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Les acides Ginkgolique et Niflumique sont les nouveaux modulateurs de récepteur à la glycine

Ginkgolic and Niflumic acids are novel modulators of glycine receptors

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RESUME

Glycine receptor is a pentameric ligand-gated neuronal receptor that possesses an ion pore permeable for Cl^- and represents an important component of inhibitory neurotransmission in the CNS of vertebrates. Glycine receptors participate in the control of motor activity, respiration, inflammatory pain sensation, perception of visual and auditory stimuli. Development of efficient modulators of glycine receptors will allow a precise control of their activity, which is especially important in the case of glycine receptor pathologies, such as hyperekplexia.

The aim of our study was to identify new modulators of glycine receptors and determine electrophysiological profiles of their interaction with different subunits of the receptor. Using patch-clamp technique we have monitored the amplitude of glycine-evoked ionic currents upon application of studied compounds. Investigation was performed on glycine receptors of different subunit composition (α1-α3, β, α1 and α2 mutants) transiently expressed in cultured CHO cell line. For the first time we have shown that ginkgolic and niflumic acids are able to modulate currents mediated by glycine receptors. We have shown that ginkgolic acid subunit-specifically potentiates α1 glycine receptors and succeeded to detect amino acids involved in this functional modulation. We have characterized niflumic acid as a voltage-dependent inhibitor of glycine receptors with higher affinity to α2 and α3 subunits than to α1. Mutation of G254A in the second transmembrane domain of α1 receptors increased the sensitivity to niflumic acid. Our data provide evidences for the pore-blocking mechanism of niflumic acid action.

Thus, in the present work we have identified ginkgolic and niflumic acids as novel modulators of glycine receptors, characterized their action on different subunits of the receptor and determined the most probable sites of interaction of the compounds with glycine receptors.

Le récepteur à la glycine est un récepteur neuronal qui appartient à la famille des canaux ligand-dépendants «cys-loop». Avec le récepteur ionotrope GABA ils fournissent la neurotransmission inhibitrice rapide dans le SNC des vertébrés grâce à leur perméabilité sélective au Cl^-. Les récepteurs à la glycine participent à différents processus physiologiques comprenant le contrôle de l'activité motrice, la respiration, la sensation de douleur inflammatoire, la perception des stimuli visuels et auditifs. Le développement de modulateurs efficaces des récepteurs à la glycine permettra un contrôle précis de leur activité, ce qui est particulièrement important dans le cas des pathologies des récepteurs à la glycine, comme l'hyperekplexie.

Le but principal de notre étude était d'identifier de nouveaux modulateurs des récepteurs à la glycine et de déterminer les profils électrophysiologiques de leur interaction avec les différentes sous-unités du récepteur. En utilisant la technique du patch-clamp, nous avons enregistré les changements des amplitudes des courants ioniques évoqués par la glycine lors de l'application des composés étudiés. L'étude a été effectuée sur des récepteurs à la glycine de compositions en sous-unités différentes (α1-α3, β et α1 ou α2 mutants) exprimés de manière transitoire dans la lignée cellulaire CHO. Pour la première fois, nous avons montré que les acides ginkgoliques et niflumiques sont capables de moduler efficacement les courants médiés par les récepteurs à la glycine. Il a été déterminé que l'acide ginkgolique, en concentrations submicromolaires, potentialise les récepteurs à la glycine de type α1 et n'influence pas d'autres sous-unités du récepteur. En utilisant la mutagenèse dirigée, nous avons réussi à détecter les acides aminés responsables d'une telle action sélective de l'acide ginkgolique. Nous avons également découvert que l'acide niflumique est un inhibiteur tensiodépendant des récepteurs à la glycine avec une affinité plus élevée pour les sous-unités α2 et α3 que pour α1. La mutation G254A dans le deuxième domaine transmembranaire du récepteur α1 a augmenté sa sensibilité à l'acide niflumique. Nos données démontrent que l'effet de l'acide niflumique sur les récepteurs à la glycine passe par le blocage des canaux des récepteurs.

Ainsi, en utilisant l'analyse électrophysiologique, la mutagenèse dirigée et l'expression de protéines spécifiques dans un système hétérologue, nous avons identifié les acides ginkgoliques et niflumiques comme nouveaux modulateurs de récepteurs de la glycine, caractérisé leur action sur différentes sous-unités du récepteur et déterminé les sites importants pour la potentialisation ou l'inhibition des récepteurs à la glycine par ces composés. Cette approche est très prometteuse et ouvre de nouvelles voies vers des futures actions thérapeutiques.

CONTENT

INTRODUCTION

Functioning of the central nervous system (CNS) is regulated by interaction of structures that provide excitatory and inhibitory neurotransmission. One of the major roles in this process belongs to ligand-gated “cys-loop” receptors. This receptors family contains both anion- (glycine and GABA receptors) and cation-selective (serotonin 5HT3 and nicotinic acetylcholine receptors) channels.

Glycine receptors provide fast inhibitory drive in the CNS of vertebrates and participate in the control of numerous physiological processes such as locomotion, respiration, perception of visual and audio signals. They are localized on motoneurons and dorsal horn neurons of spinal cord, in brainstem nucleis, retina, inner ear and hippocampus (Harvey et al., 2004; Haverkamp et al., 2003; Brackmann et al., 2004). Glycine receptors dysfunction can lead to the development of hyperekplexia, inflammatory pain sensitization and epilepsy (Schaefer et al., 2012; Meier et al., 2005; Eichler et al., 2008; Harvey et al. 2004). Besides that, activity of glycinergic system can significantly influence the state of nervous tissue after an ischemic episode (Tanbe et al., 2010; Yao et al., 2012).

Regarding an exclusive role of glycine receptors in the functioning of CNS a search for new efficient modulators of their activity is of great importance. In addition, determination of the mechanisms of glycine receptor interaction with pharmacologically active molecules will help to understand better its structure and function.

New cellular systems for glycine receptors research represents a particular interest, especially taking to the account a fast development of technologies for generation of neurons from human fibroblasts. This technique will allow studying mutant glycine receptors that cause hyperekplexia using induced neurons directly from patients.

Thus, the main aim of our investigation was to search for new modulators of glycine receptors and to determine a possibility to use neurons generated from human fibroblasts for studying glycine receptors.

In order to achieve our goals we have considered the following tasks:

1. To investigate the influence of ginkgolic acid, a component of *Ginkgo biloba* extract, on glycine receptors of different subunit composition. To identify amino acids crucial for ginkgolic acid interaction with glycine receptors.
2. To study the electrophysiological profile of niflumic acid action on glycine receptors of different subunit composition. To determine the locus of the acid interaction with glycine receptor.

3. To characterize the ionic channels, expressed by neurons generated from human fibroblasts.

Current work has been performed using the following experimental techniques:

1. Cultivation of CHO cells line that was chosen as heterologous system for glycine receptors expression.
2. Transient transfection of CHO cells with cDNA of different subunits of glycine receptor (α1, α2, α3, α1β, α2β).
3. In order to probe sites responsible for glycine receptor interaction with ginkgolic and niflumic acids we have performed point mutations of α1 and α2 subunits.
4. Using the patch-clamp technique we have studied modulation of the amplitude of currents mediated by the glycine receptors of varying subunit composition upon application of ginkgolic and niflumic acids
5. Generation of neurons from human fibroblasts and their cultivation were performed by *PhD Badja (Medical Genetics and Functional Genomics, INSERM, Aix-Marseille University).*
6. Using the patch-clamp technique we have determined functionality of neurons generated from human fibroblasts and identified several types of ligand-gated receptors expressed by these differentiated cells.

