced MA-dependent α-syn clearance in vitro and has been suggested to be a viable therapeutic strategy in a PD context [318,319]. More interestingly, pomegranate extract was shown to activate TFEB in vitro, increasing the nuclear localization of the transcription factor, and thus activated the MA process as well as mitophagy process [320].

mTOR-independent pathways have been also extensively explored to activate MA and induce α-syn clearance in the context of PD. Among the mTOR-independent autophagy enhancers, trehalose, a disaccharide found in invertebrates, was used to activate MA processes and showed efficiency to enhance clearance of WT and mutated forms of α-syn in cellular models [321–323]. Trehalose showed in vivo beneficial effects on the clearance of α-syn aggregates through MA activation associated with neuroprotective effects and prevention of motor deficits in AAV-human-A53T α-syn rats [237]. Similarly, a short intake of trehalose for one week was sufficient to induce autophagy in mice brains and to decrease insoluble α-syn levels in a transgenic mouse model overexpressing human A53T α-syn [324]. They showed that MA-activation seemed to occur through the phosphorylation of the autophagic Beclin-1 modulator at serine 15 [324]. The mechanisms behind trehalose-mediated MA activation were further investigated and demonstrated that trehalose activated MA through the inhibition of the glucose transporter SLC2A, finally leading to the activation of the AMPK protein [325]. More recently, a pharmacokinetic study showed that a daily oral administration of trehalose was more efficient than administration of the same dose by drinking water in A53T α-syn rat model of PD, and prevented from α-syn-mediated neurodegeneration and motor deficits [326]. They also showed that trehalose oral administration was efficient to prevent from α-syn-induced striatal dopaminergic deficits in a macaque model overexpressing human A53T α-syn, although they failed to show beneficial effects on α-syn levels [326]. Altogether, trehalose has been identified as an interesting drug to target α-syn, but the correct dose and administration remains to be identified. Nilotinib, an FDA-approved second-generation cAbl inhibitor, was also investigated as a potential MA enhancer through a mTOR-independent pathway. Nilotinib administration in a transgenic A53T α-syn mouse induced clearance of accumulated α-syn leading to dopaminergic neuroprotection and decreased motor deficits in this model [327]. The involvement of the Nilotinib-induced MA activation in α-syn clearance was confirmed by demonstrating that in vitro inhibition of cAbl protein by Nilotinib prevented α-syn phosphorylation at the Y39 residue [328]. Clinical studies investigating the therapeutic interest of Nilotinib have been recently conducted on PD patients. The first clinical study demonstrated that administration of Nilotinib at 150 mg and 300 mg doses appeared to be safe and well-tolerated by twelve advanced PD patients with the limited observation of a reduction in blood and CSF α-syn levels [329]. A more recent large-scale clinical study enrolling seventy-five participants confirmed

these results with an optimal daily dose of Nilotinib at 200 mg for one year that has been shown to be efficient for the reestablishment of dopaminergic metabolism associated with decreased levels of oligomeric α-syn in the CSF [330]. Another mTOR-independent enhancer has been developed aiming to directly target the autophagic activator Beclin-1 protein through genetic or pharmacological approaches. Following Beclin-1 lentiviral injection in the temporal cortex and hippocampus of α-syn transgenic mice, a decrease in α-syn levels was observed through autophagy activation [331]. This activation was mediated through an enhancement of HMGB1-Beclin1 complex formation, an essential step to induce MA, which in turn increased α-syn clearance [332]. Moreover, Isorhynchophylline, an alkaloid derived from a Chinese herbal plant, was efficient to induce α-syn clearance through a Beclin-1-mediated MA activation in different cellular models, thus opening the way for alkaloid screening as Beclin-1 dependent autophagic inducers [333]. Interestingly, chronic caffeine treatment has also been studied as a mTOR-independent autophagy enhancer. Given that human coffee consumption has been associated with a reduced risk of developing PD [334], caffeine could be inducing MA through activation of the adenosine receptor [335]. Chronic caffeine drinking during four months in human A53T α-syn fibrils-injected mice reversed α-syn-induced MA defects and decreased levels of phosphorylated α-syn in the injected site [335].

Finally, an increase in activity of the lysosomal enzymes responsible for α-syn degradation could be an appealing approach to induce MA-associated clearance of α-syn. *GBA1* encodes the lysosomal enzyme GCase. Related to PD, *GBA1* mutations are the most common genetic risk factor for developing PD and GCase activity has been demonstrated to be defective in fibroblasts derived from PD patients harboring the *GBA1* mutations [336,337] or in a mouse model of Gaucher's disease that presented α-syn accumulation [338]. Interestingly, decreased GCase activity has been observed in the SN of sporadic PD patients [339] and has been shown to correlate with increased α-syn levels at the early stage of the disease [340]. Moreover, reduced GCase activity was shown to influence α-syn aggregation through stabilization of soluble and toxic oligomeric intermediates [341,342]. This phenomenon seemed to be dependent on neuron type, on the level of extent pathology and on the stages of the pathology [343]. In return, pathological α-syn was shown to decrease lysosomal hydrolase activity, including GBA1, possibly due to a disruption of proper enzyme targeting from endoplasmic reticulum to lysosomal compartment [341,344]. All these data pointed out GBA1 enzyme as a high-relevant therapeutic target to increase α-syn clearance and decrease α-syn pathological aggregation [345]. Ambroxol hydrochloride, a safe FDA-approved molecule, was proven to enhance GCase activity and to increase α-syn clearance [336,337]. WT and α-syn transgenic mice treated with Ambroxol presented increased brain activity of GCase, associated with decreased levels of total and phosphorylated α-syn protein in different brain regions of the transgenic model [346]. Based on the results obtained after Ambroxol treatment on Gaucher's disease patients [347] and on PD rodent models, a pilot study in humans has been launched recently in order to determine the effective dose and to prove the efficacy of such a strategy on seventy-five PD patients [348]. In parallel, another recent clinical trial enrolling seventeen PD patients and showed that Ambroxol crossed the BBB and increased the GCase activity in patients both with and without *GBA1* mutation [349]. The iminosugar isofagomine (IGF) was shown to increase GCase activity in vitro [350] and in mice [351] in a tissue-specific manner [352], making this molecule interesting for approaches targeting lysosomal activity of GCase. Histone deacetylase inhibitors such as IGF have been implicated in GCase activation through hyperacetylation of the chaperone Hsp90 that enabled the appropriate folding of the GCase protein and the elimination of the mutated forms of the protein [353]. A study succeeded in confirming that oral administration of IGF in WT α-syn overexpressed transgenic mice increased α-syn clearance through lysosomal activation and improved motor performances, supporting the interest of using pharmacological chaperones in a PD context [354]. Using high-throughput screening, two GBA1 chaperone compounds, NCGC607 and NCGC758, have been tested in iPSC-derived dopaminergic neurons from PD patients and induced improved GCase activity as well as decreased levels of α-syn and GCase substrates [355,356]. In the same line of evidence, using a Gaucher's disease mice model, administration of another GCase

modulator S-181 that stabilizes wild-type GCase protein was sufficient to decrease lipid substrates and α-syn in mice brains [357]. Targeting another lysosomal hydrolase, direct overexpression of Cathepsin D gene into mammalian cells and human α-syn-transgenic *C. elegans* induced neuroprotective effects against α-syn aggregation and toxicity [358].

Finally, combined approaches have been developed to enhance MA through both mTOR-dependent and mTOR-independent pathway in order to improve α-syn clearance. A synergistic effect using Rapamycin and Trehalose treatment on dopaminergic neuroprotection was shown in the MPTP mouse model of PD through active enhancement of MA process [359].

In conclusion, enhancing the degradation of α-syn species mediated by the UPS, CMA, MA or lysosomal enzymes using multiple approaches and targets are appealing therapeutic strategies in a PD context. Given that proteasomal and autophagic activity are decreased in an age-dependent manner and in pathological conditions, collective efforts should be pursued to find efficient drug candidates to enhance these pathways independently or synergistically. Likewise, future investigations must be focused on combined therapies in order to increase the promising of such therapies in a PD context.

8. Anti-Oxidative Strategies

Natural and endogenous antioxidants have been evaluated in vitro and in vivo as therapeutic agents for preventing and delaying the development of PD. Indeed, they have shown indirect protective effects against oxidative-induced neuronal death, and/or direct interaction with different forms of α-syn decreasing its toxic aggregation.

8.1. Polyphenols

Polyphenols, a group of chemical substances present in plants, fruits, vegetables or tea, have been studied for their antioxidant property associated to the capacity to prevent and to reduce the protein aggregation. Within anti-oxidant polyphenols, there are two families that have been shown to impact α-syn aggregation: phenolic acid derivatives and flavonoids.

Amongst phenolic acid derivatives, curcumin, chemically known as diferuloylmethane, was shown to have beneficial effects in neurodegenerative diseases, including PD, through its anti-oxidant, anti-inflammatory and anti-protein aggregation properties [360]. It was found that curcumin could inhibit α-syn fibril formation and destabilize preformed fibrils [361,362]. The anti-aggregative effect of curcumin occurred through interaction of the molecule to the hydrophobic NAC domain of α-syn via both hydrophobic and hydrogen bonding [363,364]. Curcumin interacted with both α-syn monomers and oligomers via its phenolic groups [365], but was found to preferentially bind oligomeric intermediates [360]. Conformational changes of α-syn affected the extent of binding of curcumin to α-syn and its potential in inhibiting oligomers or fibrils [360,364,366]. In addition, curcumin significantly decreased the cytotoxicity of preformed α-syn oligomeric species in SH-SY5Y cell line [367] and in PC12 cells [368]. Moderate doses of curcumin increased the level of phosphorylated forms of α-syn at cortical presynaptic terminals and improved motor behavior performance in mice overexpressing human GFP-tagged WT α-syn [369]. Its anti-aggregative effect on α-syn was also shown in dopaminergic neurons of a rat model of lipopolysaccharide-induced PD [370]. Whereas some studies suggested the interest of curcumin to treat PD, its potential efficacy is limited owing to its poor stability and bioavailability. For this reason, many nanoformulations or stable curcumin analogs were evaluated against α-syn aggregation, fibrillation, and toxicity. Curcumin pyrazole derivatives reduced the toxicity of both WT and mutant A53T α-syn by preventing fibrillation and disrupting preformed fibrils [371]. In the same way, the biphenyl analogs of dehydrozingerone and O-methyl-dehydrozingerone reduced α-syn aggregation [372]. Additionally, a curcumin-glucoside derivative prevented oligomers and inhibited fibril formation in a dose-dependent manner [373]. Amine-functionalized mesoporous silica nanoparticles of curcumin showed interaction with α-syn species and prevented fibrillation [374]. A nanoformulation containing curcumin and piperine with glyceryl monooleate nanoparticles has been shown to prevent α-syn oligomerization and fibrillation [375]. Another nanoformulation

prepared with lactoferrin by sol-oil chemistry reduced α-syn expression in dopaminergic SK-N-SH cells treated with rotenone [376]. Combining curcumin with β-cyclodextrin showed a synergistic inhibition of α-syn aggregation and degraded the preformed aggregates into monomers at very low concentrations [365,377]. Finally, an analog, the liposomal nanohybrid of curcumin with polysorbate 80-modified cerasome ameliorated motor deficits and improved dopamine expression by promoting α-syn clearance in the MPTP mouse model [378]. Gallic acid (GA), another type of phenolic acid chemically known as 3,4,5-trihydroxybenzoic acid, is found in its free form or as part of the hydrolysable tannins in many plants [379]. GA and its structurally similar benzoic acid derivatives also showed anti-aggregating effects [380]. In PD models, GA was shown to inhibit α-syn fibrillation and toxicity and to disaggregate fibrils of α-syn [380,381]. GA also bound to soluble and non-toxic oligomers and stabilized their structure. It has been shown that the number of hydroxyl groups and their presence on the phenyl ring in these structural derivatives of GA were responsible for binding and inhibiting α-syn fibrillation [381]. Finally, tyrosol, a simple phenol present in Extra Virgin Olive Oil, was also shown to be effective by reducing α-syn inclusions in a *C. elegans* model of PD. Moreover, in this in vivo model, Tyrosol had a protective effect on dopaminergic neurons, reduced ROS level and promoted the expression of specific chaperones and antioxidant enzymes [382].

Within polyphenols, flavonoid compounds have also been shown to impact α-syn aggregation. The flavonoid Baicalein, isolated from the roots of *Scutellaria baicalensis Georgi*, a Chinese herbal medicine [383] is a well-known potent antioxidant. Several studies demonstrated the efficiency of Baicalein to prevent α-syn oligomerization and fibrillation [384,385]. Baicalein had a strong effect in inhibiting α-syn oligomer formation and in disaggregating pre-formed oligomers at low concentrations [386,387]. Baicalein inhibited the formation of high molecular weight α-syn oligomers and protected against neurotoxicity in Hela and SH-SY5Y cell lines [388]. By tightly binding to α-syn, Baicalein stabilized its natively unfolded conformation [384] whereas another study specified that Baicalein-stabilized oligomers were β-sheet-enriched [389]. In another study, Baicalein reduced α-syn in the media of dopaminergic cell lines overexpressing A53T α-syn [390]. The Baicalein derivative N′-benzylidene-benzohydrazide also inhibited oligomer formation [391]. Baicalein in combination with β-cyclodextrin synergistically inhibited α-syn aggregation and disaggregated preformed fibrils even at very low concentrations [365]. Two studies also reported the in vivo protective effects of Baicalein on decreasing α-syn aggregation [392,393]. Baicalein prevented the transition from α-syn monomers to oligomers, associated with behavioral improvement and neuroprotection in the striatum of rotenone-induced PD in rats [392]. Furthermore, Baicalein attenuated MPTP-induced α-syn aggregate formation in the injected SN of mice [393]. This strong inhibition was mostly due to the formation of Schiff base [384] and the presence of vicinal dihydroxyl group on the phenyl ring of Baicalein that could be responsible for its anti-aggregative action [388]. Integrating evidence from in vitro and in vivo studies, Baicalein appears to be a potential drug candidate to inhibit α-syn aggregation, fibrillation, and propagation in neurons. Interestingly, another polyphenol, Cuminaldehyde, isolated from Iranian cumin, was found to be superior to Baicalein. It inhibited α-syn fibrillation [394] and blocked protein assembly into β-structural fibrils by the interaction with its amine and aldehyde groups with α-syn [395]. In the same line of evidence, one of the most popular catechins, (−)-Epigallocatechin 3-gallate (EGCG), a flavanol compound predominantly present in green tea, inhibited α-syn aggregation and fibrillation in a concentration-dependent manner [396]. In primary cortical neuron cultures challenged with oxidative injury, EGCG inhibited fibrillation of α-syn and apoptosis [397]. EGCG also inhibited α-syn aggregation using PD post-mortem tissue [398]. The molecular mechanisms by which EGCG blocks amyloid aggregation are not yet completely understood. However, EGCG bound the natively unfolded polypeptides directly and prevented their conversion into toxic intermediates [399]. Moreover, it can induce a conformational change by binding directly with β-sheet-rich aggregates without disassembling them into monomers or small diffusible oligomers [400–402]. It was also notably suggested that EGCG had the potential to bind to the oligomeric state of α-syn, destabilizing it and blocking the membrane affinity of α-syn [403]. EGCG was later showed to exhibit its protective effect

by facilitating the conversion of active oligomers, which could exert membrane disruption and cellular toxicity, into amyloid fibrils [404]. The effects of EGCG have also been related to metal homeostasis, which will be discussed later in this review [405]. Finally, some studies suggested the hydrophobic binding and Schiff base formation with Lysine residues as important features of its mechanism of action [400,406]. Although EGCG has been shown to be neuroprotective in MPTP-induced animal models of PD, there is no proof about its direct protective effect against α-syn aggregation toxicity in an in vivo model [398]. However, when EGCG was conjugated with nanoparticles, it allowed a neuroprotective effect and a considerable inhibition of α-syn aggregation in a mouse model of PD [407]. Finally, Theaflavins, another polyphenol present in fermented black tea, had effects comparable to EGCG but it appeared less vulnerable to oxidation by air and exhibited better activity in oxidizing environments compared to EGCG [408].

8.2. Other Antioxidant Strategies

Besides the use of polyphenols, other antioxidant strategies have been investigated for their potential to reduce α-syn aggregation. Among them, coenzyme Q10 (coQ10) is an important anti-oxidant in both mitochondria and in lipid membranes [409,410]. coQ10 administration protected against MPTP toxicity in mice [411,412] and reduced α-syn aggregation [412,413]. A first positive human trial for coQ10 was reported [414] but, in the end, the investigational drug associated with Vitamin E was unlikely to demonstrate efficacy over placebo for this indication in a phase III (NCT00740714). The mechanisms by which coQ10 protects dopaminergic neurons against degeneration are not well understood. However, coQ10 as an antioxidant attenuated changes in H_2O_2 measured in PD cybrid cells by increasing complex I activity and inhibiting α-syn oligomerization [415]. Another molecule, ginseng, also known as red ginseng (*Panax ginseng, Araliaceae*), is a well-known medicinal plant and popular source of saponins. Several studies identified the biological active components of ginseng, the ginsenosides, that were shown to play many protective roles [416]. The most frequently used and studied ginsenosides, Rg1, Rg3, and Rb1, were investigated for their effect on α-syn aggregation in vitro [417,418]. Ginseng extract reduced dopaminergic cell loss, microgliosis, α-syn aggregate buildup, and improved locomotor activity in a PD rat model [419]. In another in vivo study, Rg1 attenuated neurodegeneration in the MPTP mouse model of PD and reduced oligomeric and phosphorylated α-syn in the SN [418]. An alternate anti-oxidant molecule, Apelin, was proved to be a neuroprotective peptide, which was first extracted and purified from bovine stomach [420]. In vitro, Apelin-36 was neuroprotective in MPP^+-treated SH-SY5Y cells [421]. Furthermore, in MPTP-induced PD mice, they demonstrated that the neuroprotection induced by Apelin-36 could be explained by its effect on the reduction of oxidative stress and nitrated α-syn expression. Apelin-36 also promoted autophagy and inhibited ASK1/JNK/caspase-3 apoptotic pathway [422]. Finally, the activation of the transcription factor Nrf2/antioxidant response element (ARE) pathway has shown to protect against neurodegeneration by decreasing both oxidative stress and protein aggregation [423]. Nrf2 reduced α-syn toxicity by a time-dependent, cell-autonomous mechanism. Nrf2 accelerated the clearance of α-syn, shortening its half-life and leading to lower overall levels of α-syn [424]. Scopoletin, an active principle obtained from *Morinda citrifolia*, also presented antioxidant property [425] by quenching free radicals, reversing apoptosis in rotenone-treated SH-SY5Y cells and prevented α-syn aggregation in rotenone-treated rats through activation of DJ-1/Nrf2/ARE pathway [426]. Additionally, a synthetic morpholine-containing chalcone, KMS99220, reduced α-syn aggregation in GFP A53T α-syn-overexpressing cells. In MPTP-treated mice, oral administration of KMS99220 prevented degeneration of the nigral dopaminergic neurons, induced the Nrf2 target genes, and prevented the associated motor deficits [427].