In the present work for the first time we have shown that ginkgolic acid is a positive specific modulator of α1 glycine receptors. Using point mutagenesis we have revealed amino acid residues responsible for subunit-selective action of ginkgolic acid on glycine receptors. For the first time we have demonstrated that niflumic acid inhibits ionic currents mediated by glycine receptors in a voltage-dependent manner and exhibits main features of the open channel blocker. We have, as well, identified amino acid residue that influence this process. We have shown that neurons generated from fibroblasts, by the novel, highly efficient method that does not require a use of feeder-cells, are functional and express glycine receptors on their surface.

LITERATURE REVIEW

article I

Molecular physiology of glycine receptors in vertebrate nervous system

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The review article contains a short summery of our current knowledge about glycine recepors molecular physiology. It is composed of several sections that consider (i) distribution and various function of glycine receptors in the mammalian nervous system; (ii) glycine receptors in the process of nervous system development; (iii) their role in inflammatory pain sensitization; (iv) functioning of different subunits of glycine receptors; (v) their structure; (vi) profiles of pharmacological modulation; and (vii) description of hyperekplexia – hereditary disease caused by mutations of glycine receptors.

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Molecular Physiology of Glycine Receptors in Vertebrate Nervous Systems

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Glycine receptors are anion-selective channels supporting rapid synaptic transmission in vertebrate central nervous systems. Along with acetylcholine, nicotine, $GABA_A$, and serotonin ($5\text{-}HT_3R$) receptors, glycine receptors are members of the cys-loop pentameric ligand-sensitive receptor family. One β and four α subunits have been cloned from various species. Because of their specific distribution and molecular functional features, they perform a variety of physiological functions – from controlling motor activity through regulating neuron differentiation to processing sensory information and modulating pain sensitivity. The aim of the present review was to assess the overall picture formed by many years of studies of glycine receptors, briefly presenting the main functions of these transmembrane proteins, their distribution, and their molecular organization. Particular attention is focused on results of recent studies of the molecular physiology of these receptors, explaining how amino acids and molecular domains are responsible for modulating the receptors and how impairments to their functions lead to pathological sequelae.

Keywords: glycine, synaptic transmission, cys-loop receptors, amino-selective channels, neuropathology.

Glycine is the simplest amino acid in biological organisms. Apart from its main role – as a structural "brick" in protein macromolecules – glycine has another extremely important function, as a neurotransmitter operating in synapses in the nervous system. Studies in the 1960s and 1970s demonstrated that fast inhibitory synaptic transmission in vertebrate nervous systems is provided by two main systems: the GABAergic and the glycinergic. The neurotransmitter in the former is γ-aminobutyric acid (GABA), while the neurotransmitter in the latter is glycine. Ionotropic GABA receptors are present mainly in synapses in the brain, while glycine receptors dominate in the spinal cord and brainstem. These receptors are colocated in some areas of the nervous system [14, 155, 162]. Furthermore, GABA and glycine can be present in the same synaptic vesicle [162] and are released from presynaptic terminals simultaneously [79].

The physiological functions of the glycinergic system are very diverse: from controlling motor activity and generating rhythms to processing sensory information. The main function is to transmit inhibitory spikes in the spinal cord, supporting the rapid regulation of motor activity [45]. The functioning of glycine receptors depends on their location in the nervous system, their subunit composition, their regulation by second messengers (protein kinases, phosphatases, calcium ions), and ion concentrations in the intracellular and extracellular spaces [100, 106].

This review will address the physiological functions of the glycine receptor family, their molecular organization, and impairments to the structures of these macromolecular complexes resulting in pathological sequelae.

The first evidence that glycine might play a role as an inhibitory neurotransmitter was obtained in the 1960s. Electrophysiological experiments using iontophoretic application of various compounds to spinal cord neurons demonstrated that glycine evoked decreases in the activity of these neurons [34]. Biochemical studies showed that the glycine concentrations in spinal cord tissues were much greater than those in other parts of the nervous system [4, 5]. This suggested that glycine has a role as a major neurotransmitter in the spinal cord. Furthermore, studies identified an antagonist

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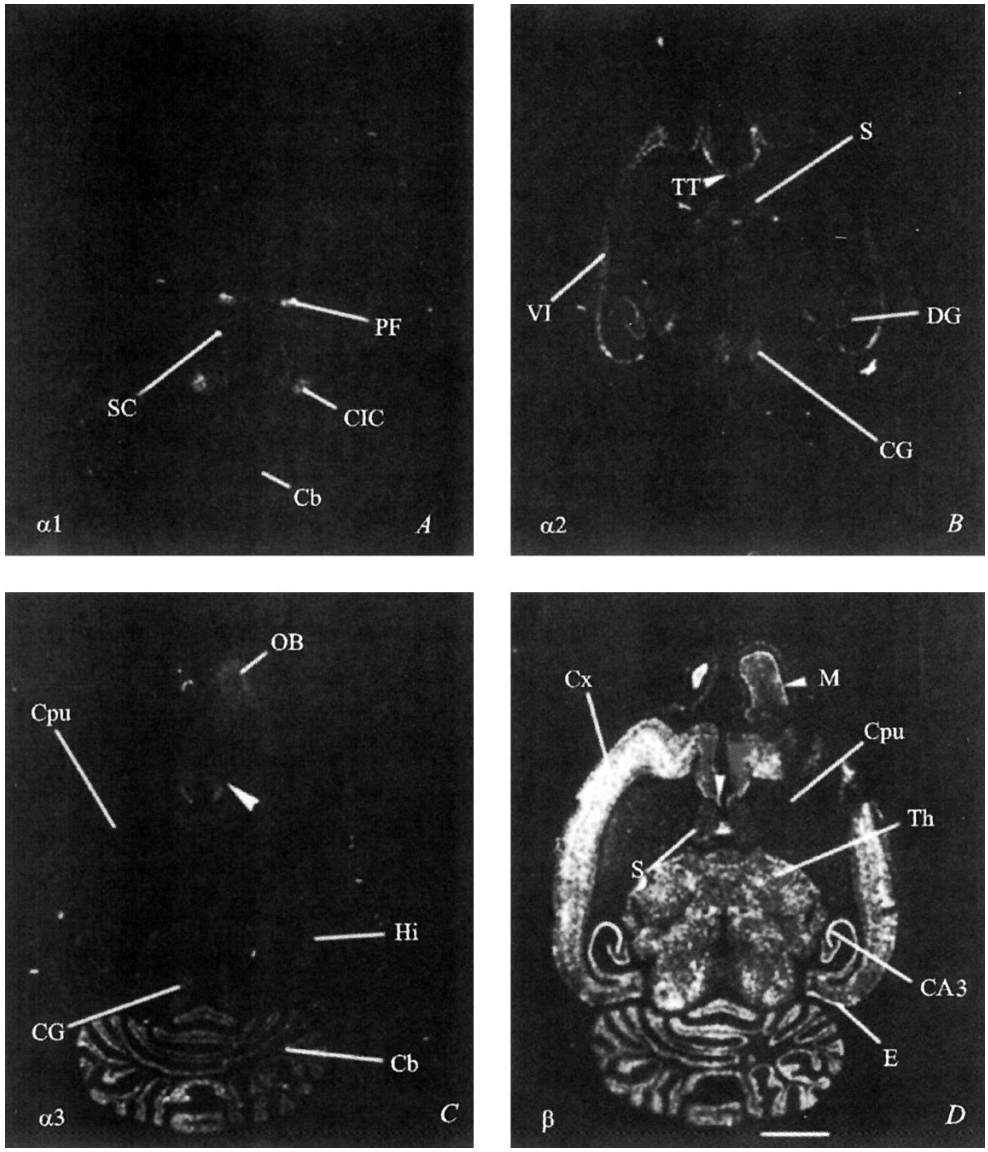


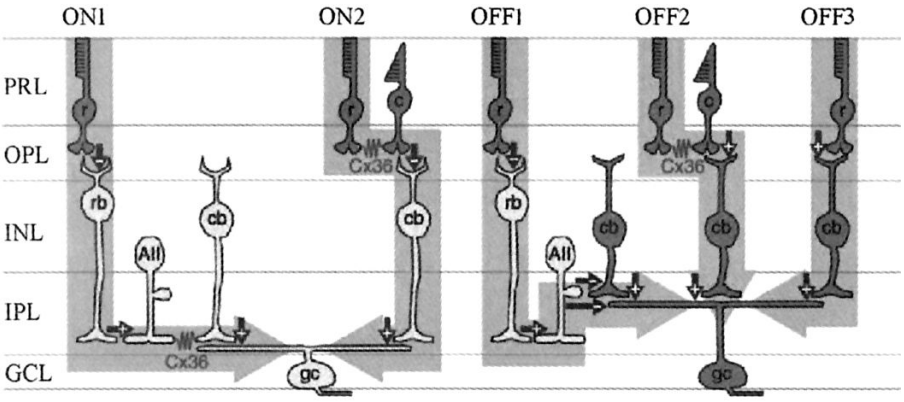
Fig. 1. Distribution of glycine receptors in the adult rat brain. In situ hybridization of glycine receptor mRNA in horizontal sections of the brain [109]. CA3 – hippocampal zone; Cb – cerebellum; CG – central gray matter; CIC – central nucleus of the inferior colliculus; Cpu – putamen; Cx – cortex; DG – dentate fascia; E – entorhinal cortex; Hi – hippocampus; OB – olfactory bulb; PF – parafascicular nucleus; S – nasal septum; Th – thalamus; TT – taenia tecta (spinal rudiment of hippocampus); VI – cerebral cortex layer VI.

– strychnine – which provided highly efficient and selective inhibition of the action of glycine in spinal neurons of anesthetized cats [33]. At the next stage in studies of glycine as a neurotransmitter, glycine was shown to be synthesized by neurons [145]. Conclusive evidence was obtained in experiments on rat spinal cord sections incubated with radioactive carbon-labeled glycine ([^{14}C]glycine). Stimulation of postsynaptic terminals was found to lead to glycine release, and this effect decreased significantly in calcium-free bathing medium [69, 70].

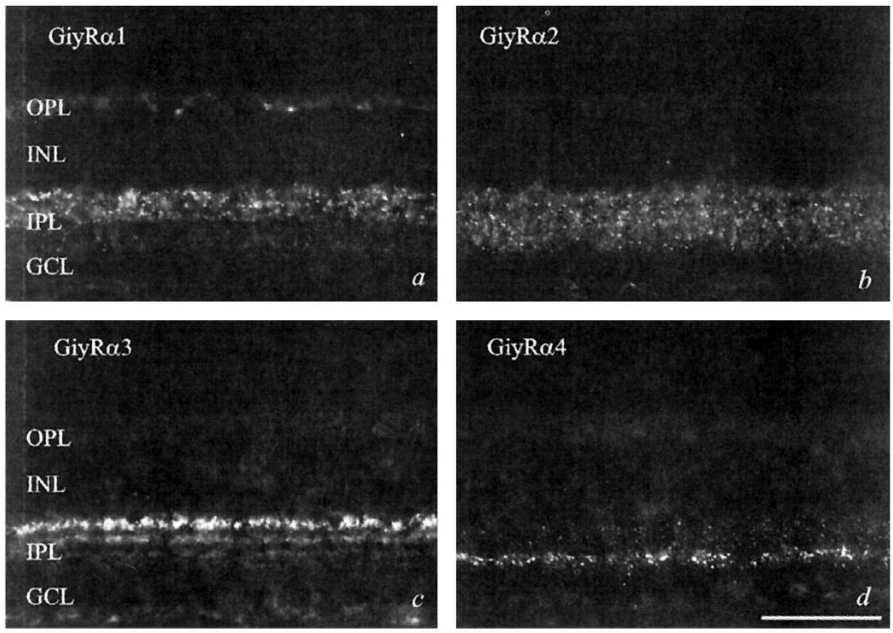
These results provided convincing evidence that glycine is a neurotransmitter in the nervous system, mainly located in the spinal cord with a hyperpolarizing action on postsynaptic cells. The discovery of the specific antagonist strychnine [33, 176] provided the basis for biochemical isolation of glycine receptors and became a starting point for active studies of their molecular properties and the physiological role of glycinergic transmission. A more detailed history of the development of concepts of the role of glycine as a neurotransmitter has been provided elsewhere [16].

The Distribution and Various Functions of Glycine Receptors in the Mammal Nervous System

Investigator shave long held the belief that glycine receptors are located mainly in the spinal cord and brainstem,



A. Photoreceptor signal pathways



B. α subunits in the retina

Fig. 2. Distribution of glycine receptor subunits in the mammalian retina. *A*) Spinal pathways of the retina. Rods are connected with ON ganglion cells via two tracts(ON1 and ON2) and with OFF ganglion cells by three (OFF1–OFF3). ON cells depolarize in response to light, while OFF cells depolarize in the dark. Glutamate is the main neurotransmitter in all the synapses of these pathways except synapses between AII-amacrine cells, which release glycine and induce hyperpolarization of OFF cone bipolar cells and OFF ganglion cells. r – rods; c – cones; rb – cone bipolar cells; cb – containing bipolar cells; AII – amacrine cells; gc – ganglion cells; PRL – photoreceptor layer; OPL outer plexiform layer; INL – inner nuclear layer; IPL – inner plexiform layer; GCL – ganglion cell layer (from [39]). *B*) Distribution of glycine receptor subtypes in the mouse retina: *a*) high immunoreactivity of α1 subunits at the surface of the inner plexiform layer; *b*) immunofluorescence of α2 subunits in the inner plexiform layer; *c*) expression of α3 subunits in the inner plexiform layer; *d*) immunoreactivity of α4 subunits in the retina (from [165]).

though recent decades have shown them to be present in many other areas of the central nervous system: the auditory and vestibular nuclei [46, 92], retina [163, 180], hippocampus [17, 35, 170], forebrain [101], dentate fascia [26], amygdaloid body [114, 115], hypothalamus, nucleus accumbens, substantia nigra [111], cerebellar cortex, and other parts of the brain [7, 35, 109] (Fig. 1).