To conclude, antioxidants have a strong power to inhibit α-syn aggregation by direct interaction with the protein for polyphenols, as well as by its other beneficial properties, such as antioxidative, anti-inflammatory and pro-autophagic activities. These specific properties can have indirect effects on the reduction in protein aggregation, activating antioxidative enzymes or pathways and decreasing

oxidative stress. Polyphenols interact directly with the monomeric or oligomeric forms of α-syn mainly through the hydroxyl groups by forming hydrogen bonds with residues of α-syn [385]. By such hydrophobic interactions, polyphenols stabilized the natively unfolded conformation of α-syn, and disaggregated preformed fibrils into monomers or soluble and non-toxic oligomers [384,400]. Even if no clinical studies confirm their beneficial effects, antioxidants, by their anti-aggregative and neuroprotective efficacy in vitro and in vivo, seem to be interesting candidates for PD therapy.

9. Metal Dyshomeostasis

Divalent metals such as iron, copper, and zinc have been shown to interact with α-syn via multiple metal binding sites and affect its stability and aggregation [41,43,428–430]. To target therapeutically the interactions between metals and α-syn, different strategies have been used including chelation, metalloproteases and several metal-interfering molecules.

9.1. Targeting Iron Homeostasis

Iron has been primarily studied for its effects on PD pathology progression, particularly in its interaction with α-syn and induction of dopaminergic cell loss [352,431,432]. α-syn phosphorylation and expression were increased both in SH-SY5Y cells and in rats when exposed to higher amounts of iron, suggesting the key role of iron in regulating α-syn expression and S129 phosphorylation [433].

Chelation-based therapy, a strategy based on the depletion and/or sequestration of metals using chelators, has mostly been studied in the case of iron and has showed some promising findings. Deferiprone is an iron chelator that has the capacity of crossing the BBB, making it of great interest in the study of chelation for PD. Deferiprone decreased neuronal cell loss in human dopaminergic neurons in vitro and in MPTP mice by decreasing oxidative stress without interfering with iron-dependent mechanisms [434]. Additionally, deferiprone was able to rescue behavioral deficits and trended to decrease α-syn accumulation in iron-fed A53T α-syn-overexpressing mice [435]. Phase 1 clinical trials of this drug also showed that 12 months of deferiprone administration decreased disease progression in PD patients compared to the placebo group [434]. Deferiprone was also tested in a phase 2 clinical trial using 22 PD patients where it showed to decrease iron and have little side effects on the patients [436]. Similarly, intranasal administration of iron chelator deferoxamine in α-syn overexpressing rats improved motor defects, decreased both the number and size of α-syn aggregates, and reduced inflammation. However, this molecule did not rescue the loss of dopaminergic neurons in this animal model [437]. 8-Hydroxyquinoline (8-HQ)-derived chelators have also shown great potential for treating neurodegeneration and α-syn aggregation. The beneficial effects of clioquinol, an 8-HQ derivative, were first demonstrated in MPTP-intoxicated mice [438]. Treatment with clioquinol led to a decrease in iron and induced alleviation in oxidative stress, increase of striatal dopamine loss and neuroprotection. Furthermore, clioquinol improved cognition, motor functions and dopaminergic cell loss in transgenic A53T α-syn-overexpressing mice [439]. A concomitant treatment of clioquinol and L-DOPA in A53T α-syn-transgenic mice not only prevented neurodegeneration but also improve motor symptoms [440]. Q1 and Q4 are other 8-HQ derivatives that target specifically mitochondrial and cytosolic iron pools respectively. Oral administration of these iron chelators in MPTP-treated mice were able to protect from mitochondrial and cytosolic iron increase, against oxidative stress, and dopaminergic cell loss [441]. Other 8-HQ derivatives such as VK-28, M30, and HLA20 were also tested in different models to determine their therapeutic potential and were capable of reducing oxidative stress, iron accumulation, and α-syn accumulation [442,443]. D-607 is another iron-chelating molecule that is capable of activating D2/D3 dopamine receptors [444]. In vitro, D-607 was able to increase cell viability in PC-12 exposed to 6-OHDA. In fly expressing WT or mutant α-syn, D-607 protected from α-syn toxicity by reducing multimeric species with a slight increase in monomeric α-syn. Neuroprotective effects of D-607 were also assessed in MPTP-injected mice and treatment with this iron-chelator inhibited dopaminergic cell loss. These in vitro and in vivo data demonstrated the neuroprotective effects of D-607 by reducing α-syn toxicity and dopaminergic

loss [445]. Recently, PBT434, another chelator binding iron, has been tested in neuroblastoma cells and was able to inhibit iron-mediated oxidative stress as well as aggregation of α-syn. In the same study, they tested the effects of PBT434 in three in vivo PD models: 6-OHDA, MPTP and human A53T α-syn transgenic mice. In these mouse models of PD, PBT434 was able to rescue dopaminergic cell loss in the SN, which translated by an improvement in motor behavior. Most importantly, PBT434 decreased the levels of α-syn in either MPTP and human A53T α-syn mice [446]. This molecule shows promise as it is a low-binding chelator and could thus have less side-effects compared to other chelators.

Another strategy that has been tested for iron-targeting is the use of Rosmarinic acid (RA), an ester of caffeic acid. RA has been shown to have multiple biological roles including anti-inflammatory, antioxidative and antiviral activities. In 2010, this molecule demonstrated its protective effects in vitro as it was able to antagonize the neurotoxic effects of MPP^+ exposure in dopaminergic cells [447]. Recently, these neuroprotective properties were also observed in a MPP^+/MPTP context tied to iron-induced α-syn aggregation. In cells, RA protected cells against iron-induced toxicity and decreased α-syn aggregation. In mice, treatment with RA protected against the decrease in tyrosine hydroxylase and superoxide dismutase expression, inhibited the increase in mesencephalic iron content, and inhibited the increase in α-syn mRNA induced by iron [448].

Finally, another approach has been to target α-syn expression at the RNA level. The 5′-untranslated region (5′-UTR) of *SNCA* is well structured and contains an iron-response element (IRE) region that is capable of regulating its translation. At low iron concentrations, the IRE is bound by the iron response protein (IRP) whereas at high iron concentrations, IRP binds to iron and frees the IRE which induces translation of *SNCA*. A study attempted to create a small molecule to target the *SNCA* IRE to regulate its translation in PD models. They designed a compound, synucleozid, that was capable of targeting α-syn mRNA 5′UTR in a neuronal cell line, inducing a decrease in protein expression and a protective effect in cells [449]. This strategy of targeting the SNCA IRE by this small molecule could prove to be efficient in reducing α-syn and thus its toxicity and aggregation, but in vivo studies remain to be seen.

9.2. Targeting Zinc and Copper Homeostasis

Besides iron, zinc and copper are essential healthy cellular elements and have been shown to also interact with α-syn. Copper has been shown to bind α-syn with the most affinity, despite mutations in certain residues, and can accelerate α-syn aggregation [42,450,451]. In addition, zinc dyshomeostasis due to the loss of its transporter ATP13A2 was showed to induce an accumulation of α-syn and reduce its exosomal transport [452,453]. In another study, overexposure to zinc in WT rats prompted a PD-like pathology. This zinc-induced Parkinsonism showed a loss of dopaminergic cells, motor impairment, aggregation of α-syn and impairment of UPS-mediated degradation. Altogether, zinc exposure induced consequences that were similar to those seen in sporadic PD and were reversible by treatment with L-DOPA [454].

As previously stated, the polyphenol EGCG has been shown to bind to α-syn and inhibit its fibrillation in PC12 cells [455]. Following these results, another study aimed at determining if EGCG had an effect on copper-mediated α-syn aggregation. α-syn overexpressing PC12 cells treated with both copper and EGCG exhibited a decrease in cell loss, in ROS production, and in α-syn accumulation [456]. EGCG was determined to be able to bind copper, thus inhibiting the copper binding on α-syn. This was also studied in the case of iron in PC12 cells, where EGCG had similar chelating effects on iron, thus inhibiting metal binding on α-syn [457]. Combined, these studies show the potential beneficial effects of EGCG on metal-induced α-syn accumulation and aggregation.

Another potential therapy that could be used to target metals in PD is by regulating the expression or function of metal-binding proteins. Metallothioneins (MT) are small copper/zinc binding proteins that are instrumental in homeostasis of these two metals. Dexamethasone, a glucocorticoid which acts on the MT gene promoter and can thus activate MT mRNA expression, rescued mice from dopaminergic cell loss and inflammation after MPTP exposure [458]. Dexamethasone was also used in SH-SY5Y cells to increase MT expression and found that this suppressed copper-induced α-syn aggregate formation in vitro [459].

To conclude, modifying metal distribution in the brain via chelators or other molecules has proven to inhibit neurodegeneration, oxidative stress, and α-syn accumulation. Targeting metals in PD remains a relatively novel approach, with only one iron-based compound that has crossed into clinical trials. Nonetheless, promising studies are underway, with the goal of reducing α-syn aggregation without affecting the other proteins to which they bind as well. Another caveat of targeting metals is their importance within cellular mechanisms in the brain but also throughout our whole organism. Thus, it is important to keep in mind that metal therapies should beware of the high risks of side-effects.

10. Challenges and Open Questions

The present review describes a myriad of strategies. We have been careful in, so far, not rating them according to a degree of confidence, giving the feeling that they hold a similar translational potential, which they obviously do not. Amazingly, some have reached clinical development with, what we consider, a very limited package of evidence. Although this is in agreement with the concept of clinical equipoise that relieves the need to achieve the impossible ideal of preclinical certainty that a therapeutic strategy will work in patients, the selection of interesting candidates should be based on the soundest clinically driven preclinical validation [460].

How can one establish a confidence rating system and what variables shall be integrated into such a system? We will not solve here an issue that is the cornerstone of the industrial therapeutic development. However, a number of steps should be, in our opinion, fulfilled. The first criterion shall be the demonstration of the presence of the affected mechanisms in human samples, whether they are post-mortem tissues and/or biological fluids. The chosen cellular and, later, animal models shall exhibit comparable changes reminiscent of what happens in the human pathology. It is striking to observe that these basic considerations are not even fulfilled in most studies. In addition, the vast majority of the preclinical studies involve only one PD animal model, raising immediately the concern of the actual recapitulation of the pathology/pathogenesis by a single animal model. Nowadays, our field has the unique opportunity to use different animal species and different triggers for inducing different aspects of α-syn pathology, starting, from a historical point of view, from neurotoxin, transgenic animals, viral-based models, and finally the use of different inocula containing α-syn aggregates (recombinant or human brain-derived). The adoption of several and intrinsically different animal models should become standard for the community to cross-validate a positive result, in a single laboratory as well as between independent laboratories.

Similarly, the issue of the experimental design that leads to the demonstration of efficacy of given therapeutic strategies in these models is astonishingly not taken into account in most (if not all) translational studies. While PD is a progressive neurodegenerative disorder, a large majority of α-syn-related therapeutic candidates have been tested using a prophylactic exposure or a concomitant administration. While PD patients are likely to receive a neuroprotective agent following diagnosis—that is, when the extent of dopamine neuron degeneration is already approximately 50% [461,462]—therapeutic candidates are tested in association with, or even weeks before, α-syn-related triggers of pathology. What is the relevance of such an administration protocol with regard to the natural progression of PD? It is not at all surprising that, despite the strength of the available PD models, they have not identified a neuroprotective agent that has been shown to be efficacious in PD patients, even in non-human primate models.

The definition of the actual therapeutics objectives are also critical. With the example of Alzheimer's disease, one should be very cautious in using the terminology "neuroprotection", "disease-modifying",

etc. What does one try to achieve with one given strategy? Truly protecting the neurons from degenerating? Slowing down the prion-like spread of the α-syn aggregates? Decreasing the monomeric α-syn load? Dampening the phospho-α-syn pathology? Most papers are unclear about the true objective and importantly about how the communicated result can translate into an exploitable clinical trial endpoint.

Another level of complexity refers to the strategies for brain drug delivery employed, i.e., gene therapy or pharmacological drugs in disease models of pathogenicity. For these, critical points have to be fulfilled, such as no toxic or adverse effects, a suitable and efficient biodistribution, and evidence of target engagement in vivo. The booming field of synucleinopathies, if it does not build upon past failures, is likely to meet the same issues as other neurodegenerative conditions. It is thus time for building large consortiums of academic labs and to establish, in coordination with clinicians, the minimal package of data that would convince us to move an appealing preclinical finding into real life, i.e., in the clinic.

11. Conclusions

Given the central role of α-syn in PD pathology and progression, α-syn met the criteria to be a tantalizing and evident therapeutic target for PD. In this review, we discussed the potential strategies that are currently being investigated to reduce or block α-syn accumulation and propagation. Whether the strategies target α-syn directly (via gene silencing, immunotherapy or small molecules) or indirectly (via its clearance), they all aimed at restoring cellular homeostasis by bringing α-syn back to its physiological levels, non-aggregative and toxic state or by inhibiting the propagation of pathological forms of α-syn. Despite the various strategies described here, individual challenges remain for each approach. Many research efforts have been made in the various technologies/methodologies aiming to target α-syn, giving rise to multiple clinical trials currently underway (Table 1). Within these many strategies, some currently seem more promising than others, or have at least progressed more rapidly in clinical trials. These include predominantly immunization, anti-aggregative molecules and an increase in α-syn clearance, compared to the less developed PTM targeting and anti-oxidant strategies. Ultimately, we could envision that one possible solution could be combining different strategies, both direct and indirect, to target α-syn accumulation at different steps and both intracellularly and extracellularly. Nevertheless, scientists face multiple obstacles with clinical trials including BBB crossing, solubility, biodistribution, administration and toxicity. Finally, another challenge for all strategies is the lack of knowledge of the physiological roles of α-syn, as well as the absence of valid biomarkers for α-syn species accumulation. These biomarkers could have the potential to not only diagnose pre-symptomatic patients, but also to stratify patients for future trials according to their PD type (i.e., familial or idiopathic). Such could be the case for isoforms of apoE, which have been differentially implicated during PD progression [463,464]. Despite these challenges, it seems that it is only a matter of time before α-syn-based therapeutic strategies are successful in slowing PD progression.

Table 1. Drug-based clinical trials targeting α-synuclein accumulation directly or indirectly.

	Molecule	Mechanism	Clinical Trial Phase	Year	Location of Trial	Reference
Immunotherapy	**PRX002**	Monoclonal antibody targeting C-terminal sequence of α-syn (amino acids 118–126)	1 (healthy volunteers)	2016	United States	NCT02095171 [190]
			1 (healthy volunteers and PD patients)	2017	United States	NCT02157714 [191]
			2 (PD patients)	Active	United States	NCT03100149
	MEDI1341	Monoclonal antibody targeting C-terminal sequence of α-syn	1 (healthy volunteers)	Recruiting	United States, United Kingdom	NCT03272165
	BIIB054	Monoclonal antibody targeting N-terminal aggregated forms of α-syn	1 (healthy volunteers and PD patients)	2018	United States	NCT02459886 [198]
			2 (PD patients)	Recruiting	United States, Japan	NCT03318523 NCT03716570
	BAN0805	Antibody targeting protofibrils of α-syn	1 (healthy volunteers)	Recruiting	United States	NCT04127695
	PD01/PD03 Affitopes	Vaccines targeting the C-terminal sequence of α-syn via small peptides	1 and 2 (healthy volunteers and PD patients)	2018	Austria	NCT01568099 NCT02216188
Clearance	**Nilotinib**	Tyrosine kinase Abelson (cAbl) inhibitor	1 (PD, PDD and DLB patients)	2016	United States	NCT02281474 [329]
			2 (PD patients)	2019	United States	NCT02954978 [330]
	Ambroxol	Pharmacological chaperone of β-glucocerebrosidase	2 (PDD patients)	Recruiting	Canada	NCT02914366 [348]
			2 (PD patients)	2020	United Kingdom	NCT02941822 [349]
Small molecules	**ANLE138B**	Small molecule targeting oligomeric forms of α-syn	1 (healthy volunteers)	Recruiting	United Kingdom	NCT04208152
	NPT200-11	Small molecule targeting the C-terminal region of α-syn	1 (healthy volunteers)	2016	United States	NCT02606682
Anti-oxidants	**CoQ10 + Vitamin E**	Antioxidant activity	3 (early PD patients)	2013	United States	NCT00740714
Metals	**Deferiprone**	Chelation of iron	1 (PD patients)	2012	France	NCT00943748 [434]
			2 (PD patients)	2019, Recruiting	France, Canada, Austria	NCT02728843 NCT02655315

Abbreviations: α-syn, α-synuclein; PD, Parkinson's Disease; DLB, Dementia with Lewy Bodies; PDD, Parkinson's Disease Dementia.

Author Contributions: M.T. prepared parts on immunotherapy and metal dyshomeostasis, M.-L.A. on α-syn synthesis and clearance, E.F. on anti-aggregative molecules and anti-oxidants, and F.L. on α-syn as a therapeutic target and post-translational modifications. M.T. combined and finalized the first draft. M.T., M.-L.A., E.F. and F.L. collectively corrected the draft of the Review, including text and figures. B.D. established the structure of the review. B.D. and E.B. did the final editing. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by Fondation de France Grant number 00066525, a France Parkinson Grant and an IDEX Emergence Grant number OPE-2018-410 (B.D.). M.T. is recipient of an MSER fellowship (France). M.-L.A. is recipient of a France Parkinson Foundation fellowship, E.F. is recipient of an ANR grant (ANR-17-CE18-0026-01), F.L. received support from the EU/EFPIA/Innovative Medicines Initiative 2 Joint Undertaking (IMPRiND grant No 116060). This work has received support from the EU/EFPIA/Innovative Medicines Initiative 2 Joint Undertaking (IMPRiND grant No 116060). The LABEX Brain, the University of Bordeaux, and the Centre National de la Recherche Scientifique provided infrastructural support.

Acknowledgments: We apologize to the authors of several high-quality scientific articles that contributes significantly to the development of the field, which could not be cited due to space limitations.

Conflicts of Interest: The authors declare no conflict of interest.

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Received: 30 April 2020 | Revised: 9 October 2020 | Accepted: 28 November 2020

DOI: 10.1111/nan.12678

Neuropathology and Applied Neurobiology WILEY

ORIGINAL ARTICLE

L-DOPA regulates α-synuclein accumulation in experimental parkinsonism

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Abstract

Aims: Widespread accumulation of misfolded α-synuclein aggregates is a key feature of Parkinson's disease (PD). Although the pattern and extent of α-synuclein accumulation through PD brains is known, the impact of chronic dopamine-replacement therapy (the gold-standard pharmacological treatment of PD) on the fate of α-synuclein is still unknown. Here, we investigated the distribution and accumulation of α-synuclein in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) non-human primate model of PD and determined the effect of chronic L-DOPA treatment on MPTP-induced α-synuclein pathology.