In the retina, different glycine receptor subtypes are located in bipolar, amacrine, and ganglion cells, forming a quite complex and highly efficient functional system [61, 64, 163, 165, 166]. The complexity of the activation cascade in ganglion cells, which are responsible for the perception of light and darkness, can be assessed from the simplified scheme of retinal signal pathways presented in Fig. 2, *A*.

The dendrites of glycinergic cells have small territories and mediate local interactions between different sublayers of the inner retinal (plexiform) layer (between the ON and OFF sublayers) [72]. The highest concentration of clusters of α1 subunits is seen in the OFF sublayer of the inner plexiform layer, which corresponds to synapses between AII amacrine cells and the endings of OFF bipolar cell axons [141]. The retina is characterized by a high concentration and uniform distribution of α2 subunits, which are expressed mainly by amacrine and ganglion cells [62]. Synapses, whose membranes bear glycine receptor α3 subunits, form four sublayers in the inner plexiform layer. α3 glycine receptors are present in the dendrite membranes of AII amacrine cells [61] and type A ganglion cells [108]. Synapses including α4 subunits form a narrow immunoreactive strip in the inner plexiform layer [64] (see Fig. 2 *C*).

Thus, the characteristic feature of the distribution of glycine receptor subunits in the retina is the high level of specificity in relation to cell types and retinal layers, which is required for clear functioning of a complex neuronal system of this sensory organ.

Glycine receptors and the development of the nervous system. During embryonic development, extrasynaptic glycine α2 receptors are dominant in the nervous system [9, 109, 167]. The characteristics of glycinergic transmission in the developing brain are determined by the typical high intracellular chloride concentration, such that GABA and glycine have depolarizing actions on neuron membranes [169]. Glycine-induced depolarization of embryonic neurons can lead to the opening of voltage-dependent calcium channels and increases in intracellular calcium concentrations [137], triggering various cascades of intracellular reactions, particularly stimulation of synaptogenesis [86]. Studies of the role of glycine receptors in the formation of the nervous system have shown that local increases in calcium concentrations in postsynaptic terminals lead to accumulation of the armature protein gephyrin on the cytoplasmic side of the plasma membrane. Gephyrin, which has a binding site for the glycine receptor β subunit, binding laterally diffusing β subunit-containing heteromeric receptors, thus promoting their clustering and the formation of functional glycinergic synapses [86].

Many neurotransmitters function as signal molecules regulating neuron migration processes during embryogenesis [66]. Glycine is no exception. Activation of α2 glycine receptors in embryonic cortical neurons evokes increases in the intracellular calcium concentration. This leads to modulation of the contractility of actomyosin complexes, thus supporting the migration of cortical interneurons during embryogenesis [6].

Receptor-activated ion channels are also involved in the development of other zones of the nervous system. Taurine, whose concentration in the brain during embryogenesis is quite high, activates production of rod photoreceptors. Activation can be blocked by strychnine and bicuculline, which are glycine and GABA antagonists. Furthermore, knockout of α2 glycine receptors produces a sharp decrease in the number of photoreceptors and increases in the numbers of other types of retinal cells [177]. These data indicate an important role for α2 glycine receptors during the formation of the retina in vertebrates.

Glycine receptors can also have a presynaptic location, regulating glutamatergic transmission. Activation of presynaptic glycine receptors in the brainstem and subsequent membrane depolarization increases the frequency of glutamate release from presynaptic terminals [156]. Glycine receptors are also located in the presynaptic terminals of hippocampal mossy fibers, where they are presumptively responsible for regulating glutamate release during the embryonic development of this type of fiber [91].

Glycine receptors in pain regulation. The intensity of signals transmitted by the spinal cord to the pain sensitivity center of the brain does not depend only on the intensity of the peripheral signal; the local neural network of excitatory and inhibitory interneurons also has a significant influence. Glycine α3 receptors play an important role in controlling pain sensitivity, which is formed in a center located in the dorsal horns of the spinal cord.

Prostaglandin E_2 (PGE_2) is a mediator of inflammation. It is released in response to tissue damage and the subsequent inflammatory process leads to the phosphorylation of glycine α3 receptors and their deactivation [178]. This results in the release of dorsal horn neurons from the inhibitory action of glycine and an increase in pain sensitivity. Inactivation of the gene encoding the α3 receptor (*Glra3*) does not produce either inhibition of glycine currents in the dorsal horns on the spinal cord or increases in pain sensitivity in response to PGE_2 [57].

Thus, the role of glycine receptors in the nervous system is far from restricted to controlling motor activity. Because of the specific distribution of their different subunits, glycine receptors are involved in visual perception, modulation of pain sensitivity, the migration of cortical neurons, and regulating the excitability of neural networks in different parts of the central nervous system.

Subunits. Glycine receptors were first isolated from the rat spinal cord using affinity chromatography and the highly selective glycine receptor antagonist strychnine [130]. Three proteins with molecular weights of 49, 58, and 93 kD were detected. The first two were identified as glycine receptor α1 and β subunits. Subsequent experiments demonstrated that the 93-kD protein displayed specific binding with the β subunit and tubulin, thus playing an important role in glycine receptor clustering [87]. This was named gephyrin.

Four subtypes of glycine receptor α subunit, which share 90% homology (α1, α2, α3, α4) [9, 54], were isolated from mammalian brains, along with a β subunit, which is 47% homologous with the α1 subunit [53]. An analogous set of subunits was characteristic of the nervous system of