Methods: We measured the density of α-synuclein and tau immuno-positive neurons in the substantia nigra, putamen, hippocampal CA1 region, temporal cortex and dentate nucleus of control, MPTP and MPTP+L-DOPA-treated monkeys. Moreover, we also extracted and quantified Triton-X (TX) soluble and insoluble α-synuclein in putamen and hippocampus samples from a separate cohort of control, MPTP and MPTP+L-DOPA-treated monkeys.

Results: MPTP-induced α-synuclein accumulation in NHP model of PD was not limited to the substantia nigra but also occurred in the putamen, hippocampal CA1 region and temporal cortex. Tau was increased only in the temporal cortex. Moreover, increased intraneuronal TX insoluble α-synuclein was truncated, but not in the structural form of Lewy bodies. The MPTP-induced increase in α-synuclein levels was abolished in animals having received L-DOPA in all the brain regions, except in the substantia nigra.

Conclusions: Dopamine replacement therapy can dramatically ameliorate α-synuclein pathology in the MPTP NHP model of PD. Therefore, patient's dopaminergic medication should be systematically considered when assessing α-synuclein as a biomarker for diagnosis, monitoring disease progression and response to disease-modifying treatments.

KEYWORDS
levodopa, macaque, MPTP, Parkinson's disease, protein aggregation, tau, α-synuclein

INTRODUCTION

Widespread accumulation of aggregation-prone proteins is a key feature of neurodegenerative disorders. In Parkinson's disease (PD) and related-disorders designated as synucleinopathies, misfolded α-synuclein accumulates either in Lewy bodies (LBs) in neurons in PD and dementia with Lewy bodies or in oligodendroglial cytoplasmic inclusions in multiple system atrophy. In addition to α-synuclein,

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these cytoplasmic inclusions contain numerous components, including other aggregation-prone proteins such as tau [1,2]. The spatial and temporal evolution of α-synuclein accumulation over the course of PD has been described, suggesting an ascending progression from the brainstem, later invading subcortical and cortical territories in the most advanced stages of the disease [3]. Although this scheme has been confirmed in subsequent studies, deviation from this proposed pattern has also been reported (for review, see [4]). Whether this relates to the intrinsic heterogeneity of the disease process, clinical subtypes associated with unconventional patterns of α-synuclein accumulation, concurrent ageing-related formation of LBs or external factors remains a matter of debate.

Given its pivotal role in the pathogenesis of PD, α-synuclein is considered as a promising biomarker [5]. As such, it is critical to identify if symptomatic treatments used in PD may affect the pattern and extent of α-synuclein accumulation. This remains an open question due to inherent limitations. Indeed, pathological studies on PD brains are unlikely to be able to reveal any effect of a given symptomatic treatment on protein aggregation due to clinical and pathological heterogeneity, combined with a large variety of drugs used over the course of the disease and incomplete or limited clinical records of drug intake.

The administration of the parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is the gold-standard for modelling PD in non-human primates (NHPs), recapitulating many of the motor and non-motor clinical features of the disease [6,7]. With regards to protein misfolding and accumulation, exposure to MPTP leads to a secondary accumulation of α-synuclein in the substantia nigra [8–10], that can still be detected over 10 years after administration of the toxin, suggesting that this neurotoxin leads to long-lasting alterations of α-synuclein homeostasis [11]. These seminal studies have however focused on the substantia nigra pars compacta (SNpc) and did not investigate other regions of the central nervous system relevant to the pathophysiology of PD. Furthermore, these studies did not consider other aggregation-prone proteins such as tau and did not investigate the potential effect of dopamine replacement therapy (DRT).

The aim of this study was to investigate in the MPTP NHP model of PD the possible occurrence of a widespread synucleinopathy as occurring in the human disease and to determine if the gold-standard pharmacological treatment (i.e. L-DOPA) may influence some neuropathological features such as neuronal loss in the substantia nigra, astrogliosis and the fate of aggregation-prone proteins such as α-synuclein and Tau.

MATERIALS AND METHODS

Animals

Brain tissue samples were obtained from the non-human primate brain bank at the Institut des Maladies Neurodegeneratives (Bordeaux, France). Experiments were carried out in an AAALAC-accredited facility and in accordance with the European Union Directive (2010/63/EU) on the protection of animals used for scientific purposes. Experiments were conducted on 28 female rhesus monkeys (*Macaca mulatta*, Xierxin, Beijing, China, mean age = 5.4 ± 1 years, mean weight = 3.8 ± 0.4 kg). Animal cares were supervised by veterinarians skilled in NHP healthcare and maintenance. Animals were housed in individual primate cages under controlled conditions of humidity (50 ± 5%), temperature (24 ± 1°C) and light (12 h light/12 h dark cycles, lights on 8:00 AM), food and water were available ad libitum. Even though animals were housed individually, the disposition of cages allowed each animal to have visual contacts and interact with monkeys housed in the adjacent cages. Stimulations for play were provided including rubber toys and mirrors that the monkeys used to view themselves and to get a greater look around the room. A radio was played daily from 8.00 AM–10.00 AM to 3.00 PM–5.00 PM to provide stimulation.

Experimental protocol

Ten animals were kept as untreated controls (control group). The remaining 18 animals were treated daily with MPTP hydrochloride (0.2 mg/kg, i.v., Sigma) dissolved in saline according to a previously described protocol [12,13]. Following stabilisation of the MPTP-induced syndrome, animals received either vehicle (MPTP, $n = 8$), or were chronically treated twice daily with 20 mg/kg levodopa for 110 days (MPTP +L-DOPA, $n = 10$). All animals were killed by sodium pentobarbital overdose (150 mg/kg, i.v.) 1 h after the last dose of vehicle or L-DOPA, the brains were removed quickly after death and bisected along the midline. For the histopathological study ($n = 5$ untreated controls, 3 MPTP, 5 MPTP +L-DOPA), brain hemispheres were post-fixed in formalin before paraffin embedding. For the western blot study ($n = 5$ untreated controls, 5 MPTP and 5 MPTP +L-DOPA), the two hemispheres were immediately frozen by immersion in isopentane (−45°C) and then stored at −80°C. Coronal 300-μm-thick sections were cryostat-cut and punches of brain tissue were taken for several brain regions including the striatum and the hippocampus [14–18].

Brain processing

Immunohistochemical studies were performed with procedures and antibodies used in human pathology, as described below. On 4-μm-thick coronal sections, a horseradish peroxidase method was used with the following antibodies: anti-tyrosine hydroxylase (TH), (monoclonal mouse antibody, 1:10,000, Chemicon international), anti-human-specific α-synuclein clone LB509 (monoclonal mouse antibody, 1:100; Invitrogen by life technologies), anti-tau (polyclonal rabbit anti-human 1:1000; Dako), phosphorylation-dependant tau antibody (Tau AT8, PHF-tau monoclonal, mouse anti-human, clone AT8, 1:100; Pierce Biotechnology), anti-Glial Fibrillary Acidic Protein (GFAP, polyclonal rabbit, 1:4000; Dako).

Sections were deparaffinised in toluene and rehydrated in graded series of ethanol. Antigen retrieval was not required for anti-Tau or

anti-Tau AT8 labelling whereas sections labelled with GFAP were pre-treated with proteinase K (Dako, Trappes) and those labelled with LB509 α-synuclein were pressure-cooked in sodium citrate buffer (10 min in 0.01 M, pH 6.0). Non-specific binding was blocked with a universal blocking reagent (Biogenex) for LB509 α-synuclein, GFAP and Tau or with 5% normal goat serum in PBS for anti-Tau AT8 for 30 min at room temperature. For TH labelling, non-specific binding was blocked with 3% normal horse serum in PBS for 60 min. Sections were incubated in primary antibodies overnight. Subsequently, sections were transferred in 3% H2O2 in PBS for 5 min to quench endogenous peroxidase activity and treated with a ready-to-use goat anti-mouse or goat anti-rabbit EnVision-HRP enzyme conjugate (Dako, France) for 30 min. For TH staining, sections were incubated with the biotinylated horse anti-mouse/rabbit IgG (1:200) secondary antibody for 2 hours and then with the avidin-biotin-peroxidase complex (ABC Elite Kit, PK-6200, Vector Labs) for a further 90 min. Immunoreactions were revealed using the highly sensitive diaminobenzidine plus (DAB+) and the 3-amino-9 ethyl carbazol plus (AEC+) (both from Dako) as substrates chromogens. Finally, sections were counterstained with Mayer's hemalum and mounted in an aqueous medium for microscopy (Aquatex; Merck). Negative immunohistological control demonstrated the absence of a signal when omitting the first antibody. Pictures were taken with a Leica microscope (Leica DM6000B) at x20 magnification.

To assess the localisation of α-synuclein, a double immunostaining was performed with anti-tyrosine hydroxylase (TH), (polyclonal rabbit antibody, 1:2000, Institute Jacques Boy, France) and anti α-synuclein, clone LB509, (monoclonal mouse antibody, 1:100; Invitrogen by life technologies) antibodies. The same protocol for deparaffinisation and antigen retrieval in sodium citrate buffer was used as above. Then, 4 μm midbrain sections were blocked with 5% normal goat serum in PBS and sequentially incubated overnight with the primary antibodies. Secondary antibodies were Alexa Fluor 488-labelled goat anti-mouse for α-synuclein, and Alexa Fluor 568-labelled goat anti-rabbit for TH (both from Invitrogen). To lower the intensity of autofluorescence, slides were incubated for 10 min with 0.1% Sudan Black B (Sigma-Aldrich) in 70% ethanol. After thorough washing in PBS, slides were mounted in fluorescence mounting medium (Agilent). Immunofluorescence was visualised using a Zeiss Axioplan 2 epifluorescent microscope at ×40 magnification.

Quantitative analysis

To minimise the inherent variability in the immunochemical procedures, all sections from all animals were processed simultaneously for a given antibody and brain region. The number of immunopositive cells was analysed using a computerised image analysis system (Mercator V6.50, Explora Nova) linked to a Leica microscope type DM 6000B. Quantitative analysis was carried out on the whole-structure area and results were expressed as an average of immunopositive cells per mm^2. α-Synuclein immunofluorescence intensity was assessed on 15 α-syn-positive dopaminergic neurons in each animal. For cell fluorescence intensity, ImageJ software v1.52a was used to measure pixel intensity and area with respect to background intensity and cell surface, respectively, according to the following formula: Intensity of stained cell—(sample of background/area of sample background) X area of stained cell.

Sequential α-synuclein extraction and Western blot analysis

Tissue was homogenised in Triton-X (TX) extraction buffer (50 mM Tris-base pH 7.6, 150 mM NaCl, 1% Triton-X-100, 2 mM EDTA) containing protease and phosphatase inhibitors [19]. The lysate was sonicated and then centrifuged (120,000*g* for 60 min at 4°C) and the supernatant was collected (TX soluble fraction). The pellet was then washed three times with 1 M PBS/1% TX, centrifuged (13,000*g* for 15 min) and re-suspended in SDS extraction buffer (50 mM Tris pH 7.6, 150 mM NaCl, 1% Triton-X-100, 0.5% Na-deoxycholate, 1% SDS), sonicated and centrifuged (120,000*g* for 60 min at 4°C) and the supernatant was collected (TX insoluble fraction). The samples (30 μg) were run on 15% SDS-PAGE gels. Primary antibodies included antibodies against α-syn [monoclonal syn-1, (1:1000; BD Biosciences)] and β-actin (1:2000; Millipore Sigma A5441) as a loading control. The intensity of the immunoreactive bands was estimated by densitometric quantification using ImageJ (relative density, RD) and then normalised to the corresponding β-actin levels.

Statistical analysis

Data are presented as mean ± SEM. Assumptions about normality of distribution and homogeneity of variances were analysed using the Shapiro–Wilk and Brown–Forsythe tests respectively. Data were analysed using one-way ANOVA followed by post hoc Tukey tests for multiple comparisons or using Kruskal–Wallis followed by Dunn's multiple comparisons tests whenever appropriate. These analyses were completed using Graphpad Prism V 8.0. A probability level of 5% ($p < 0.05$) was considered significant.

Data accessibility

The data are available from the corresponding author upon request.

RESULTS

L-DOPA treatment does not affect MPTP-induced neurodegeneration and astrogliosis

We first aimed to determine if L-DOPA could have an impact on neuronal loss in the substantia nigra. As expected, MPTP intoxication induced a significant loss of TH-positive neurons in

the substantia nigra ($F_{(2,10)}$ = 46.40, p < 0.0001, Figure 1). The extent of TH-immunoreactive cell loss was similar between MPTP (79.9%, p < 0.0001) and MPTP +L-DOPA-treated monkeys (76.8%, p < 0.0001). Counting of Nissl-stained neurons confirmed neurodegeneration ($F_{(2,10)}$ = 12.46, p < 0.01), both in MPTP (−66%, p < 0.01) and in MPTP +L-DOPA-treated animals (−80.4%, p < 0.01). All MPTP-treated monkeys exhibited a mild parkinsonian syndrome that was alleviated in animals receiving L-DOPA (data not shown), as previously reported [13,20–22].

Because astrogliosis is a prominent feature of PD that is recapitulated in MPTP models [23], we next investigated if L-DOPA could affect the number of reactive astrocytes. MPTP intoxication induced significant astrogliosis in the nigrostriatal pathway compared with controls, as shown by increased densities of GFAP-positive astrocytes in the substantia nigra ($F_{(2,10)}$ = 10.42, p < 0.01, Figure 2A) and putamen (H = 9.11, df = 2, p < 0.01, Figure 2B). Astrogliosis was also significant in MPTP +L-DOPA-treated monkeys compared with controls in the substantia nigra (p < 0.01) but not in the putamen. In the temporal cortex, MPTP +L-DOPA-treated animals displayed increased counts of GFAP-positive astrocytes compared with controls ($F_{(2,10)}$ = 6.54, p < 0.05, Figure 2D). No significant effects of MPTP or MPTP +L-DOPA were found in CA1 (H = 3.53, df = 2, p = 0.17, Figure 2C) or in the dentate nucleus (H = 2.34, df = 2, p = 0.32, Figure 1E). The density of GFAP-positive astrocytes was not significantly different between MPTP and MPTP +L-DOPA-treated monkeys in any of the regions investigated, indicating that chronic L-DOPA treatment had no effect on MPTP-induced astrogliosis. Microscopic illustrations of the density of GFAP-positive astrocytes in the different regions of interest are depicted for control (Figure 2F–J), MPTP (Figure 2K–O) and MPTP +L-DOPA-treated (Figure 2P–T) monkeys.

MPTP and L-DOPA differentially affect α-synuclein and tau accumulation

MPTP intoxication and subsequent chronic L-DOPA treatment differentially affected α-synuclein immunoreactivity (Figure 3). In the substantia nigra, there was a significant difference on the density of α-synuclein immuno-positive neurons between groups (H = 6.76, df = 2, p < 0.05, Figure 3A). Post hoc analysis indicated increased α-synuclein staining in MPTP-treated monkeys compared to controls (p < 0.05). A significant effect was found for the density of α-synuclein positive fibres in the putamen (H = 7.47, p < 0.05, Figure 3B), where α-synuclein immunostaining was significantly elevated in MPTP-treated monkeys compared with the MPTP +L-DOPA group (p < 0.05). A similar effect was found in CA1 (H = 11.76, p < 0.01, Figure 3C) and post hoc analysis indicated that MPTP-treated monkeys displayed increased α-synuclein immunoreactivity compared with control and MPTP +L-DOPA groups (p < 0.01). In the temporal cortex the density of α-synuclein-immunopositive fibres was also significantly different between groups (H = 8.366, df = 2, p < 0.01, Figure 3D), with the MPTP group displaying higher density of α-synuclein-positive fibres compared with the control and MPTP +L-DOPA groups (p < 0.05). Conversely, no significant effect was found in the dentate nucleus (H = 3.33, p = 0.23, Figure 3E). Remarkably, the marked α-synuclein immunostaining following MPTP was abolished in animals having received L-DOPA in all regions except in the substantia nigra (Figure 3Q–T). Double immunofluorescence for α-synuclein and tyrosine hydroxylase revealed no cytoplasmic accumulation of α-synuclein in controls (Figure 4A–C) and confirmed that α-synuclein accumulation in the substantia nigra of MPTP and MPTP +L-DOPA-treated animals occurred in the remaining dopaminergic neurons (Figure 4D–I). Quantification of α-synuclein fluorescence intensity in remaining nigral dopaminergic neurons revealed that albeit L-DOPA was not able to clear α-synuclein, immunofluorescence intensity was significantly reduced in MPTP +L-DOPA compared with the MPTP group (p < 0.05, Figure 4J).