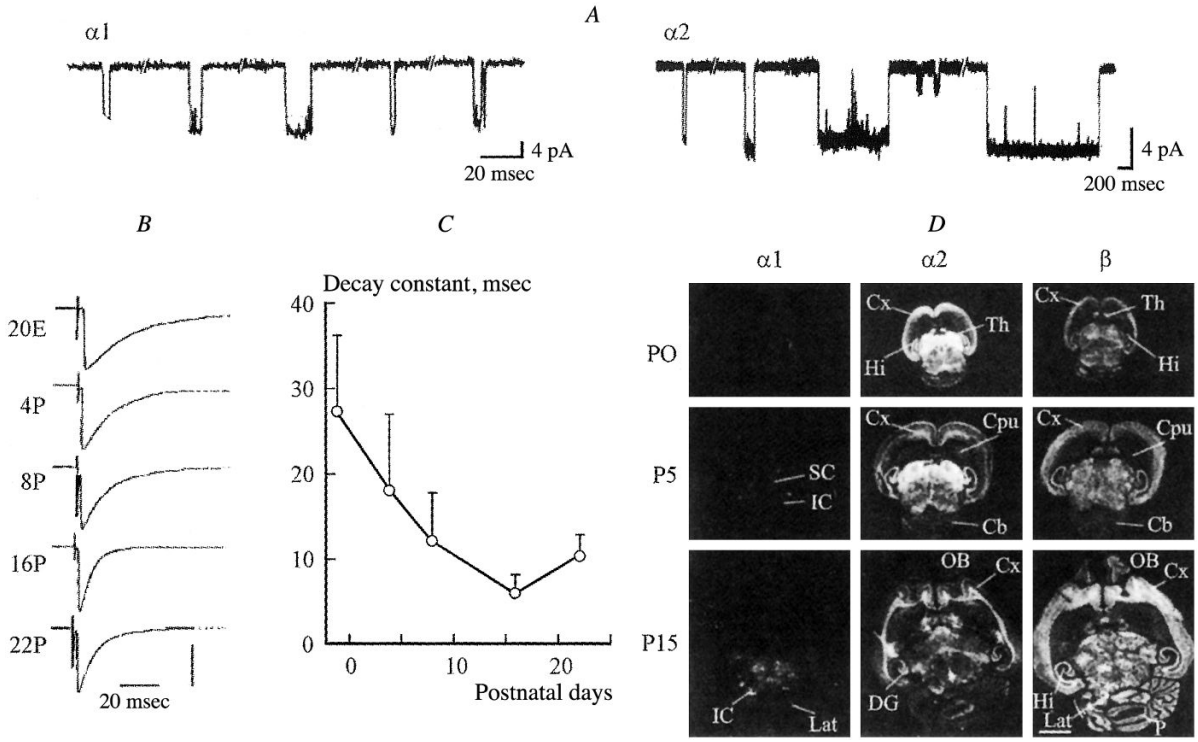


Fig. 3. Kinetics of glycine receptor α1 and α2 subunits and glycinergic synaptic currents during postnatal development. *A*) Currents through individual glycine channels recorded in the outside-in configuration. Expression in *Xenopus* oocytes [152]; *B*, *C*) glycinergic inhibitory postsynaptic currents (IPSC) of posterior horn neurons; *B*) averaged IPSC recorded at different points during embryonic (E) and postnatal (P) development. Calibration: 100 pA for E20 and P4; 140 pA for P16 and P22; 200 nA for P8. Holding potential: –83 mV; *C*) changes in IPSC decay kinetics during development. Averaged data from 11–13 neurons (from [152] with modifications); *D*) level of expression and distribution of glycine receptors in the rat brain during development. Sections at postnatal developmental stages P0, P5, and P15 are shown. Cb – cerebellum, CPu – caudate putamen; Cx – cortex; D – diencephalon; DG – dentate gyrus; Hi – hippocampus; IC – inferior colliculus; La – lateral amygdaloid nucleus; Lat – lateral cerebellar nucleus; M – midbrain; OB – olfactory bulb; P – Purkinje cell layer; S – septum; SC – superior colliculus; T – telencephalon; Th – thalamus; arrows show the inner layers of the infralimbic cortex. Scale: 3.4 mm (from [109]).

the zebrafish *Danio rerio* [37, 41, 75, 76]. Glycine receptor subunits were detected in all parts of the central nervous system in zebrafish and their distribution was generally similar to that in mammals [74].

α1, α2, and α3 subunits can form functional homomeric and heteromeric (in combination with β subunits) receptors, while β subunits are unable to form functional homomeric receptors [15, 52]. An important property of the β subunit was the presence of a gephyrin binding site in the cytoplasmic domain – gephyrin being an armature protein involved in the formation of synaptic clusters [85, 88, 89, 116].

The properties of homomeric and heteromeric receptors are somewhat different. The conductivity of homomeric receptors has been shown to be about double that of heteromeric receptors (80–100 and 40–50 pS respectively) [15]. Furthermore, homomeric receptors are highly sensitive to the blocking action of picrotoxin (effective dose 10 μM), while the sensitivity of heteromeric α and β receptors to this blocker is 100–500 times lower [105, 133]. This results from the differences in the amino-acid composition of the domains forming the ion-conducting channels [172, 179].

It should be noted that homomeric glycine receptors formed by different subunits display different kinetics: the duration of the opening state of ion channels formed by α1 subunits is significantly shorter than that of channels formed by α2 subunits (Fig. 2, *A*) [152]. This property has important physiological value, as changes in the levels of expression of these subunits during postnatal development (decreases for α2 and increases for α1) (Fig. 3, *D*) affect the kinetics of glycinergic synaptic currents (Fig. 3, *B*, *C*).

Studies using an in situ hybridization method demonstrated that each subunit was characterized by a specific location in the spinal cord, brainstem, and particular areas of the brain [109]. The α1 subunit is dominant in the central nervous system of adult mammals. Its expression level is high in the brainstem nuclei, spinal cord, thalamus, and hypothalamus. The predominantly synaptic localization of α1 subunits is evidence for the formation of heteromeric α/β receptors, as the β receptor is responsible for synaptic anchoring of glycine receptors [10, 83, 116].

During embryonic development, the α2 subunit is the most widespread glycine receptor subunit in the central ner-

vous system [9, 109, 167] (Fig. 3, *D*). The high sensitivity of most embryonic α2 receptors to picrotoxin [92, 110, 164] and their high permeability [110, 152] provide evidence of that they are mostly homomeric. Located outside of synapses, they probably support tonic transmission, mediating nonvesicular glycine release [45]. However, at three weeks after birth, the α2 subunit expression level decreases sharply, and its distribution becomes synaptic in nature (Fig. 3, *D*). In the adult body, most glycinergic receptors are formed by α1-subunits, α2 subunits being seen only in the retina [161], the nuclei of the olfactory analyzer, and some brain areas [109]. It should be noted that this receptor is not critically required for the normal functioning of the central nervous system, as knockout of the α2 glycine receptor subunit does not lead to any changes in the behavioral reactions of the phenotype [107].

Expression levels of α3 and α1 subunits have similar dynamics, though the concentration of α3 subunits is much lower than that of α1 subunits [109]. The locations of α3 subunits were confirmed in several parts of the central nervous system, though the most detailed studies of their distribution were in the retina [61] and nociceptive neurons in layers I and II of the posterior horns of the spinal cord (colocation with gephyrin points to a synaptic localization) [57].

Glycine receptor α4 subunits have received little study. These subunits have been shown to be located in the spinal cord, dorsal ganglia, and sympathetic ganglia in chicks [58], and in the retina in mice [64].

α and β subunit coexpression leads to the formation of heterodimeric glycine-activated receptors. The stoichiometry of this receptor has long remained controversial and has thus far not been determined convincingly. For a period, the general view was that heteromeric glycine receptors had the composition 3α:2β [24, 94, 95]. Later studies using mutant and radioactively labeled α and β subunits showed that the 2α:3β combination is more likely [55]. The 2α:3β combination is also supported by experiments using scanning atomic force microscopy, with analysis of the number of specific antibodies binding to glycine receptors [173]. However, another recently published report using single-molecule tomography and stepwise photodecolorization suggested the combination 3α:2β [44]. Despite the fact that this study was the most convincing, strict evidence of the stoichiometry of heteromeric glycine receptors requires additional investigations.

As noted previously, one of the key properties of β subunits is their ability to bind the protein gephyrin, which is responsible for forming clusters of glycine and some GABA receptors in synaptic membranes [90]. This provides further support for the view that synaptic glycine receptors are heteromeric. In addition, the β subunit – or, more precisely, the M2 segment – is a determinant of picrotoxin resistance [133, 179].

Thus, the existence of five glycine subunit receptors – four α and one β – forming both homomeric and heteromeric receptors, has now been demonstrated. This variety of subunit compositions allows glycine receptors to take part in a wide spectrum of processes occurring in the nervous systems in vertebrates. As different receptor subtypes have different physiological characteristics, different localizations, and differences in expression levels during development, they can support the highly effective functioning of this specific system controlling neural networks in the body.