Because Tau pathology is frequent in PD [24], we investigated the effects of MPTP and subsequent L-DOPA treatment on Tau accumulation. Contrary to the effect on the accumulation and extent of α-synuclein, MPTP intoxication and subsequent chronic L-DOPA treatment led to only modest effects on tau immunoreactivity (Figure 5). In the nigrostriatal pathway, MPTP and MPTP +L-DOPA induced a non-significant increase in the number of tau immunoreactive neurons in the substantia nigra (H = 3.14, p = 0.21, Figure 5A) and in the putamen ($F_{(2,10)}$ = 1.95, p = 0.19, Figure 5B). No significant effects of MPTP and MPTP +L-DOPA were found in CA1 ($F_{(2,10)}$ = 0.52, p = 0.60, Figure 5C) or in the dentate nucleus (H = 1.80, df = 2, p = 0.43, Figure 5E). Conversely, there was a significant effect in the temporal cortex ($F_{(2,10)}$ = 4.7, p < 0.05). Post hoc analysis

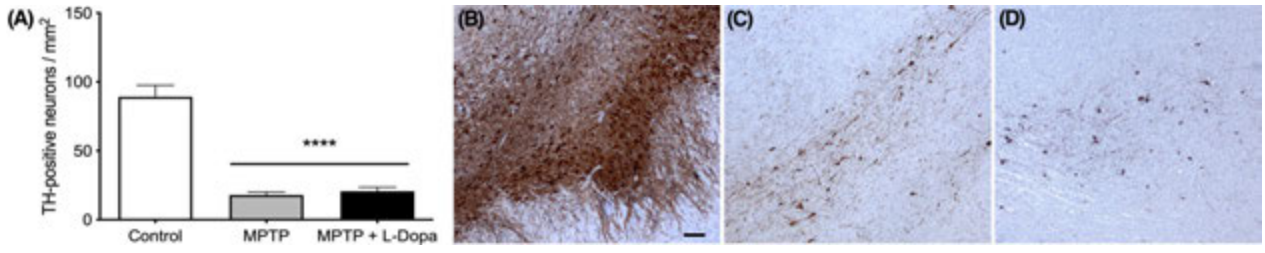

FIGURE 1 Effects of MPTP and L-DOPA on neuronal loss in the SNpc. Counting of TH-immunopositive neurons in the SNpc (A). Representative images of TH immunostaining in the SNpc of control (B), MPTP (C) and MPTP +L-DOPA (D) treated monkeys. Scale bar = 200 μm. ****p < 0.0001

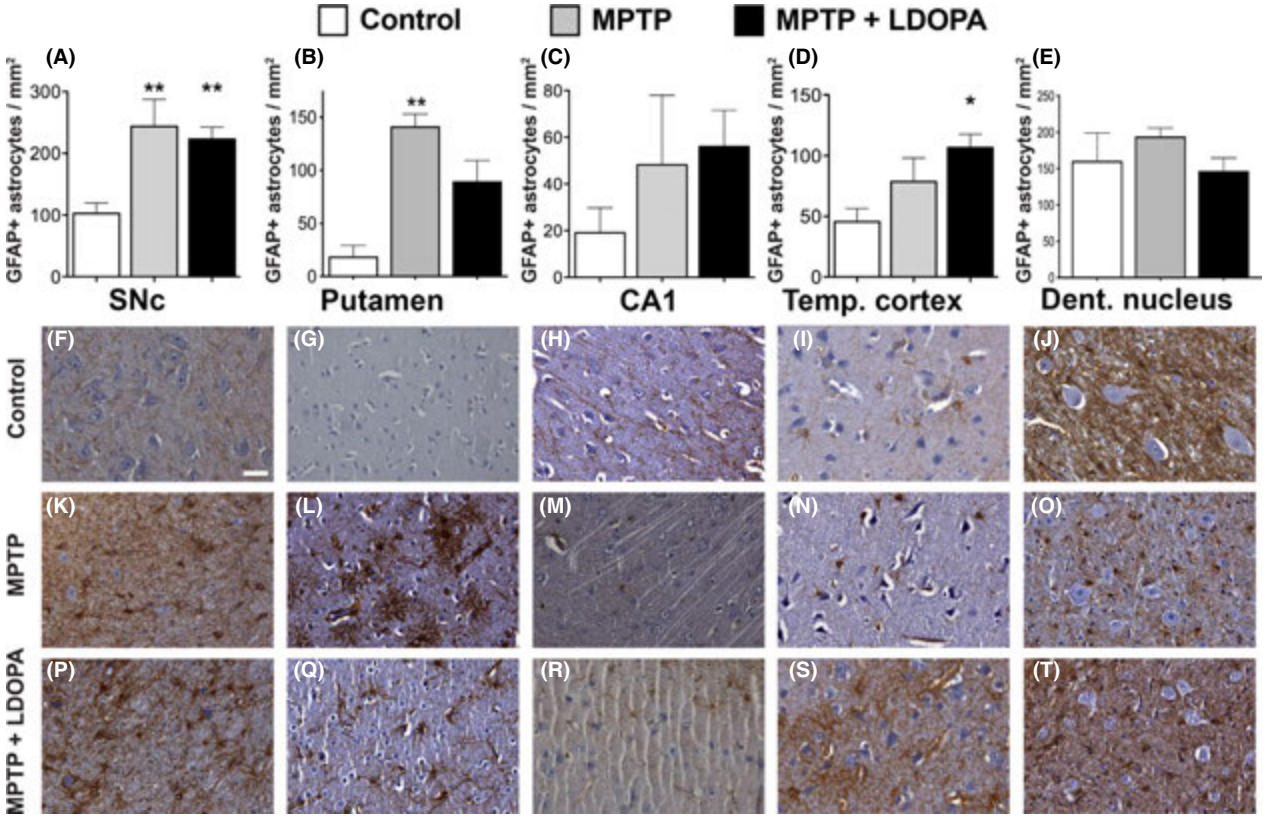

FIGURE 2 Effects of MPTP and L-DOPA on astrogliosis. Astrogliosis, as determined by the density of GFAP immunopositive astrocytes in the SNpc (A), putamen (B), CA1 (C), temporal cortex (D) and dentate nucleus (E). Representative images of GFAP immunostaining in the SNpc (F, K, P), putamen (G, L, Q), CA1 (H, M, R), temporal cortex (I, N, S) and dentate nucleus (J, O, P). Scale bar = 50 μm. *$p < 0.05$; **$p < 0.01$ compared with the control group

indicated a significantly increased number of tau immunoreactive neurons in MPTP-treated monkeys ($p < 0.05$) compared with controls (Figure 5D). Additional investigation of the effect of MPTP and chronic L-DOPA administration on tau was performed using the AT8 antibody that recognises tau phosphorylated at serine 202 and threonine 205 (data not shown). Counts of AT8-immunopositive neurons revealed no significant effects of MPTP and MPTP +L-DOPA in the substantia nigra ($H = 3.10$, $p = 0.22$), CA1 ($F_{(2,10)} = 0.21$, $p = 0.81$), temporal cortex ($F_{(2,10)} = 0.58$, $p = 0.57$) and dentate nucleus ($H = 2.75$, $p = 0.27$), whereas in the putamen, there was a trend towards an increase in tau AT8-positive neurons in MPTP and MPTP +L-DOPA-treated animals ($H = 4.94$, $p = 0.07$).

To further investigate the effect of chronic L-DOPA treatment on α-synuclein homeostasis, putamen and hippocampus samples from a separate cohort of control, MPTP and MPTP +L-DOPA-treated monkeys were subjected to a sequential protein extraction protocol in order to quantify triton-X (TX) soluble and TX insoluble α-synuclein by western blot (Figure 6). α-synuclein immunoblots performed on TX-soluble and TX-insoluble fractions revealed a signal at the expected weight of 14 kDa (full length α-synuclein) and a faint signal at 12 kDa (truncated α-synuclein) that could be quantified upon overexposure. No high molecular weight assemblies of α-synuclein were detected. In the putamen, there was no significant effect of MPTP or MPTP +L-DOPA on full-length α-synuclein ($F_{(2,12)} = 1.83$, $p = 0.20$, Figure 6A and C), whereas a significant effect was found for the 12 kDa band ($F_{(2,12)} = 20.12$, $p < 0.0001$, Figure 6A and D) in the TX soluble fraction. Post hoc analysis revealed a significant decrease in α-synuclein in MPTP +L-DOPA-treated animals compared with controls and with the MPTP group ($p < 0.01$). Interestingly, in the TX-insoluble fraction, there was a significant effect of treatment on full length α-synuclein ($F_{(2,12)} = 10.05$, $p < 0.01$, Figure 6B and E), with MPTP +L-DOPA-treated monkeys showing significantly less α-synuclein than their MPTP-treated counterparts ($p < 0.05$) and controls ($p < 0.01$). A significant effect was also observed on 12 kDa α-synuclein ($F_{(2,12)} = 8.95$, $p < 0.01$, Figure 6B and F), with reduced 12 kDa α-synuclein in the MPTP +L-DOPA compared with the MPTP ($p < 0.01$) and control ($p < 0.05$) groups.

In the hippocampus, there was a significant effect on full length α-synuclein in the TX-soluble fraction ($F_{(2,12)} = 12.17$, $p < 0.01$, Figure 6G and I), with MPTP +L-DOPA-treated monkeys showing significantly more α-synuclein than their MPTP-treated counterparts ($p < 0.01$) and controls ($p < 0.05$). No significant effect was detected for the 12 kDa band ($F_{(2,12)} = 2.47$, $p = 0.12$). In the TX-insoluble fraction, no significant differences were found among the three groups for full length α-synuclein ($F_{(2,12)} = 1.49$, $p = 0.26$). However, there was a significant effect of 12 kDa α-synuclein ($F_{(2,12)} = 10.92$, $p < 0.01$). Post hoc analysis revealed that 12 kDa α-synuclein was significantly increased in MPTP-treated monkeys compared with

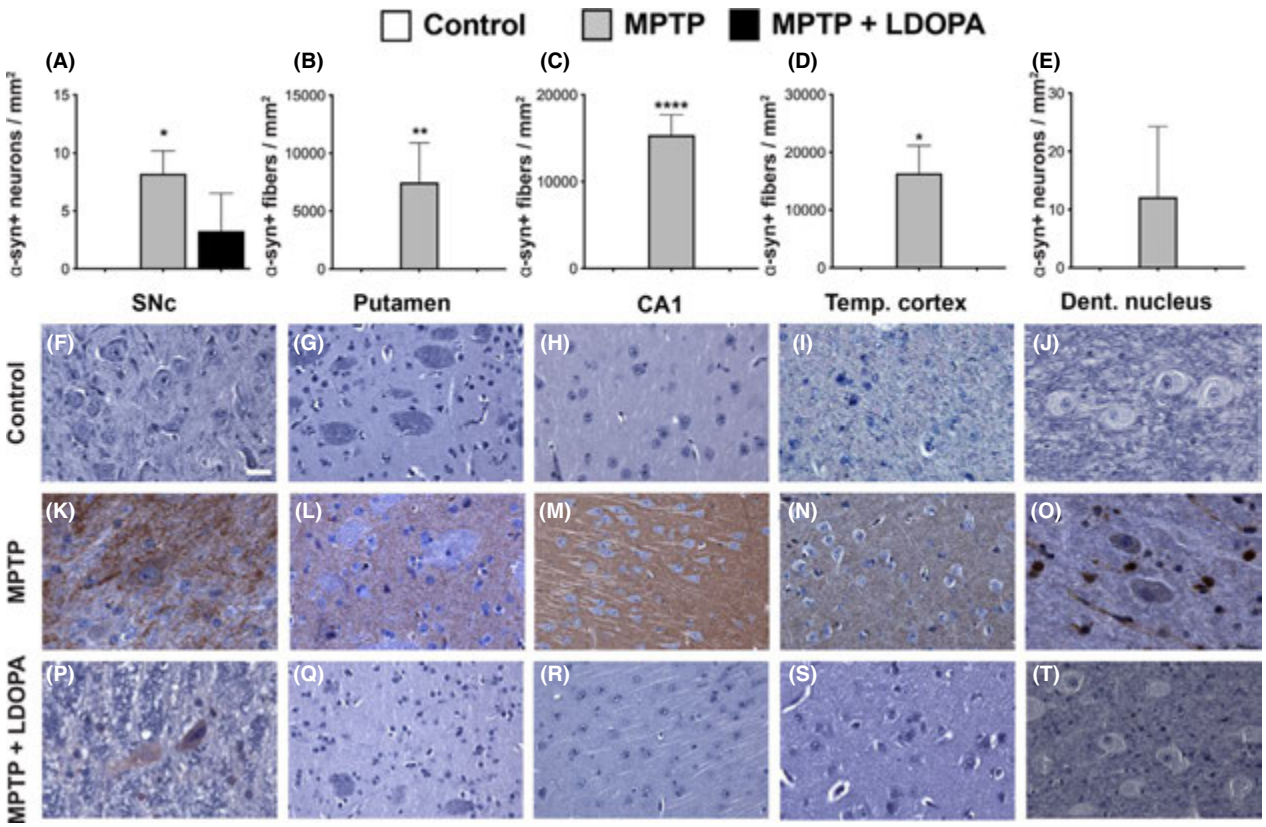

FIGURE 3 Effects of MPTP and L-DOPA on α-synuclein accumulation. α-Synuclein accumulation, as determined by the density of α-synuclein immunopositive neurons in the SNpc (A), fibres in the putamen (B), CA1 (C) and temporal cortex (D) and neurons dentate nucleus (E). Representative images of α-synuclein immunostaining in the SNpc (F, K, P), putamen (G, L, Q), CA1 (H, M, R), temporal cortex (I, N, S) and dentate nucleus (J, O, T). Scale bar = 50 μm. *$p < 0.05$ vs control group; **$p < 0.05$ vs MPTP group, ****$p < 0.01$ vs control and MPTP+L-DOPA groups

controls ($p < 0.05$) and MPTP +L-DOPA-treated animals ($p < 0.01$, Figure 5L).

DISCUSSION

The spatial and temporal evolution of α-synuclein accumulation over the course of PD has been well-described [3]. However very little is known about the impact of long-term exposure to DRT (i.e. the gold-standard pharmacological treatment of PD) on the propagation/distribution/accumulation of α-synuclein. Since it has already been shown that intoxication with MPTP induces α-synuclein aggregation in the SNpc of NHPs [8–11], we therefore investigated in MPTP-treated monkeys the fate of α-synuclein and tau in several regions of the central nervous system relevant to the pathophysiology of PD and examined the consequences of chronic DRT on the pattern and extent of these aggregation-prone proteins. Therefore, characterising the impact of DRT on their fate could aid in developing and validating reliable biomarkers to assess the progression and severity of the disease.

As reported previously, MPTP-treated animals showed large neuronal loss and pronounced astrogliosis in the SNpc (Figures 1 and 2) [23]. Moreover, we showed that MPTP-induced astrogliosis was not limited to the substantia nigra but also occurred in the putamen (Figure 2B) and the temporal cortex (Figure 2D). So far, it has been demonstrated that MPTP—possibly due to its inhibitory action on the complex I of the mitochondrial respiratory chains [25,26]—induced α-synuclein accumulation in the surviving midbrain dopaminergic neurons of NHPs [8–11] and mice [27,28]. In line with these studies, we found a significant increase in the density of α-synuclein immuno-positive neurons in the SNpc of the MPTP-treated monkeys (Figure 3A and 4). Moreover, we demonstrate for the first time in MPTP-treated monkeys that the density of α-synuclein increased in the putamen (i.e. a motor-related/subcortical structure, Figure 3B) as well as in neurons of non-motor and cortical regions, such as the hippocampal CA1 (Figure 3C) region and temporal cortex (Figure 3D). In PD, LBs in which α-synuclein is a major constituent are widely distributed in the central nervous system, including brainstem, subcortical and cortical areas [2,3,29,30]. Moreover, their distributions are most likely correlated with the motor and non-motor symptoms of PD [2,3,30,31]. A major difference between idiopathic PD and the MPTP-primate models of PD is the presence of insoluble, high molecular weight assemblies of α-synuclein in LBs in PD, but not in animal models of PD [8,11]. Here, and in line with previous results, we did not find LBs in the different brain regions of MPTP-treated animals.

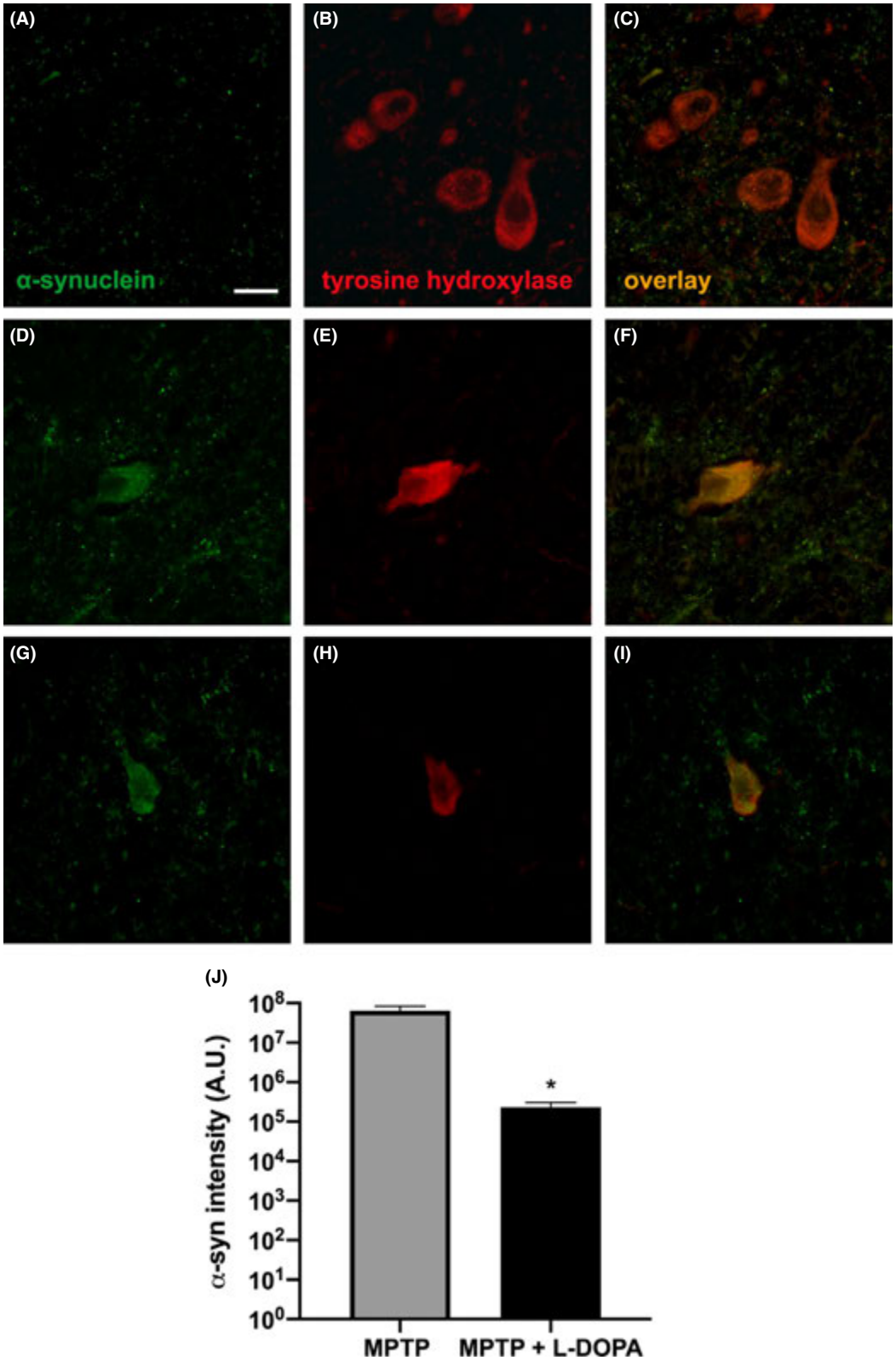

FIGURE 4 Phenotypic identity of α-synuclein accumulating neurons in the SNpc. Double immunofluorescence for α-synuclein (A, D, G) and tyrosine hydroxylase (B, E, H) and overlay (C, F, I), in control (A-C), MPTP (D-F) and MPTP+L-DOPA (G-I) treated monkeys. Scale bar = 30 μm. Quantification of α-synuclein fluorescence intensity in remaining nigral dopaminergic neurons of MPTP and MPTP +L-DOPA treated animals (J). *$p < 0.05$

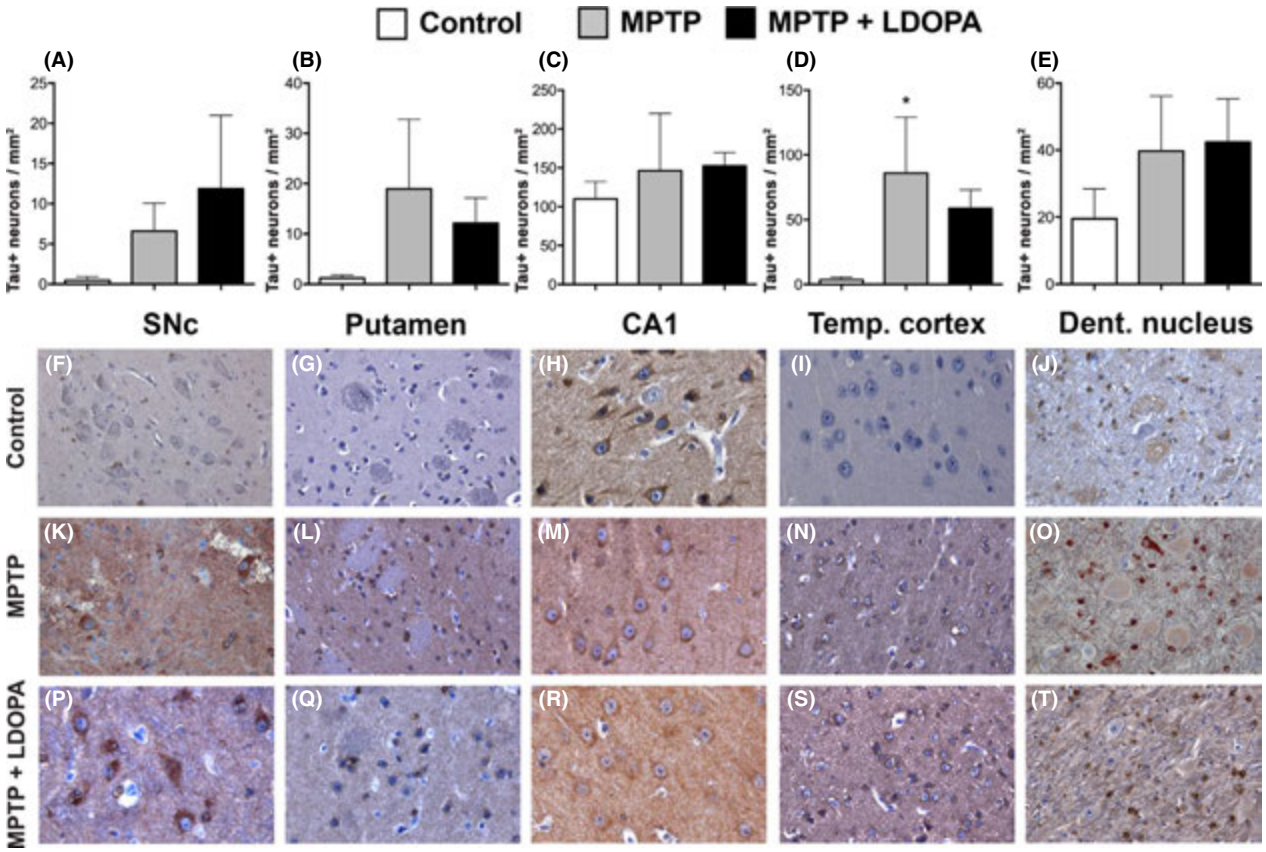

FIGURE 5 Effects of MPTP and L-DOPA on tau accumulation. Tau accumulation, as determined by the density of tau immunopositive neurons in the SNpc (A), putamen (B), CA1 (C), temporal cortex (D) and dentate nucleus (E). Representative images of tau immunostaining in the SNpc (F, K, P), putamen (G, L, Q), CA1 (H, M, R), temporal cortex (I, N, S) and dentate nucleus (J, O, T). Scale bar = 50 μm. $^{*}p < 0.05$ compared with the control group

Nevertheless, we still demonstrated the accumulation of α-synuclein in non-motor cortical regions, indicating that the widespread synucleinopathy classically observed in PD patients is somewhat recapitulated in the MPTP NHP model of PD.

Under physiological conditions, α-synuclein is a soluble protein widely distributed in the brain and especially expressed in the presynaptic nerve terminals [2,32]. However, during the disease process, α-synuclein changes its conformation to form oligomers and high molecular weight insoluble aggregates. Previous studies have shown that the clearance of α-synuclein in the substantia nigra is mainly related to autophagy-lysosomal pathway (reviewed in [33]), in particular through the chaperone-mediated autophagy (CMA), as α-synuclein is a CMA-substrate. Autophagy is impaired in the substantia nigra in experimental models of parkinsonism (including MPTP models) and in PD, as shown with deficits in several lysosomal proteins such as LC3, LAMP1, LAMP-2a, cathepsin-D, glucocerebrosidase or ATP13A2 [34–39].

Remarkably, post-translational modifications, such as phosphorylation, ubiquitination, nitration and truncation are possible enhancers for α-synuclein aggregation [40–42]. In accordance with the aggregation propensity of truncated α-synuclein, our biochemical results revealed an increase in the TX insoluble fraction of the truncated (not the full-length) α-synuclein in hippocampus samples of MPTP-treated monkeys. However, we did not find high molecular weight assemblies of α-synuclein in our experimental conditions that might reflect aggregated forms of α-synuclein as occurring in LBs. Overall, these biochemical results corroborate the histopathological results that showed that MPTP-induced α-synuclein accumulation in NHPs is not limited to the substantia nigra and that the increased intraneuronal α-synuclein immunoreactivity was insoluble but not in the structural form of LBs. Furthermore, the lack of effect of MPTP on total α-synuclein levels (Figure 6) together with the widespread increased immunoreactivity (Figure 3) suggests that an anatomical redistribution of the protein may possibly occur, leading to its accumulation in cell bodies or processes.

Although α-synuclein is a major constituent of LBs, other aggregation-prone proteins such as tau, play a role in the pathogenesis of LBs [1,2]. Indeed, tau is also the major structural component of neurofibrillary tangles (NFTs). It has already been showed that tau and α-synuclein co-exist in LBs, and that NFTs are present around LBs [1,43–45], thus indicating that tau aggregation and NFTs are also significant hallmarks of PD. However, whether tau accumulation, tau aggregation and NFTs occur in the MPTP animal models of PD remains relatively unexplored. Here, we only observed a slight increase in the density of tau immuno-positive neurons in the temporal cortex of MPTP-treated monkeys (Figure 5D), thus indicating

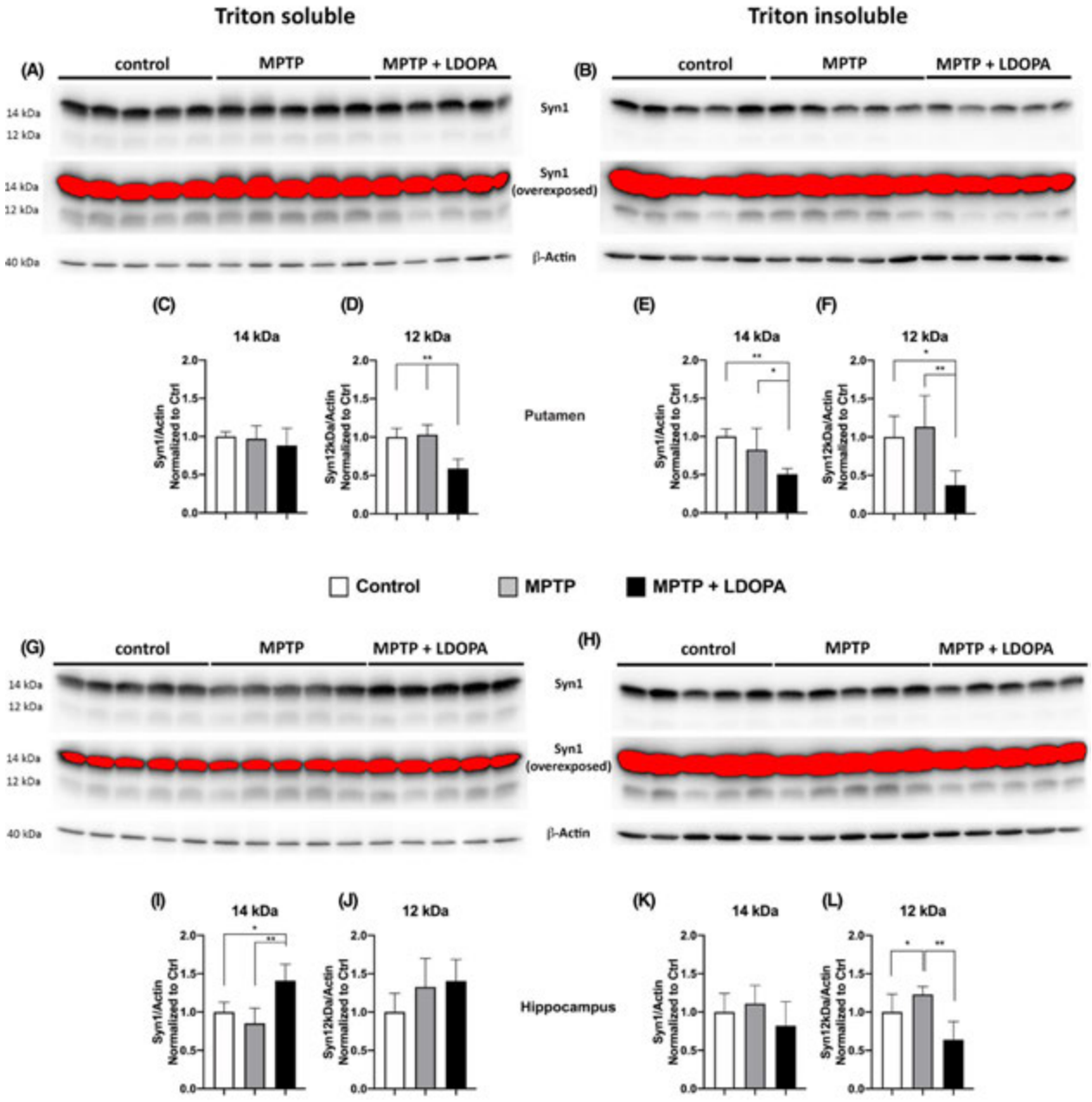

FIGURE 6 Effects of MPTP and L-DOPA on α-synuclein homeostasis and insolubility. α-Synuclein immunoblot levels following sequential protein extraction of putamen (A, B) and hippocampus (G, H), in triton soluble (A, G) and insoluble fractions (B, H) and quantification of full-length (14 kDa) and truncated (12 kDa) α-synuclein in the putamen (C–F) and hippocampus (I–L). n = 5 per group. Data represent mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$

that MPTP intoxication induced larger effects on α-synuclein than tau accumulation. Importantly, earlier in vitro studies demonstrated that the hyperphosphorylation of tau may promote its aggregation [46]. Moreover, tau is usually phosphorylated in LBs and NFTs [43–45]. Phosphorylation of tau at Threonine 231, Serine 202/Threonine 205 and Serine 396 has already been reported in Alzheimer disease [47,48]. Remarkably, in the MPTP mouse model of PD, MPTP-induced tau hyperphosphorylation—which was α-synuclein-dependent - was found at Serine 396/404 [27]. Moreover, an increase in tau phosphorylation at Serine 202/Threonine 205, which might occur through GSK3β pathway, was also recently observed in the MPTP mouse model of PD in which phosphorylated tau and α-synuclein co-localised in SNpc and hippocampus and formed plaques in SNpc [48]. In contrast with this latter study, despite α-synuclein accumulation, we did not find significant increases in the density of phosphorylated tau (detected using the AT8 antibody) immuno-positive neurons in the SNpc, putamen, hippocampal CA1 region, temporal cortex and dentate nucleus of the MPTP-treated monkeys. Therefore, our results suggest that although changes in α-synuclein homeostasis might be necessary for phosphorylated tau accumulation and aggregation in

PD brains [27], α-synuclein accumulation does not necessarily lead to phosphorylated tau accumulation and aggregation.

Earlier in vitro studies have showed that dopamine, L-DOPA and other catecholamines (e.g. adrenaline and noradrenaline) inhibit α-synuclein fibrillation [49–51] and its degradation through chaperone-mediated autophagy [52]. In aerobic conditions, these catecholamines tend to oxidise and their oxidative products appear to be even more effective for inhibiting α-synuclein fibrillation [50]. In line with these results, it has been demonstrated that the capacity of dopamine and L-DOPA to prevent the formation of α-synuclein fibrils was cancelled by the addition of antioxidants in the growth medium [49]. Since quinones and aminochromes are two of the most stable classes of oxidative products derived from dopamine and L-DOPA, they could be at the source of α-synuclein fibril inhibition induced by dopamine and L-DOPA in these studies [50]. Indeed, it has been proposed that inhibition α-synuclein fibrillation is due to a covalent modification of α-synuclein by dopamine-derived orthoquinone [49].

Although the relationship between distinct conformations of α-synuclein and neurodegeneration is still unclear and debated, if α-synuclein fibrillation process is involved in neuronal death [53,54], then disaggregating α-synuclein fibrils may reverse or slow down PD progression. However, despite the unique value of NHP models in translational research, the effect of DRT (L-DOPA+carbidopa) on propagation/distribution/accumulation of pathological insoluble α-synuclein in the brain of the MPTP NHP model of PD was still unexplored. In this study, we found that α-synuclein immunostaining was no longer detected in the putamen, hippocampal CA1 region, temporal cortex and dentate nucleus of the MPTP-treated monkeys with L-DOPA, thus indicating that DRT wiped out MPTP-induced alterations of α-synuclein immunoreactivity in several brain regions of the central nervous system relevant to the pathophysiology of PD (Figure 3B–E). However, α-synuclein immuno-positive SNpc neurons were still detected in the MPTP+L-DOPA group (Figure 3A), even though the intensity of α-synuclein immunofluorescence was reduced in animals treated with L-DOPA (Figure 4). The marked deleterious effect of MPTP on autophagy in the SNpc may have contributed to the reduced clearance of α-synuclein in this region. In contrast, we did not find any effect of L-DOPA on the density of tau immuno-positive neurons in any brain regions investigated (Figure 5). In line with these results, our biochemical results revealed that L-DOPA mainly affected the TX insoluble fraction of α-synuclein (Figure 6) from both putamen and hippocampus samples, so that the TX insoluble fraction of the full-length and the truncated α-synuclein decreased in putamen samples (Figure 6E and F respectively), whereas only the truncated α-synuclein decreased in the hippocampus samples (Figure 6L). Accordingly, it has been shown that catecholamines (probably via their oxidative product as discussed above) not only inhibited α-synuclein fibril formation but also disassembled existing fibrils generated in vitro and α-synuclein deposits in tissue samples from SNpc of mice treated with the herbicide paraquat [50]. Similarly, it has been recently showed that administration of L-DOPA reduced phosphorylated α-synuclein accumulation in the SNpc of mice injected with preformed synthetic α-synuclein fibrils in the striatum [55]. Together with these studies performed in mice, our results obtained in NHPs reveal the ability of L-DOPA to mitigate α-synuclein pathology, either triggered by neurotoxins [50] or by α-synuclein fibrils [55]. Although further experiments are needed to understand why DRT might be less effective on α-synuclein pathology in the SNpc compared to others brain regions, our results strengthen earlier in vitro and in vivo reports. Moreover, our study is the first to demonstrate in vivo that DRT can dramatically ameliorate α-synuclein pathology in the MPTP NHP model of PD.

Our results may also have implications regarding the possible effects of DRT on the natural course of the disease that are tremendously difficult to apprehend in clinical trials. The earlier vs later L-DOPA (ELLDOPA) trial that evaluated the effects of DRT on the progression of the disease suggested a possible neuroprotective effect of L-DOPA [56]. However, in the Levodopa in Early Parkinson's Disease (LEAP) trial, no evidence for a disease-modifying effect of L-DOPA was found [57]. What these trials show, in light of our results, is that L-DOPA does not protect from synucleinopathy as a *primum movens*. In the MPTP monkey model, the observed synucleinopathy is secondary to the neurotoxin insult and this secondary reaction is sensitive to L-DOPA. In idiopathic PD, one can assume that the primary synucleinopathy is a triggering factor and no evidence for sensitivity to L-DOPA has been shown so far.

The histopathological hallmark of PD is the presence of fibrillar aggregates referred to as Lewy bodies (LBs), in which α-synuclein is a major constituent [2,30]. Accordingly, α-synuclein is regarded as a promising biomarker of the disease [5,40]. Indeed, α-synuclein can be measured in peripheral tissue and body fluids (e.g. cerebrospinal fluid, plasma/serum, blood and saliva) [40]. Although we are still unable to directly image aggregates of α-synuclein, their structural and functional consequences can be detected by current imaging strategies (e.g. MRI, SPECT, PET) [5]. In this context and given the translational significance of the NHP studies, our results are fundamental and indicate that patient's dopaminergic medication should be systematically taken in consideration when assessing α-synuclein as a biomarker for accurate diagnosis, monitoring disease progression and response to treatment. In fact, special attention should be paid to the patient's full medical record and not only DRT since other drugs widely used in PD therapy (e.g. MAO-B inhibitors) may also influence α-synuclein homeostasis.

ACKNOWLEDGEMENTS

The Université de Bordeaux, Université de Poitiers, Centre National de la Recherche Scientifique and Institut National de la Santé et de la Recherche Médicale provided infrastructural support. The sequential α-synuclein extraction was performed in the Biochemistry and Biophysics Platform of the Bordeaux Neurocampus at the Bordeaux University funded by the LABEX BRAIN (ANR-10-LABX-43) with the help of Y. Rufin.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

EB, POF and BD designed the research; MHC, MT and QL performed the research; POF, MT and MD analysed the data; MD, POF and EB wrote the paper; All authors read and approved the final version of the manuscript.

ETHICAL APPROVAL

Experiments were carried out in an AAALAC-accredited facility and in accordance with the European Union Directive (2010/63/EU) on the protection of animals used for scientific purposes. Experiments were performed after acceptance of the study design by the Institute of Lab Animal Science (Chinese Academy of Science, Beijing, China) IACUC for experiments on non-human primates.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/nan.12678.

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How to cite this article: Deffains M, Canron MH, Teil M, et al. L-DOPA regulates α-synuclein accumulation in experimental parkinsonism. *Neuropathol Appl Neurobiol*. 2020;00:1–12. https://doi.org/10.1111/nan.12678

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Review

A New Rise of Non-Human Primate Models of Synucleinopathies

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Abstract: Synucleinopathies are neurodegenerative diseases characterized by the presence of α-synuclein-positive intracytoplasmic inclusions in the central nervous system. Multiple experimental models have been extensively used to understand better the mechanisms involved in the pathogenesis of synucleinopathy. Non-human primate (NHP) models are of interest in neurodegenerative diseases as they constitute the highest relevant preclinical model in translational research. They also contribute to bringing new insights into synucleinopathy's pathogenicity and help in the quest and validation of therapeutical strategies. Here, we reviewed the different NHP models that have recapitulated key characteristics of synucleinopathy, and we aimed to highlight the contribution of NHP in mechanistic and translational approaches for synucleinopathies.