Structure. Glycine receptors are members of the cys-loop ionotropic receptor superfamily [119]. Results of studies in recent years provide evidence that cys-loop receptors are widely distributed among biological organisms – from single-celled organisms through mollusks and insects to mammals [82, 154]. In the mammal nervous system, the cys-loop receptor superfamily includes nicotine, acetylcholine, serotonin (5-HT_3), glycine, and $GABA_A$ receptors. The ionotropic channels of these receptors are homo- and heteromeric assemblies of five protein subunits forming the central pore of the channel [80, 157, 158]. They all have structural features in common: a large extracellular N-terminal domain consisting of more than 200 amino acids, four transmembrane domains (TM1–TM4) joining loops of different lengths (the cytoplasmic loops making up TM3 and TM4 consist of almost a hundred amino acid residues) and a short extracellular C-terminal (Fig. 4, *A*). The N-terminal domain of each subunit has a conserved region of 13 amino acids, delimited by cysteine. Covalently linked, the cysteines form a cys-loop between the ligand-binding and transmembrane domains of the protein subunit.

Understanding of the molecular structure of cys-loop channels has increased significantly, due to several important results obtained in recent years. First was the discovery of acetylcholine-binding protein (AChBP) from the freshwater mollusk *Lymnaea stagnalis* and solution of its crystal structure with a resolution of 2.7 Å [21]. Although this protein is not associated with an ion channel, it is a pentamer with a structure similar to that of the extracellular macromolecular clusters formed by the N-terminals of nAChR and glycine receptors. This allows the structure of AChBP to be used for homologous modeling of the receptor centers of the whole family of cys-loop channels. Second was the identification of the structure of the complete acetylcholine channel from *Torpedo* with a resolution of 4 Å [158]. This clarified the topology of cys-loop channels and determined the positions of many of the atoms and side chains. Third was the high-resolution (1.94 Å) solution of the crystal structure of the extracellular domain of the α1 subunit of the mouse nAChR bound to α-bungarotoxin [38]. This clarified many of the details of the molecular organization of this region of the AChR. Fourth was the determination of the crystal structure of two prokaryotic channels with homology to cys-loop receptors. One channel, from the bacterium *Erwinia chrysanthemi* (ELIC), was crystallized in the closed state, allowing its structure to be solved with a resolution of 3.3 Å [67]. The crystal structure of another channel (GLIC), from the bacterium *Gleobaceter violaceus*, was solved in the

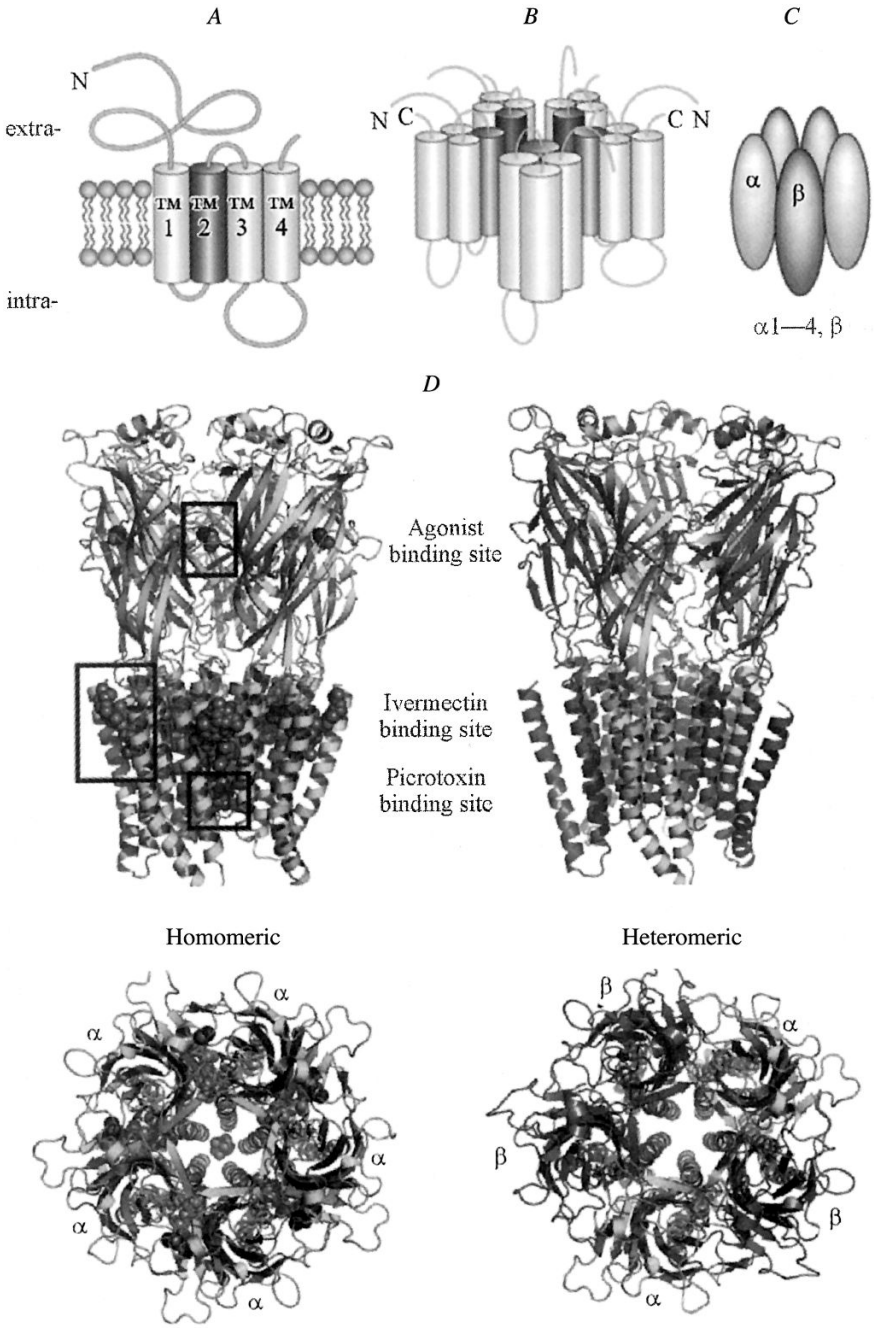


Fig. 4. Structural organization of ligand-activated cys-loop channels using the glycine receptor as an example. *A*) Glycine receptor subunits consisting of the long outer N-terminal domain, four transmembrane segments (TM1–TM4), the long intracellular loop and the extracellular C-terminal; *B*) diagram showing the organization of the ion channel formed by the TM2 domains; *C*) the glycine receptor is a pentamer consisting of α and β subunits (from [123] with modifications); *D*) structure of homomeric α1 and heteromeric α1/β glycine receptors (from [45]), though the stoichiometry requires clarification (see [44]).

open state with a resolution of 2.9 Å [13]. This allowed the two states of the channel to be compared and concepts of the molecular details of changes in conformation leading to opening of the ion pore to be understood. We will dwell in a little more detail on the architectural-functional organization of these proteins.