Keywords: non-human primates; synucleinopathy; neurodegeneration; α-synuclein; animal model

Citation: Teil, M.; Arotcarena, M.-L.; Dehay, B. A New Rise of Non-Human Primate Models of Synucleinopathies. *Biomedicines* **2021**, *9*, 272. https://doi.org/10.3390/biomedicines9030272

Academic Editor: Marc Ekker

Received: 20 January 2021
Accepted: 4 March 2021
Published: 9 March 2021

1. Introduction

Synucleinopathies are neurodegenerative diseases characterized by α-synuclein (α-syn)-positive intracytoplasmic inclusions, which accumulate in the central nervous system (CNS) but are also found in the peripheral and enteric nervous (ENS) systems. The synucleinopathy family includes Parkinson's disease (PD), Dementia with Lewy Bodies (DLB), and Multiple system atrophy (MSA). Each pathology differs from the other clinically and neuropathologically by the different brain areas most affected and the specific cell type targeted by α-syn accumulation. Parkinson's disease, the most prevalent synucleinopathy [1], is characterized by a parkinsonian motor syndrome associated with (i) a striatal dopaminergic deficit due to the loss of dopaminergic neurons in the substantia nigra pars compacta (SN) and (ii) the presence of α-syn-positive intracytoplasmic inclusions present in the neurons and widespread in the CNS, called Lewy Bodies (LB) [2,3]. The second most prevalent synucleinopathy, DLB [4], differs from PD clinically with the appearance of early cognitive impairments in parallel with motor symptoms and neuropathologically with amyloid plaques in cortical areas in addition to widespread LB accumulation in the brain [5]. MSA is a rarer synucleinopathy in terms of prevalence [6]. MSA is clinically separated into two phenotypes: MSA-parkinsonian with a parkinsonian syndrome associated with nigrostriatal dopaminergic loss, and MSA-cerebellar with cerebellar syndrome with olivopontocerebellar loss. MSA differs from PD and DLB by the presence of α-syn-positive intracytoplasmic inclusions accumulated in oligodendrocytes named Glial Cytoplasmic Inclusions (GCI) [7].

To better understand synucleinopathies' pathophysiology and propose therapeutic strategies to alter the disease progression, appropriate animal models should be used. An ideal model to study synucleinopathy must recapitulate the different characteristics of the disease, including the course of the disease, the neurodegeneration, and the distribution and accumulation of α-syn-positive intracytoplasmic inclusions. Among experimental models, non-human primates (NHP) to study neurological disorders can present different advantages. Primates present two distinct phylogenetic groups: Old World monkeys and

advantages. Primates present two distinct phylogenetic groups: Old World monkeys and New World monkeys. Both groups are studied in research and present various benefits in terms of laboratory use and human similarities. Phylogenetically, Old World monkeys are the closest species to humans, including macaques and baboon monkeys [8]. Humans are also close to the New World monkeys' group, including squirrel monkeys and marmosets, as they shared a common phylogenetic ancestor [8]. NHPs are also closest to humans in terms of anatomical complexity, physiological systems, genetics, and cognitive and social behavior [9–12]. As aging is the major risk factor for synucleinopathies [1], and knowing that these pathologies are progressive, the long lifespan of NHP and the age-dependent accumulation of the neuronal pigment neuromelanin [13,14] are also critical criteria to take into consideration. Even if more costly, NHPs thus provide a higher preclinical model for research in neurosciences. Even if we are aware that studies on other animal models have led to critical advances on synucleinopathies [15,16], we aimed here to present the many NHP models proposed, in chronological order, to study synucleinopathies and to highlight their contribution in understanding the pathogenesis of synucleinopathy (Figure 1).

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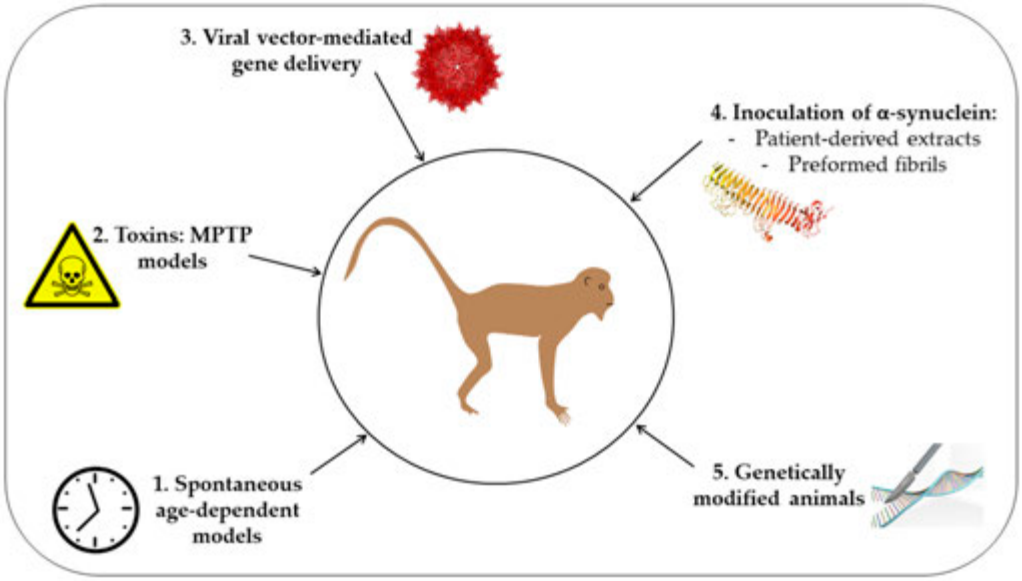

Figure 1. Schematic representation of the different approaches used to generate non-human primate models of synucleinopathy. 1. Spontaneous age-dependent models; 2. Toxins: MPTP models; 3. Viral-mediated gene delivery; 4. Inoculation of α-synuclein using either patient-derived extracts or preformed fibrils; 5. Genetically-modified animal models.

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2. Spontaneous Aged-NHP Models

As synucleinopathies are specific to human primates and as aging remains the most compelling and prominent risk factor for the development of synucleinopathies [1], a spontaneous aged NHP profile was explored to model synucleinopathies. In 1998, the study of Emborg and collaborators on young (3–5 years) and aged (26–28 years) rhesus monkeys (*Macaca mulatta*) reported that aged monkeys display motor decline relative to young individuals, with a decrease in general activity, difficulties in achieving fine motor task coordination and the appearance of clinical signs associated with PD profile (bradykinesia, tremor, balance disturbance, stooped posture) [17]. These motor disturbances were associated with a spontaneous reduction of the number of Tyrosine Hydroxylase (TH)-positive neurons and Dopamine Transporter (DAT) immunoreactive cells in the SN. Nevertheless, the magnitude of nigrostriatal dopamine loss observed in this study was insufficient to cause a more prominent parkinsonian syndrome and was proposed as a useful model for human aging and early PD. Although the nigrostriatal dopamine decline in aged NHP was demonstrated here, no report was done regarding the synucleinopathy in aged individuals. In 2007, Chu and Kordower filled the gap and examined the expression of α-syn in individual neurons across a wide range of normal aging NHP [18]. For this purpose, they used

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rhesus monkeys separated into young (2–12.1 years), middle-aged (15–23 years), and aged (24–34.1 years) groups. Using unbiased stereological counting of nigral α-syn-positive cells, they showed an increase in α-syn-positive neurons by 169% and 215% in the SN of middle-aged and aged monkeys, respectively, compared to young monkeys, demonstrating that accumulation of α-syn in nigral cells was strongly associated with aging. They also found a significant positive correlation between the increase of α-syn immunoreactivity and the decrease in TH immunoreactivity in nigral neurons, suggesting a strong association between the accumulation of α-syn in nigral neurons and the loss of dopamine phenotype. Using proteinase K (PK) treatment, as a readout for the pathological property of α-syn, they reported that age-dependent α-syn accumulation in nigral neurons is soluble and non-aggregated. These data proposed aged rhesus monkeys as a natural and spontaneous model for early PD, given that these animals present a strong increase of soluble α-syn levels associated with a decrease in dopaminergic nigrostriatal phenotype. Using *Microcebus murinus* primates, Canron and collaborators investigated the presence of α-syn in different brain areas of young (1.42–3.07 years) and aged (8.21–10.32 years) animals [19]. They showed that intracytoplasmic α-syn accumulation occurred in the anterior olfactory nucleus, in the cortex, and in the two PD-related regions, SNpc and striatum, of aged but not young monkeys. Interestingly, they observed pathological S129-phosphorylated α-syn in the cerebellum, hippocampus, thalamus, red nucleus, olfactory tubercle, cortex, SNpc, and striatum of old mouse lemur primates. In contrast, no immunostaining was detected in young individuals. Specifically, they noticed that S129-phosphorylated and nitrated α-syn accumulated in the dopaminergic neurons of the SN of aged animals. They thus proposed aged mouse lemurs as a spontaneous model for age-associated α-syn pathology.

In a more recent study, it was also shown that α-syn oligomeric species increase in cytosolic fractions from striatum and hippocampus of middle-aged (10–12 years) and aged (15 years) cynomolgus monkeys (*Macaca fascicularis*) compared to young (3–4 years) individuals [20]. Mitochondrial oligomeric α-syn was demonstrated to increase significantly with age in the striatum, the hippocampus, the cerebellum, and the occipital cortex. Levels of S129-phosphorylated α-syn were also shown to increase according to age, specifically in the striatum and the hippocampus. In 2016, Kimura and collaborators focused on expanding the α-syn profile in normal aging, studying macaque monkeys (Cynomolgus, Japanese, and Rhesus) ranging from 10 to 31 years [21]. They observed a topological pattern of α-syn expansion in the brain according to age, which originated in the midbrain dopaminergic regions such as the SN and the ventral tegmental area, then reached the striatum for the dopaminergic nigrostriatal system and the nucleus accumbens for the dopaminergic mesolimbic system. They also observed an expansion of α-syn occurrence in the mesocortical dopamine system with the appearance of α-syn accumulation in the prefrontal cortex, which increases with age, expanding through motor areas of cortical regions such as the primary motor cortex. They thus demonstrated an age-dependent topological accumulation of α-syn along with the dopaminergic connectivity that can be compatible with the topological expansion of the LB pathology attributed to PD patients. They also noticed the presence of α-syn accumulation in the amygdala and hippocampus, regions related to dementia associated with human DLB pattern. Nevertheless, no LB inclusions or clinical symptoms were observed in aged monkeys. Thereby, they indicated that aged macaques could be useful as a model for studying presymptomatic stages for PD or DLB pathology.

Although dopaminergic neurodegeneration occurs spontaneously according to age in NHP and is associated with accumulation and expansion of α-syn pathology, no LB formation has been observed with aging. Moreover, few of the aged animals used in these studies provide clear clinical phenotypes that resemble human PD, DLB, or MSA pathologies. However, aged NHPs seem to be a relevant model to study normal aging and very early presymptomatic stages of synucleinopathy. Technical difficulties and high costs to allow the NHP to age, and a lack of advanced α-syn pathology considerably decrease the interest in using aged NHP as a spontaneous model for synucleinopathy.

3. MPTP-Intoxicated NHP Model

The discovery that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intoxication was able to induce parkinsonism [22] was critical in the development of modeling PD in animals [23]. Acute or chronic MPTP intoxication was shown to induce an oxidative insult through the blockade of the complex I of the mitochondrial respiratory chain by its metabolite, MPP^+ [24]. This toxic injury specifically induced dopaminergic nigrostriatal lesion associated with motor deficits in different NHP species, including squirrel monkeys (*Saimiri sciureus*) [25], rhesus monkeys (*Macaca mulatta*) [26,27], or marmosets (*Callithrix jacchus*) [28]. For decades, MPTP-intoxicated NHP thus became the gold-standard model to study PD. In 1986, using a chronic protocol to stabilize MPTP intoxication in squirrel monkeys, Forno and collaborators observed a lesion in the locus coeruleus, in addition to the nigral dopaminergic lesion, that provides a supplemental similarity with PD patients' brains [29]. The similarity with PD brains was also accentuated by the presence of few neuronal eosinophilic bodies in the medulla and nucleus basalis of Meynert in one aged MPTP-intoxicated animal and in the SN and dorsal raphe nuclei in another, which resembled LB inclusions at this time. However, they were less dense and with a weak peripheral halo. In 2000, Kowall and collaborators chose to assess the presence of α-syn pathology in MPTP-intoxicated baboons (*Papio anubis*) [30]. They confirmed the specific dopaminergic lesion in the central region of SN. For the first time, they highlighted a redistribution of α-syn from axons to neuronal cell bodies and dendrites, where they observed α-syn aggregates in the MPTP-intoxicated monkeys compared to the controls. They suggested that oxidative stress induced by MPTP toxin induces aggregation of α-syn as the initial stage of LB inclusion formation. Five years later, Purisai and collaborators showed that α-syn expression was upregulated two to three times, one week after acute MPTP intoxication in aged squirrel monkeys (over 12y) and persisted at one month after intoxication [31]. This upregulation was associated with a redistribution of α-syn protein localization from the neuronal fibers to the cell bodies in the SN one month after MPTP intoxication. Moreover, the number of α-syn-positive neurons in the SN increased in the MPTP-intoxicated animals and was strongly representative of the remaining dopaminergic and neuromelanin-positive neurons. This work highlighted two different directions in the relationship between α-syn accumulation and dopaminergic lesions. The authors first proposed that long-term lasting α-syn upregulation was triggered solely by an acute MPTP insult and might participate in dopaminergic neuronal cell loss, suggesting a toxic gain-of-function of α-syn in the pathology. On the other hand, as the increase of α-syn cells occurred in the surviving dopaminergic neurons, the data highlighted a compensatory response of neuronal cells to toxic injury. They also concluded that these two mechanisms could act at different timepoints in the pathology with a first α-syn response to protect dopaminergic neurons that were reversed into pathological mechanisms by other undetermined factors. The same team went further and focused on the induced synucleinopathy characteristics after acute MPTP injury in middle-aged squirrel monkeys [32]. They confirmed that MPTP insult induced α-syn upregulation and nigral dopaminergic cell loss one-month post-intoxication. They also highlighted that the increase in α-syn immunoreactivity was specifically neuronal. No α-syn was localized in glial cells in MPTP-intoxicated animals, suggesting that MPTP-monkeys could not be used as a MSA pathology model [32]. For the first time, they showed that nitrated and S129-phosphorylated α-syn accumulated in neuromelanin-containing neurons and dystrophic neurites. They also demonstrated that neuronal α-syn was soluble using PK treatment, whereas 15% of neuritic α-syn were PK-resistant and possibly aggregated. These data suggested that the MPTP insult induced oxidative stress that might lead to α-syn modifications such as nitration and phosphorylation, which could be key events in the aggregative process of α-syn observed in damaged axons. They hypothesized that the accumulation of aggregated α-syn in damaged axons might dysregulate the normal synaptic distribution of α-syn protein and contribute to its accumulation in dopaminergic neuronal cell bodies as the initial stage of LB formation. Nevertheless, no LB were observed in the MPTP-intoxicated monkey. These data also pointed

out the importance of age in developing α-syn pathology induced by MPTP insult on NHP. To test the hypothesis that LB formation required a long period after MPTP intoxication to be initiated, Halliday and collaborators assessed the α-syn-related pathology presented in middle-aged cynomolgus monkeys (*Macaca fascicularis*) treated ten years before chronically with MPTP for over two years [33]. Animals presented sustainable parkinsonism lasting for ten years, associated with a strong dopaminergic neuronal loss and increased intraneuronal α-syn and S129-phosphorylated α-syn, which accumulated in the remaining nigral neurons [33]. Nevertheless, LB were still not observed in these animals. This work highlighted that, in addition to the length of intoxication, the age of the intoxicated animal must also play a role in the severity of the α-syn-related pathology. In 2018, Huang and collaborators adopted a specific MPTP modeling-recovery-MPTP remodeling strategy on aged MPTP-intoxicated rhesus macaques (17 and 21 years) to stabilize parkinsonian syndrome without any recovery of the animals [34]. They found a significant negative correlation between the number of nigral TH-positive cells and S129-phosphorylated α-syn aggregation in the SN of these aged MPTP monkeys. These data confirmed that using MPTP intoxication in aged monkeys is a relevant new model to understand mechanisms underlying α-syn-related pathology. Using a large cohort of young rhesus monkeys (*Macaca mulatta*) chronically treated with MPTP, it was recently shown that α-syn-related pathology was not only present in the dopaminergic neurons of the SN but widespread to motor-related structures, such as the putamen, and to non-motor cortical regions, such as the hippocampal CA1 and the temporal cortex [35]. More interestingly, by treating some MPTP animals with the gold-standard pharmacological treatment L-DOPA, they demonstrated that L-DOPA abolished the MPTP-induced α-syn accumulation in the putamen and the cortical areas and decreased the amount of α-syn-positive neurons observed in the SN. These data highlighted the relevance of employing MPTP-intoxicated NHP in a translational approach that aimed to halt MPTP induced-α-syn pathology. Finally, it has been shown that MPTP-intoxicated monkeys can be useful to study peripheric α-syn pathology associated with PD. Human autopsy studies have consistently shown that LB are found in the ENS in nearly every case examined [36]. In 2020, Li and colleagues showed that total, S129-phosphorylated and oligomeric α-syn amounts are increased in the SN and the striatum as well as in the colon of middle-aged MPTP-intoxicated cynomolgus monkeys (10–12 years) [37]. They also observed dopaminergic neurodegeneration in the ENS and alterations in the expression of two enzymes involved in α-syn phosphorylation/dephosphorylation. Interestingly, they did not only report for the first-time development of α-syn pathology in the ENS of MPTP-intoxicated NHP but also found a positive correlation between levels of α-syn in the CNS and ENS. As they studied the tissues at only one-month post-intoxication, the α-syn pathology and dopaminergic lesion present in the CNS and ENS resulted from independent local response to intravenous MPTP injection and not a consequence of a long-distance propagation of α-syn pathology. Nevertheless, this work demonstrated the relevance of the MPTP NHP model to bring new information in an attempt to understand the mechanisms underlying central and peripheric PD pathogenesis.

Using MPTP-intoxicated monkeys, these studies first pointed out the role of a potential environmental and external toxic insult in the induction of neurodegeneration and α-syn pathology, two important characteristics involved in the pathogenesis of synucleinopathy. Specifically, they showed that MPTP increases oxidative stress through the blockade of the complex I of the mitochondrial respiratory chain by its metabolite MPP+ and modifies α-syn expression and protein localization, and promotes pathological α-syn aggregation. Notably, age and duration of intoxication with MPTP in NHPs were shown to be the key players to obtain an experimental model with a stable clinical pattern associated with dopaminergic neurodegeneration and α-syn-related pathology. Thus, aged and intoxicated NHP could represent a relevant model to study the synucleinopathy, recapitulating two important criteria to model in particular PD, with a specific dopaminergic lesion and a widespread neuronal accumulation of pathological α-syn forms. Nevertheless, no advanced LB-like pathology was observed in the MPTP-intoxicated monkey, despite a clear and stable

clinical parkinsonian syndrome associated with a robust dopaminergic lesion. In the future, the MPTP modeling-recovery-MPTP remodeling strategy adopted by Huang and al [34] should be considered in middle-aged monkeys to enable a safe and stable induction of parkinsonian motor syndrome after a long-term period of intoxication, as well as strong neurodegeneration that might be associated with advanced synucleinopathy, including LB formation. Nevertheless, the high toxic sensitivity of old monkeys to MPTP and the technical and pecuniary issues to let the monkeys age considerably slow down the interest in using aged MPTP-intoxicated monkeys as a model for synucleinopathies.

4. Viral Vector-Mediated Models

With the development and fine-tuning of viral production, certain studies have aimed at injecting viral vectors to overexpress either wild-type (WT) or mutant α-syn to induce pathology in NHPs. The goal of these viral vector-mediated models is to replicate more closely the pathology in NHP, mainly by causing not only loss of dopaminergic neurons, but also an accumulation of α-syn in neurons or oligodendrocytes. Besides, this viral-mediated overexpression has the advantage of targeting specific regions or cell types in the brain, making their use of interest in the study of all synucleinopathies.

4.1. Viral Vector-Based Models of Parkinson's Disease

Intracerebral injections of adeno-associated viruses (AAV) were first validated in rodents, particularly in rats, before being tested in NHP [38,39]. Building on their study in rats, Kirik and colleagues injected AAV1/2 to overexpress GFP, WT-α-syn, or A53T-mutant α-syn unilaterally in neurons in the right SN of common marmosets [40]. Three weeks post-injection, they verified their AAV's correct expression in control (GFP) monkeys and found that their virus was indeed specifically expressed in neurons. Following this, they waited four months post-injection to assess their various AAV effects on neurodegeneration and α-syn. In A53T-α-syn marmosets, they observed a loss of TH immunostaining in the SNpc with a decrease in VMAT-2. They also observed the presence of TH-positive fragmented neurites and α-syn-positive inclusions. This was accompanied by behavioral changes with a bias in the head position test on the ipsilateral side, starting at six weeks post-injection. This study was followed by using AAV2/5, also under the same neuronal promoter, targeting GFP, WT-α-syn, and A53T-mutant α-syn in a larger group of common marmosets [41]. The long-term effects of these unilateral injections in the SN were assessed one year after the AAVs injection. During the one-year live phase, they observed contralesional motor deficits in A53T-α-syn injected monkeys, with worsening general motor coordination over time. Post-mortem analysis showed that these monkeys displayed loss of TH immunostaining in the SN of WT and A53T-α-syn injected monkeys. They also observed total and S129-phosphorylated α-syn-positive inclusions, with the added presence of ubiquitin in these inclusions in the A53T-injected group. Both of these studies showed that both WT and A53T-mutant α-syn had deleterious effects when overexpressed in marmosets, but the A53T-mutant was more potent in inducing a PD-like pathology. These two first studies in marmoset monkeys were essential in demonstrating the ability to overexpress α-syn via viral vectors in NHPs, leading to decreased dopaminergic neurons and the formation of α-syn inclusions, associated with motor behavior.

Other approaches have consisted of injecting lentiviral vectors to force the expression of A53T-mutant α-syn in the SN of rhesus monkeys [42]. Yang and colleagues wanted to assess both the effect of injecting lentiviral vectors containing mutant α-syn and if this effect depended on the monkeys' age. After first verifying their lentivirus's correct expression in control and A53T-injected monkeys, they observed the initial formation of small α-syn aggregates in long neuronal processes, similar to Lewy neurites, in the A53T-injected monkey. They next injected monkeys of different ages (2y, 8y, and over 15y) with either PBS or the A53T lentivirus. After eight weeks, A53T monkeys demonstrated the formation of Lewy neurites and astroglial activation, which were more abundant in older monkeys than in younger monkeys. These monkeys also presented axonal degeneration and TH

immunostaining loss in the SN, specifically in A53T-injected monkeys. As previously stated, age has been shown to impact the accumulation of α-syn in the brain. This study demonstrated that monkeys' age plays a role in neuropathology when combined with lentiviral overexpression of A53T-α-syn.

Similarly, Bourdenx and colleagues wanted to assess whether age was a factor in developing PD neuropathology. For this purpose, they injected an AAV2/9 to overexpress the A53T-α-syn mutant using a neuronal promoter in the SN of young and old marmoset monkeys [43]. Eleven weeks post-injection, they first observed decreased TH immunostaining in sham-operated old animals in the SN and striatum. They observed a decrease in TH immunostaining in the SN and the striatum in both young and aged monkeys in the injected side. Concerning α-syn pathology, both young and old monkeys demonstrated an increase in total and S129-phosphorylated α-syn. Surprisingly, old monkeys presented less α-syn phosphorylation than young animals. Here, they showed that overexpression of α-syn induced a decrease in dopaminergic neurons and fibers accompanied by α-syn accumulation. Still, the age of monkeys did not impact this pathological progression. These last two studies aiming at determining the part of age in α-syn accumulation have shown quite diverging results. On the one hand, Yang and colleagues demonstrated that A53T overexpression impacts older monkeys more severely, while Bourdenx and colleagues did not observe this same effect of age. This could be due to the difference in species used, with one study using rhesus monkeys and the other using common marmosets or lentivirus compared to AAV.

More recently, Koprich and colleagues injected AAV1/2 A53T-α-syn in the SN of cynomolgus macaques [44]. In this study, they used different parameters to determine the conditions in which sustained expression of α-syn would induce neurodegeneration. In a first experiment, when injecting the virus at four sites of the SN, no dopaminergic neuron loss was observed. To optimize their AAV effects, they used higher titers or larger volumes in their second experiment. Both high titers and larger volumes were able to induce the loss of dopaminergic neurons in the SN. Nonetheless, the injection of a higher titer of virus had a more substantial effect on the decrease of DAT and dopamine than the injection of larger volumes. On the contrary, injecting larger volumes of virus induced higher levels of putaminal α-syn. Altogether, this study showed the impact of both the titer and volume of injection in monkeys, demonstrating once more that diverging results between experiments could not only be due to the species used but also to the injection itself.

4.2. Viral Vector-Based Models of Multiple System Atrophy

Given the promising studies using viral vectors to induce PD pathology, certain studies aimed to use viral vectors to generate other synucleinopathies, and more specifically, MSA. In mice, several studies have successfully overexpressed α-syn specifically in oligodendrocytes and recapitulated certain aspects of the neuropathology, including some motor deficits [45–47]. In a first study, Bassil and colleagues injected AAV1/2 with α-syn driven by an oligodendroglial promoter in both rats and macaque monkeys [48]. After putaminal injection of α-syn, they demonstrated specific α-syn localization in oligodendrocytes, with little neuronal expression of α-syn, highlighting that such an AAV-mediated approach may be usable in NHP. This study provided the first description of AAV-mediated transgene expression in oligodendrocytes in the NHP that was achieved using a cellular promoter. Nonetheless, further longitudinal studies will determine the progression of behavioral deficits and MSA-like pathology in this species. Concomitantly, Mandel and collaborators injected AAV-Olig001 targeting either GFP or α-syn in oligodendrocytes of rhesus monkeys [49]. The authors first demonstrated the control virus's efficacy to express GFP in oligodendrocytes of the striatum after four weeks. Three months post-injection in the striatum, they observed α-syn and S129-phosphorylated α-syn aggregates in the caudate nucleus, the putamen, and corpus callosum of α-syn-injected monkeys. These phosphorylated inclusions were shown to be PK resistant, demonstrating the production of insoluble aggregates. Besides, they established that these monkeys showed demyelination of the

corpus callosum combined with microglial activation in the striatum. Following this study, Marmion and colleagues also wanted to determine the effect of high titer AAV-Olig001 targeting α-syn in cynomolgus macaques six months after injection [50]. α-Syn was shown to accumulate in the putamen and to display inclusions that were α-syn-positive and S129-phosphorylated, and Y39-phosphorylated-α-syn-positive. Given this, they concluded that AAV-Olig001-α-syn was able to induce the formation of GCI-like inclusions in macaques. These monkeys also showed neurodegeneration with decreased TH immunostaining in the SN and demyelination in the striatum. Altogether, they demonstrated that injections with their viral-vector induced an MSA-like pathology in macaques with the formation of GCI-like inclusions, neurodegeneration, demyelination, and inflammation.

Taken together, these studies proved that the use of viral vectors was able to induce a sustained expression of α-syn in the SN of various monkeys, including marmosets and macaques. Despite the multiple viruses and serotypes, driving promoters, and injection periods tested, they were able to observe sustained α-syn overexpression accompanied, for the most part, by a loss of dopaminergic neurons in the SN. Injection of viral vectors demonstrated for the first time that, by using a cell-specific promotor overexpressing α-syn, it is possible to create models for different synucleinopathies. In certain studies, these viral-vectors were combined with the use of older monkeys to establish models closer to human conditions. Nonetheless, these viral vectors have yet to recapitulate all aspects of synucleinopathies but are of high interest in the study of these diseases in NHP.

5. Patient-Derived Brain Extracts Models

In another way to model synucleinopathies, we and other groups have used patient brain-derived material to get closer to the human pathophysiology in terms of the nature of inoculated material and the amount of injected α-syn. In 2014, Recasens and collaborators used LB-enriched fractions purified from three PD patients' mesencephala, which contained amyloid-like structures and insoluble aggregated α-syn, to inject ng of pathological α-syn into mice and NHP [51]. They showed that injection of LB-enriched fractions into mice induced significant dopaminergic neurodegeneration, accompanied by accumulation of PK-resistant and S129 phosphorylated forms of endogenous α-syn in the SN, the striatum, and the neocortical areas. These data were the first proof of concept regarding the induction of the pathogenicity from the human pathogenic material towards the endogenous murine α-syn protein. Along with the demonstrated aggregation and propagation of α-syn into interconnected brain areas, this study brought strong evidence about the "prion-like" hypothesis of α-syn in synucleinopathies [52]. More interestingly, LB-derived fractions into either the SN or the striatum were injected into four rhesus monkeys (8y). One monkey of each group was previously chronically treated with MPTP three years before. Using PET imaging, they demonstrated that striatal and nigral LB-inoculated monkeys exhibit a striatal dopaminergic lesion that appeared at nine months and lasted up to twelve months. Fourteen months after the LB inoculation, they obtained a more pronounced dopaminergic cell loss in the SN of striatal-injected monkeys than the nigral-inoculated animals. They also showed that, in striatal LB-inoculated primates, PD-derived LB extracts induced a widespread increase of α-syn levels in interconnected regions suggesting a long-distance propagation of α-syn pathology, which appeared to be more local for the nigral-injected groups. Interestingly, striatal inoculation of LB fractions in MPTP-treated monkeys did not lead to α-syn pathology in the SN but instead to an aggravated increase of α-syn into striatal and efferent areas, suggesting a retrograde transmission of α-syn from the striatum to the SN. This work was the first proof-of-concept showing that LB extracts purified from patients' brains induced a pathological response in NHP, including neurodegeneration and a "prion-like" synucleinopathy. To confirm this result, a follow-up study aimed at investigating the consequences of PD-derived LB inocula on a larger cohort of NHP. The authors injected PD patient-derived LB fractions containing large and insoluble α-syn aggregates in the striatum of olive baboons (*Papio papio*) [53]. Two years after the injection, LB-inoculated NHP present nigrostriatal neurodegeneration associated with α-

syn pathology localized in different brain regions. More surprisingly, and in contrast with mice, when PD patient-derived noLB fractions, containing small aggregates and mainly soluble α-syn, were injected into NHP, they observed dopaminergic neurodegeneration to the extent of LB-inoculated monkeys, also associated with α-syn pathology localized in many brain regions. Taking advantage of a machine learning approach, the authors sorted out the twenty variables that constituted the best predictors of neurodegeneration among a dataset of 180 measured variables for the two injection groups. Interestingly, they obtained unique pathological signatures of induced pathology between LB and noLB groups, leading to the same dopaminergic lesion level. This study showed that distinct pathological α-syn species led to the same dopaminergic lesion level through different underlying mechanisms, modeling the multifactorial nature and complexity of synucleinopathies. Using the same type of patient brain-derived extracts, the same authors decided to inject LB fractions in both the stomach and ventral duodenum wall of five baboon monkeys. The underlying idea was to compare the pathology induced with the one obtained for the striatal LB-fraction injected group and to challenge the hypothesis of a caudo-rostral propagation of α-syn pathology presumed by Braak and colleagues [54]. Interestingly, they observed that enteric inoculation of LB fractions in NHP led to central dopaminergic neurodegeneration in the SN and the striatum, at the same level as for the striatal-LB-injected group, associated with the development of α-syn pathology in the CNS. These data suggested that α-syn pathology propagated in a caudo-rostral fashion from the ENS towards the CNS. More surprisingly, they found that not only did the enteric LB-injected animals induce a local α-syn accumulation in enteric neurons, but so did the striatal-LB-injected animals. Moreover, they observed a significant negative correlation between the number of nigral dopaminergic neurons in the CNS and the amount of α-syn in the ENS neurons, suggesting that the enteric α-syn pathology extent may reflect the severity of the central dopaminergic lesion. Of interest, these data demonstrated that α-syn pathology also propagated rostro-caudally from the CNS towards the ENS. Regarding the bidirectional routes of propagation of the synucleinopathy, the vagus nerve was put aside in this experimental model, and biological fluids have been considered as possible alternative routes of α-syn pathology spreading.

Injection of LB-enriched fractions in NHPs recapitulates two important neuropathological criteria to model synucleinopathies: neurodegeneration and presence and spreading of α-syn pathology. Hence, LB-enriched inoculation in NHP may provide a relevant model to better understand the mechanisms underlying the pathology in synucleinopathy. However, only subtle behavioral changes assessed by validated ethological evaluation have been observed two years after the injection of LB-fraction, due to a dopaminergic cell loss that does not reach the threshold of the appearance of the motor symptoms. This progressive model can thus be employed to mimic the early stages of synucleinopathies to decipher the underlying mechanisms and better understand the "prion-like" properties of α-syn. Assessing α-syn pathology and neurodegeneration at early timepoints after LB injection has to be performed to evaluate the pathological signature dynamics of the pathology at very early stages. Similarly, increasing LB post-injection duration could be considered to assess a more advanced picture of neurodegeneration and α-syn pathology. Finally, inoculating other types of pathogenic brain extracts such as GCI fractions derived from MSA patients could be envisaged trying to model the other synucleinopathies and mimic their own specificity. Limited access to human material remains the drawback of using NHP models based upon human brain-derived extracts. Considerable collective efforts have to be made to find innovative solutions to bypass the need for fresh human material.

6. Recombinant α-Syn Preformed Fibrils Models

Injections of α-syn preformed fibrils (PFFs) in vitro and in mice have shown their efficacy in inducing both loss of dopaminergic neurons of the SN and the formation of aggregates of insoluble α-syn [55–57]. Following the demonstration of this in rodents, Shimozawa and collaborators endeavored to determine whether this was also the case in marmoset monkeys [58]. For this purpose, they injected marmoset monkeys with mouse

recombinant α-syn fibrils in the caudate nucleus and putamen. Three months after injection, they observed abundant S129-phosphorylated α-syn structures throughout various brain regions, including the striatum, SN, cortex, amygdala, thalamus. The inclusions seen were also positive for human α-syn, ubiquitin, and p62 staining. Formation of round, LB-like, S129-phosphorylated α-syn-positive inclusions was detected in dopaminergic neurons three months post-injection. Neurodegeneration was also observed with the decrease of TH-positive staining in the SN. Colocalization of the human-specific antibody LB509 and the microglial marker Iba1 suggested that the inclusions were phagocytosed by microglial cells. Altogether, this study showed for the first time that the use of PFFs could induce the formation of LB-like inclusions and dopaminergic neurodegeneration in the SN of marmoset monkeys. More recently, Chu and colleagues used injections of PFFs in the striatum of macaque monkeys to determine the effects of human recombinant α-syn fibrils [59,60]. Cynomolgus monkeys received intrastriatal injections of PFFs, and four control monkeys received sham surgery. After 12 to 15 months, they observed loss of TH immunostaining, accompanied by increased striatal DAT immunostaining. In addition, they observed S129-phosphorylated-α-syn inclusions in the SN that demonstrated two different aspects: either a more granular aspect or a whole-cell staining with absent cytoplasm, similar to the beginning of LB formation. These nigral neurons containing α-syn inclusions had lost both their TH and Nurr1 staining, reminiscent of PD pathology. In both cases, these aggregates suggested progressive α-syn accumulation, leading to the formation of larger LB-like inclusions. Both of these studies have shown the possibility of inducing a PD-like pathology in both marmoset and macaque monkeys. Despite using mouse or human α-syn PFFs, these studies demonstrated the ability of α-syn propagation and aggregate formation in NHPs. Nonetheless, it is important to note that large quantities of α-syn fibrils had to be injected to induce these PD-like pathologies. These are not sufficient to induce the appearance of clinical symptoms. Recently, other PFF delivery methods have been tested in cynomolgus monkeys by using intranasal injections [61]. After either one, four, or seventeen months, Guo and colleagues observed an accumulation of iron, accompanied by a sparse appearance of S129-phosphorylated α-syn, which did not colocalize with iron deposits. Nonetheless, this type of administration of PFFs did not induce dopaminergic neurodegeneration. Altogether, these studies showed the attempt of growing use of PFFs in NHP models and their potential, depending on their administration, to cause a PD-like pathology.

7. Transgenic Models

The purpose of a transgenic NHP synucleinopathy model would be to have a progressive and universal model able to recapitulate aspects of the disease from birth. Given the constraints, ethical and costly, and technical challenges, even with the recent progress in transgenesis, very few studies have attempted to create transgenic synucleinopathy models. In 2014, Niu and colleagues endeavored to generate a transgenic monkey model by lentiviral vector injection in fertilized monkey eggs [62]. After expression of A53T-mutant α-syn in oocytes, 75 eggs were transferred in rhesus monkeys. These transfers resulted in 11 pregnancies and led to the birth of live newborn monkeys. Transgene expression was confirmed, and immunostaining also showed the presence of increased α-syn in the SN, striatum, and cortex, but not of S129-phosphorylated α-syn in these A53T-transgenic monkeys. The authors also noted that older monkeys developed cognitive defects and anxiety starting at 2.5 years of age. These defects implicated object recognition, dexterity, and stereotypical circling behavior of these animals, reminiscent of prodromal defects seen in PD patients. Nonetheless, these animals showed no motor abnormalities and no neurodegeneration using MRI. Compared to other models, these transgenic monkeys could be more reliable to observe phenotypes and pathological modifications and find biomarkers for PD. Given that this study only followed the A53T-transgenic monkeys for 2.5 years, it is difficult to say whether they will develop other aspects of PD in the future. Still, this study remained highly interested in observing the age-dependent factors

of PD onset. More recently, with the discovery of CRISPR-Cas9 technology, certain studies have taken advantage of the mere use of this system to develop transgenic models of synucleinopathy. Yang and colleagues injected CRISPR-Cas9 directed against the *PINK1* gene in one-cell stage embryos from rhesus monkeys [63]. After the transfer of the embryos to surrogate rhesus monkeys, eleven fetuses developed (8 *PINK1* mutants and 3 WT) and were born naturally. Of these, four mutant and one WT monkey died in the first week after birth. Of the four remaining mutant monkeys, one lived for 1.5 years, and the three others were terminated three years after birth. Certain monkeys with *PINK1* mutations displayed decreased grey matter density in the cortex, and others had decreased movement after 1.5 years. After 1.5 years, decreased neuronal immunostaining and increased astrogliosis compared to WT monkeys were observed. Electron microscopy in one mutant monkey demonstrated degeneration of neurons in the cortex, SN, and striatum. Despite not demonstrating any modifications in α-syn or its distribution, this team showed the possible use of CRISPR-Cas9 relating to PD in NHPs. Regardless of the uncommon use of transgenic NHP models in PD, it is important to note their potential importance for future studies. Given the constraints observed, it is not surprising that few studies currently exist that have created transgenic NHPs. Still, we suspect that the number of studies will grow in the next years to resolve the need to better understand intractable diseases such as PD.