The end of the N-terminal domain of each subunit is an α helix, which is followed by a series of 10 β-pleated structures (β sheets). β-Sheets form two hydrophobic zones, forming an agonist binding site. The conserved cys loop, which is part of this domain, and the loop connecting β-sheets 2 and 3 protrude towards the transmembrane do-

mains and are probably responsible for transmitting information from the ligand-binding site to the channel-activating gates in the pore [21, 31, 168]. Formation of the ligand-binding site involves the central parts of the N-terminal domains of two neighboring subunits, namely the A–C loop of the main (or "+") subunit and the D–F loop of the complementary (or "–") subunit [32]. These loops are linker regions connecting the β-pleated structures. An interaction takes place between the amino group of the ligand and phenylalanine 159 of the B loop (tryptophan 149 in the case of the nACh receptor) at the moment of binding of agonist with receptor, i.e., a cation-π interaction [132]. This leads to fixation of agonist at its binding location, along with displacement of the cys loop and the loops joining β-pleat structures 1 and 2 towards the transmembrane domains and their interaction with the TM2-TM3 linker [21, 120]. This displacement can evoke conformational changes in other domains of the receptor, particularly the TM2 domain, which forms the ion channel.

The four transmembrane domains of glycine receptor subunits are α-helixes penetrating the bilipid membrane. The five subunits making up the receptor are oriented such that their TM2 domains form a nonselective channel and their TM1, TM3, and TM4 domains surround and interact with lipids of the cytoplasmic membrane [120]. The center of each TM2 domain of the α helix is flexed, such that different parts of the channel have different widths. The flexure zone includes two hydrophobic rings formed by 9'-leucines and 13'-valines, which probably form the main channel gates [120]. According to Unwin's model, ion channel opening involves rotation of the TM2 domains, inducing destabilization of hydrophobic interactions in the channel gates. This model is based on electron microscopic analysis of images of nACh channels from the electric organ of the marine skate *Torpedo*, which yielded the structure of the receptors with a resolution of 4 Å [157, 158]. Analysis of the crystal structure of prokaryotic ion channels (analogous to cys loops channels in eukaryotes) GLIC [13] and ELIC [67, 68] suggested another model, proposing that during receptor activation, the TM2-TM3 loop shifts inwards, dragging the TM2 domain with it. This leads to concordant movement of the TM3 and TM2 domains, which is accompanied by changes in their tilting and an increase in pore diameter from 2 to 12 Å [31]. Acidic residues in the lower ring of glutamates of the TM2 domain appear within the channel, dynamically forming its cation selectivity. However, this model is based on comparison of the structures of different channels – ELIC in the closed and GLIC in the open state; the model requires further clarification. The architecture and molecular models of the opening of cys-loop channels have been presented in reviews [20, 31, 168].

Molecular Pharmacology of Glycine Receptors

Agonists. The pharmacological diversity of glycine receptor agonists is relatively small. The main agonists are amino acids, which have different efficacies in the general sequence glycine > β-alanine > taurine [143]. This ratio applies to all glycine receptor subunits. Studies on cultures of organotypical hippocampal slices showed that modulation of specific β-alanine and taurine transporter proteins leads to changes in the tonic activity of glycine receptors, suggesting an inhibitory role for these amino acids in hippocampal functioning [121]. GABA is also a very weak agonist [37, 48]. As GABA and glycine can be colocated in vesicles in the presynaptic terminals of glycinergic synapses [162], it is possible that GABA plays some particular role in regulating the functional activity of these synapses.

Antagonists. The best studied and most widely used glycine receptor antagonists are strychnine and picrotoxin.

Strychnine is a classical competitive glycine receptor inhibitor, operating independently of subunit composition. Exposure of living organisms to strychnine induces impairments to motor functions, increased muscle tone, and hyperactivation of sensory, visual, and acoustic perception. At high doses, strychnine produces convulsions and death [53, 176]. Most studies support the view that the strychnine binding site is the same as the glycine binding site or extensively overlaps it. Mutations in the B, C, D, and E loops of the extracellular domain forming the glycine binding site (G160E, Y161A, K200A, Y202A, F63A, and R131A) decrease strychnine sensitivity [55].

It should be noted that as a competitive antagonist of glycine receptors blocking their activity at nanomolar concentrations, strychnine also suppresses the activity of other ionotropic cys-loop receptors. At micromolar concentrations, it is also a noncompetitive blocker of acetylcholine receptors at neuromuscular synapses [49, 93] and is a highly effective competitive antagonist of neuronal homomeric nicotinic receptors (α7 and α9/10) [8, 113].

Picrotoxin is an alkaloid which includes two active substances – picrotoxinin and picrotin – known for their abilities to inhibit GABA and glycine receptors. The mechanism of action of picrotoxinin was long controversial – is it an allosteric antagonist or a channel blocker? As picrotoxin sensitivity is determined by the amino acid residues located in TM2 [133], blockade of the glycine receptor channel seems the more likely. However, studies using mutant and chimeric receptors demonstrated that the mechanism of action of picrotoxin is more complex than classical blockade of the ion channel and suggests an allosteric interaction site [63, 105, 127]. Theoretical modeling showed that the picrotin and picrotoxinin binding site is formed by amino acids in the 2' and 6' positions of the TM2 α subunit of the glycine receptor [17]. Experiments on receptors with amino acid point mutations supported this hypothesis. Studies showed that picrotin and picrotoxinin form hydrogen bonds with threonine in the 6' position. The chemical bond between the amino acid and picrotoxin at the 2' position does not play a decisive role; coordination of the modulator depends on the size of the amino acid. α1 homomeric glycine receptors, with glycine in the 2' position, are more sensitive

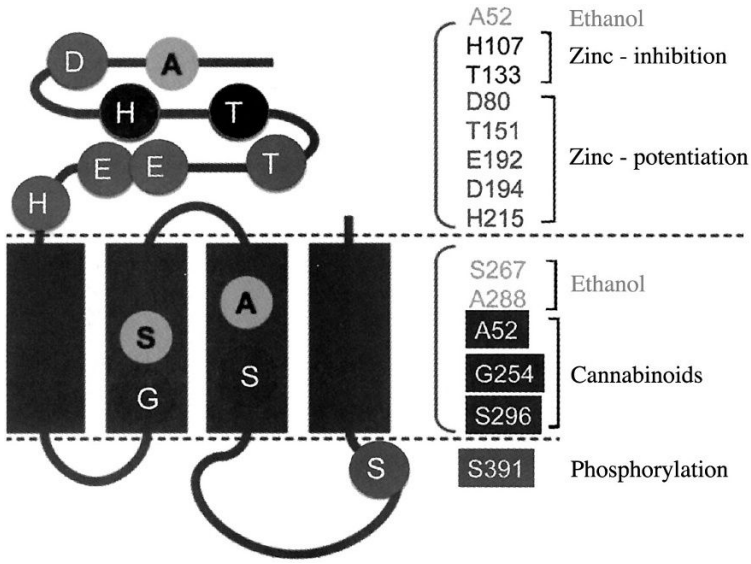


Fig. 5. Amino acids responsible for allosteric modulation of the glycine receptor. Mutations for the α1 subunit are shown. The dotted line shows the position of the plasma membrane.

to picrotoxin than α2 and α3 homomeric receptors, which have a larger amino acid in the 2' position [63, 172].

It can therefore be suggested that picrotoxin blocks glycine receptors, acting both as a blocker of the ion-conducting pathway and as an allosteric antagonist. More detailed information on the actions of antagonists has been reviewed [99, 107, 108].