8. Conclusions

Here, we have discussed the existing NHP models of synucleinopathy that have been developed and emerged in the last years. Different approaches have been used among these models, such as natural aging of animals and injections of toxins, viruses, or α-syn-based products (summarized in Table 1). Together, these NHP models present their advantages and limits, noting that most models lack certain aspects found in human pathologies [16]. The central difficulty has been observing the formation of LB, as can be found in the human pathology, with the formation of dense bodies with a peripheral halo. Given the many monkey species available and the cost they engender, the species is not always an easy choice but can play a significant role in the results observed. As seen previously, the same type of models can have different results based on the species used, particularly between New World and Old World monkeys. New World monkeys present specific differences in their α-syn sequence, including the natural appearance of the A53T mutation, which the Old World monkeys do not show [64]. Besides, the presence of neuromelanin has been detected in Old World monkeys, but not in New World monkeys, which could explain specific differential susceptibilities between species [14,65,66]. These differences between Old World and New World monkeys have been thoroughly previously discussed, along with the impact of genetic modifications in NHP models [67]. Furthermore, it is important to note that the lifespan between monkey species can differ tremendously, making the study of aging difficult. For example, marmoset monkeys live around ten years, whereas macaque monkeys live around 25–30 years [68]. Even taking all these aspects into account, the variability between injections and quantities injected, this remains difficult to compare two experimental models side by side. Moreover, NHP models render substantial costs related to the animals' maintenance and care and the difficulties of having a sufficient number of monkeys. Nonetheless, many models have shown interesting findings in NHP that mimic more closely than in rodents, the appearance of certain neuropathological features. This could be mainly due to the closer anatomical resemblance of monkeys to humans, compared to rodents. In WT rodents, no age-dependent accumulation of the neuronal pigment neuromelanin occurred. Furthermore, the α-syn protein sequence diverged between rodents and primates, with, for instance, the natural presence of the A53T mutation in rodents. It is equally important to note that other animal models, though less ethically challenging, have been unable to show such similarities with human synucleinopathy than NHP models. Among the NHP models of synucleinopathy, some teams aim to combine both aging and intracerebral injections to obtain a model closer to human PD. In some cases, this approach has successfully demonstrated the more important

effect of injections on aged animals [32,42], while in others, no such differences were observed [43]. Despite these variable effects of age, this could be an interesting strategy to study synucleinopathies in the future.

Table 1. Summary of non-human primate models of synucleinopathy.

Type of Model	NHP Species	Injection	Pathological Phenotype	Reference
Aging	Rhesus monkeys (young: 3–5y; aged: 26–28y)	NA	Clinical motor symptoms Dopaminergic cell loss (aged animals)	[17]
	Rhesus monkeys (young: 2–12y; middle-aged: 15–23y; aged: 24–34.1y)	NA	Dopaminergic neurodegeneration α-syn accumulation in the SN (middle-aged and aged animals)	[18]
	Mouse lemur primates (*Microcebus murinus*) (young: 1.42–3.07y; aged: 8.21–10.32y)	NA	α-syn accumulation in anterior olfactory nucleus, cortex, SN, and striatum S129-phosphorylated α-syn in the cerebellum, hippocampus, thalamus, red nucleus, olfactory tubercle, cortex, SNpc, and striatum (aged animals)	[19]
	Cynomolgus monkeys (*Macaca fascicularis*) (young: 3–4y; middle-aged: 10–12y; aged: 15y)	NA	Accumulation of oligomeric and S129-phosphorylated α-syn in striatum and hippocampus (middle-aged and aged animals)	[20]
	Macaques (Cynomolgus, Japanese, and Rhesus) (10–31y)	NA	Accumulation of α-syn in the SN, ventral tegmental, striatum, nucleus accumbens, prefrontal cortex, primary motor cortex	[21]
MPTP	Squirrel monkeys (*Saimiri sciureus*) (young: 5–10y; old:15–20y)	Chronic MPTP	Clinical parkinsonism Dopaminergic neurodegeneration Neuronal eosinophilic bodies	[29]
	Baboon (*Papio anubis*)	Chronic MPTP	Clinical parkinsonism Dopaminergic neurodegeneration Redistribution of α-syn from axons to cell bodies	[30]
	Squirrel monkeys (*Saimiri sciureus*) (aged: >12y)	Acute MPTP	Clinical parkinsonism Dopaminergic neurodegeneration α-syn upregulation and accumulation in the SN	[31]
	Squirrel monkeys (*Saimiri sciureus*) (aged: >12y)	Acute MPTP	Clinical parkinsonism Dopaminergic neurodegeneration S129-phosphorylated, nitrated and PK-resistant α-syn in the SN	[32]
	Cynomolgus monkeys (*Macaca fascicularis*) (14y)	Chronic MPTP	Clinical parkinsonism Dopaminergic neurodegeneration S129-phosphorylated α-syn accumulation in the SN	[33]
	Rhesus monkeys (*Macaca mulatta*) (17–21y)	MPTP modeling-recovery-MPTP remodeling	Clinical parkinsonism Dopaminergic neurodegeneration S129-phosphorylated α-syn accumulation in the SN	[34]

Table 1. *Cont.*

Type of Model	NHP Species	Injection	Pathological Phenotype	Reference
	Rhesus monkeys (*Macaca mulatta*) (5.4 ± 1y)	Chronic MPTP	Clinical parkinsonism Dopaminergic neurodegeneration α-syn accumulation in the SN, putamen, and cortical areas	[35]
	Cynomolgus monkeys (*Macaca fascicularis*) (10–12y)	Chronic MPTP in the CNS and ENS	Clinical parkinsonism Dopaminergic neurodegeneration in the CNS and ENS S129-phosphorylated and oligomeric α-syn accumulation in CNS and ENS	[37]
Viral vectors	Common marmoset (*Callithrix jacchus*) (5.5–6y)	AAV1/2-CBA GFP, A53T-α-syn or WT-α-syn (4 months)	Behavioral changes: head position bias starting at 6 weeks Dopaminergic neurodegeneration α-syn-positive inclusions	[40]
	Common marmoset (*Callithrix jacchus*) (5.5–6y)	AAV2/5-CBA GFP, A53T-α-syn or WT-α-syn (12 months)	Behavioral changes: contralesional motor deficits in A53T-α-syn monkeys + worsening general motor coordination Dopaminergic neurodegeneration α-syn and S129-phosphorylated α-syn inclusions in A53T group	[41]
	Rhesus monkeys (young: 2y; middle-aged: 8y; old: 22y)	Lentiviral A53T-α-syn (2months)	Axonal damage and dopaminergic neurodegeneration Accumulation of α-syn (particularly in older monkeys) Increased astroglial activation	[42]
	Common marmoset (*Callithrix jacchus*) (young: 2y; old: 5y)	AAV2/9-CMVie A53T-α-syn (11 weeks)	Dopaminergic neurodegeneration Accumulation of α-syn and S129-phosphorylated α-syn	[43]
	Cynomolgus monkeys (*Macaca fascicularis*) (8y)	AAV1/2-CBA/CMV A53T-α-syn High titer/low volume or low titer/large volume (4 months)	Dopaminergic neurodegeneration Higher α-syn levels by ELISA	[44]
	Rhesus monkeys (*Macaca mulatta*)	AAV-Olig001-α-syn (3 months)	Soluble and aggregated α-syn observed in the caudate and nucleus Formation of GCI-like inclusions Demyelination and microglial activation	[49]
	Cynomolgus monkeys (*Macaca fascicularis*)	AAV-Olig001-α-syn (6 months)	Dopaminergic neurodegeneration α-syn-positive GCI formation (pS129+, pY39+, LB509+) Demyelination and microglial activation	[50]

Table 1. *Cont.*

Type of Model	NHP Species	Injection	Pathological Phenotype	Reference
Patient brain-derived extracts	Rhesus monkeys (*Macaca fascicularis*)	PD brain-derived LB fraction (14 months)	Dopaminergic neurodegeneration α-syn accumulation and propagation	[51]
	Olive baboon (*Papio papio*)	PD brain-derived LB fraction PD brain-derived noLB fraction (24 months)	Dopaminergic neurodegeneration α-syn accumulation and propagation Distinct pathogenic signature	[53]
	Olive baboon (*Papio papio*)	PD brain-derived LB fraction in the CNS and ENS (24 months)	Dopaminergic neurodegeneration (CNS) α-syn accumulation and propagation (CNS and ENS) Bidirectional propagation of α-syn pathology	[54]
Preformed fibrils	Common marmoset (*Callithrix jacchus*) (2y)	Mouse recombinant α-syn fibrils (3 months)	Dopaminergic neurodegeneration S129-phosphorylated α-syn in the striatum, SN, cortex, amygdala, thalamus, and others Formation of LB-like structures	[58]
	Cynomolgus monkeys (*Macaca fascicularis*) (6–10y)	Human recombinant α-syn fibrils (12–15 months)	Dopaminergic neurodegeneration Increase in DAT staining S129-phosphorylated α-syn inclusions in the SN (granular and whole-cell inclusions) Loss of Nurr1 and TH staining in α-syn-positive inclusions	[59]
	Cynomolgus monkeys (*Macaca fascicularis*) (6–10y)	Human recombinant α-syn fibrils (1, 4, 17 months)	Accumulation of iron in microglia Weak S129-phosphorylated α-syn immunostaining No dopaminergic neurodegeneration	[61]
Transgenic models	Rhesus monkeys (*Macaca fascicularis*)	Lentiviral A53T α-syn in oocytes	Cognitive defects and anxiety starting at 2.5y Increased α-syn levels in the SN, striatum, and cortex	[62]
	Rhesus monkeys (*Macaca fascicularis*)	CRISPR-Cas9 *PINK1* in oocytes	Decrease in grey matter density in the cortex Neurodegeneration in the SN, striatum, and cortex Increased astrogliosis	[63]
NA: non-attributable; y: year(s)				

Since the discovery of its implication in LB formation, α-syn has been very much at the center of most PD and MSA models. Studies have been carried out to elucidate its ability to be a trigger, a biomarker, and/or a therapeutic target for PD. In various models, α-syn overexpression induces not only dopaminergic cell loss in the SN, but also demonstrates its ability to propagate and seed the formation of new α-syn aggregates. The main foundation of α-syn overexpressing models is based on the presence of PD patients presenting genetic duplication or triplication of the *SNCA* gene [69,70]. Although only certain of the abovementioned models use this overexpression to induce PD pathology, most recent models demonstrate α-syn accumulation in their studies despite their lack of induced α-syn overexpression. Overall, current studies have mostly focused on PD NHP models, with very few studying DLB or MSA. In the future, additional efforts should

also be placed on these two other synucleinopathies to create models that recapitulate their cell-specific α-syn accumulation and neuropathology. The nature of α-syn assemblies (i.e., oligomers, fibrils) used for intracerebral injection may be the source of variabilities at different levels mainly and not exclusively due to (i) α-synuclein inoculum preparation (protein folding, protein concentration, the composition of the vehicle, detergent-insoluble fractions vs. total brain homogenates vs. LB-bearing fractions); (ii) α-synuclein inoculum purity (molecular size, sonication protocol, and storage conditions); (iii) volume of injected material and speed of injection; and (iv) time post-injection. Altogether, the resulting anatomopathological features will be dependent per se from the injected material, and in its ability to form oligomers and/or fibrils, to self-maintain and propagate over-time in vivo. It is equally important to note that no current consensus exists that defines the observation and the criteria of synucleinopathy occurrence in models [71]. Many studies observe various aspects of α-syn accumulation in their respective studies, whether it is the presence of PK-resistant α-syn, S129-phosphorylated α-syn, or distinct α-syn conformers. Better terms and standardized methods to characterize α-syn are eagerly awaited in the field. Overall, it would be essential to agree upon certain aspects that must be demonstrated before validating an adequate model to study these synucleinopathies.

Transgenic NHP models are one of the methods that have yet to be thoroughly studied, given the complications of such experiments. Despite this, studies have shown the potential interest of these models to have a progressive installment of the disease, without requiring injections. In fact, other neurodegenerative disorders such as Huntington's Disease have generated transgenic monkey models to recapitulate main aspects of diseases [72,73]. This transgenic monkey model shows a progressive installment of the disease, with the presence and progression of aggregated huntingtin protein throughout the brain, and progressive striatal atrophy. With the limited current data on α-syn transgenic monkeys, it has yet to be demonstrated whether there will be a valid model and, more importantly, how long the disease onset will require. It is important to note that new strategies using CRISPR-Cas9 technologies should prove to be assets in developing such NHP models [74].

Despite the lack of models that recreate the full spectrum of synucleinopathy as in PD or MSA patients, NHP models have come further in recapitulating the majority of neuropathological aspects of these diseases. Together and to date, these multiple NHP models demonstrated the capacity of inducing the early stages of synucleinopathy through different approaches. Understanding these early stages of α-syn aggregation and neurodegeneration could be extremely important in having a better comprehension of the establishment of PD, DLB, and MSA. With the improvement of strategies for developing models such as the injection of viral vectors, patient-derived extracts, preformed fibrils, we have been seeing a new rise in NHP models of synucleinopathy, which should continue to progress in the next decade.

Author Contributions: M.T. prepared parts on viral-based NHP models, transgenic NHP models and PFF-injected NHP models, M.-L.A. on aging, MPTP-intoxicated NHP, and patient brain-derived extracts. M.T. and M.-L.A. collectively corrected the draft of the Review, including text and figures. B.D. did the final editing. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by Fondation de France Grant number 00066525, a France Parkinson Grant, an IDEX Emergence Grant number OPE-2018-410, and the Michael J. Fox Foundation (Project Grant No. MJFF-008814) (B.D.). M.T. is a recipient of an MSER fellowship (France). M.-L.A. is a recipient of a France Parkinson Foundation fellowship. The LABEX Brain, the University of Bordeaux, and the Centre National de la Recherche Scientifique provided infrastructural support.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We apologize to the authors of several high-quality scientific articles that contributed significantly to the development of the field, which could not be cited due to space limits.

Conflicts of Interest: The authors declare no conflict of interest.

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Titre : Etude des mécanismes et cibles thérapeutiques des synucléinopathies

Résumé : Mon projet de thèse s'inscrit dans l'étude des synucléinopathies, une famille de maladies neurodégénératives. Les trois principales synucléinopathies sont la maladie de Parkinson, l'atrophie multisystématisée et la démence à corps de Lewy. Ces maladies sont caractérisées par une perte de neurones dans des régions cérébrales spécifiques et la présence d'inclusions intra-cytoplasmiques positives pour l'α-synucléine dans les neurones (Corps de Lewy) ou dans les oligodendrocytes (Inclusions gliales cytoplasmiques). Les causes d'induction de ces maladies restent encore inconnues et les traitements curatifs sont inexistants. L'objectif de mon travail de thèse visait à étudier les mécanismes neurodégénératifs et de potentielles cibles thérapeutiques dans le contexte des synucléinopathies. Je me suis tout d'abord intéressée aux mécanismes impliqués dans la transmission de l'α-synucléine issue de patients atteints de l'atrophie multisystématisée. Ce travail nous a permis de développer un potentiel nouveau modèle de l'atrophie multisystématisée chez la souris et le primate non-humain, par la transmission de l'α-synucléine dans le cerveau. Dans un deuxième temps, nous nous sommes intéressés à des cibles thérapeutiques éventuelles pour la maladie de Parkinson dans un même modèle animal de la pathologie. Nous avons pu vérifier l'efficacité et la pertinence de trois différentes stratégies ciblant plusieurs mécanismes affectés dans la maladie de Parkinson dans le but d'induire une protection des neurones dopaminergiques de la substance noire des souris. Nous avons pu démontrer une dérégulation des niveaux de zinc au cours de la pathologie qui a suscité l'intérêt de cibler son homéostasie dans le cerveau à travers une molécule chélatrice du zinc. Ensuite, la surexpression d'un facteur de transcription impliqué dans la survie des neurones dopaminergiques ainsi que dans le stress oxydatif et le protéasome a montré son intérêt comme cible thérapeutique de la maladie de Parkinson. Enfin, une molécule anti-agrégative a aussi démontré sa capacité à induire une neuroprotection. En résumé, ces travaux montrent d'abord l'importance de l'α-synucléine dans la mise en place et la progression des synucléinopathies, mais aussi la nécessité de cibler d'autres mécanismes dérégulés dans ces pathologies pour proposer des nouvelles stratégies thérapeutiques.

Mots clés: maladie de Parkinson ; atrophie multisystématisée ; synucléinopathies ; neuropathologie ; thérapeutique

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Title: Mechanistic study and potential therapeutic targets of synucleinopathies

Abstract: My thesis focused on the study of synucleinopathies, a family of neurodegenerative diseases. The three main synucleinopathies are Parkinson's disease, multiple system atrophy, and dementia with Lewy bodies. These diseases are characterized by the loss of neurons in various brain regions and the presence of intracytoplasmic α-synuclein-positive inclusions. These inclusions are located either in neurons (Lewy bodies) or in oligodendrocytes (Glial cytoplasmic inclusions). The trigger and cause for the formation of these inclusions remain unknown, and no curative treatments currently exist. The objective of my thesis was to study the neurodegenerative mechanisms and potential therapeutic strategies of these synucleinopathies. For this, I was first interested in the mechanisms implicated in the transmission of α-synuclein from multiple system atrophy patients. This allowed us to develop a potential new model to study multiple system atrophy in both mice and non-human primates by spreading of α-synuclein within the brain. In the second part, we wanted to investigate potential therapeutic targets in the same model of Parkinson's disease. This study confirmed the efficacy and pertinence of three different strategies that target various mechanisms of Parkinson's disease to induce the protection of dopaminergic neurons of the substantia nigra in a mouse model. By modulating zinc levels, we demonstrated the importance of zinc concentrations in the brain and the therapeutic interest in targeting metal homeostasis via specific chelators. We then used viral vectors to overexpress a transcription factor implicated in dopaminergic neuron survival, oxidative stress and proteasome activity in the substantia nigra of a mouse model of Parkinson's disease. Finally, we used an anti-aggregative molecule to determine its efficacy in protecting neurons in the same mouse model. Altogether, this thesis work showed the implication of α-synuclein in triggering and propagating synucleinopathies, the importance of targeting this protein, and other dysregulated cellular mechanisms to discover potential therapies.

Keywords: Parkinson's Disease; multiple system atrophy; synucleinopathies; neuropathology; therapies

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