Modulation of Glycine Receptors

Glycine receptor activity can be regulated by interaction of modulators with different molecular domains, both extracellular and intracellular. The pharmacological diversity of glycine receptor modulators is quite extensive, from ions to complex chemical compounds. These include the divalent cations Ca^{2+} and Zn^{2+}, as well as allosteric modulators such as anesthetics, alcohols, and endocannabinoids. Glycine receptor functions are also modulated by phosphorylation, RNA editing, and, perhaps, G proteins. We will briefly discuss only a few points, noting that more detailed information has been published in reviews [19, 42, 170, 174, 181].

Zinc. Zinc (Zn^{2+}) is an important ion in the central nervous systems of multicellular organisms. Free Zn^{2+} concentrations vary from 100 to 200 nM in the cerebrospinal fluid to more than 200 mM in specific synaptic zones [28].

Zn^{2+} cations are colocated with glycine in synaptic vesicles [11, 36, 129] and are released from presynaptic terminals via a calcium-dependent mechanism [71]. The effect of Zn^{2+} on glycine receptor activity is biphasic: low concentrations (less than 10 nM) potentiate glycinergic currents, while higher concentrations inhibit them [12, 41, 59, 98].

Elucidation of the molecular mechanisms of the biphasic action of Zn^{2+} is physiologically important, as high concentrations can make a significant contribution to increasing epileptogenic activity and neuron death in ischemia, and are also involved in the development of neurodegenerative processes [28, 43], while low concentrations, enhancing the inhibitory activity of glycinergic receptors, can protect neurons from hyperarousal and glutamate-induced cell death [25, 27].

Mutational analysis identified the functional Zn^{2+} binding sites with glycine receptors and the amino acids responsible for Zn^{2+}-induced potentiation or inhibition. Amino acids D80 and D194, located at the N-terminal of the α1 subunit (Fig. 5), have been shown to be key determinants of the potentiating action of zinc. In addition, important roles in the control of Zn^{2+}-dependent potentiation are also played by residues E192 and H215, as well as T151, located in the cys loop [99] (Fig. 5). The inhibitory action of Zn^{2+} appears to arise as a result of interaction of ions with residues H107, H109, and T133 of the N-terminal domain of α1 subunits (Fig. 5), N114 of α2, and N107 of α3. This forms ion bridges between two of the receptor subunits, preventing changes in the conformation of the subunits and opening of the channel [59, 97, 104, 118].

Calcium. Divalent calcium ions (Ca^{2+}) also modulate the operation of glycine-activated channels. Increases in $[Ca^{2+}]_i$ have been shown to lead to increases in the duration of their functioning, with resultant increases in the amplitudes of integral responses to application of agonist [47, 124]. Ca^{2+}-dependent potentiation has been described in experiments on spinal cord neurons [18, 47], bipolar cells in retinal sections, and motoneurons in the nucleus of the sublingual nerve in brainstem sections [124]. This has three important properties: a) the effect develops quickly, in less than 100 msec; b) increases in intracellular Ca^{2+} concentrations lead to increases in the effectiveness of the action of glycine;

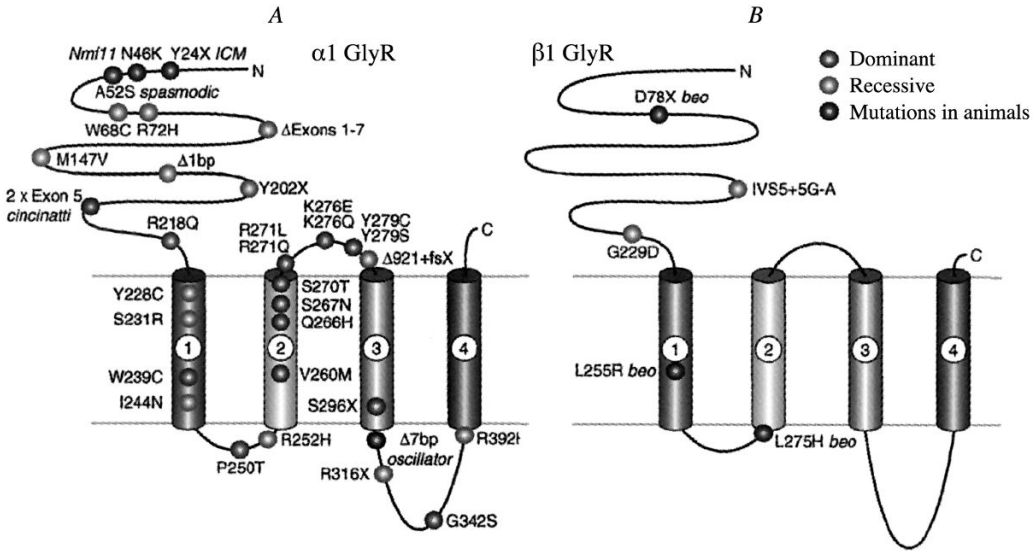


Fig. 6. Mutations of the glycine receptor inducing hyperekplexia. *A*) Glycine receptor α1 subunit; *B*) glycine receptor β subunit.

c) Ca^{2+} modulates channel operation not directly, but via a cytoplasmic mediator, perhaps a Ca^{2+}-binding protein [23].

Endocannabinoids constitute a family of endogenously produced lipid mediators, arachidonic acid derivatives, which modulate many physiological functions [81, 131]. Amides of arachidonic acid with ethanolamine (anandamides) [40] and 2-arachidonoylglycerol (2-AG) [151] are very widespread in the nervous system. Endocannabinoids have direct and retrograde actions on glycinergic synapses. Initial studies showed that endocannabinoids released via a Ca^{2+}-dependent mechanism from presynaptic terminals inhibit glycinergic transmission by a retrograde mechanism, i.e., by diffusion to presynaptic terminals and interaction with cannabinoid receptors (CB1) in the membranes of presynaptic terminals. A G protein-induced decrease in calcium influx into presynaptic terminals results in reductions in the release of glycine-containing vesicles and the amplitude of glycinergic synaptic currents [42, 124]. Studies have also demonstrated that endocannabinoids can also interact directly with glycine receptors, inducing potentiation [65] or inhibition of their activity and acceleration of desensitization [102, 103]. Most of the amino acid residues identified as determining endocannibinoid binding with glycine receptors are located in the second intracellular loop, TM2, and between the intracellular loop and TM4 (Fig. 5) [174].

Alcohols are positive modulators of glycine receptors. Glycine receptor α1 and α2 subunits contain binding sites for *n*-alcohols, propofol, pentobarbitone, and volatile anesthetics, potentiating their activity [112]. Molecules consisting of 10–12 carbons have the clearest potentiating effects. Such clear boundaries provide evidence that there is a specific limit to the binding site. Site-directed mutagenesis of the α1-glycine receptor identified two amino acid residues which might be responsible for the interaction with alcohols – S267 in TM2 and A288 in TM3 (Fig. 5) [117]. However. Aguayo et al. proposed a different mechanism of action for alcohols, whereby potentiation of glycine receptors is induced by changes in the ability of protein kinases or G proteins to interact with the large intracellular loop of the receptor [140, 175].

Phosphorylation of glycine receptors plays an important role in modulating the operation of glycinergic synapses. The intracellular parts of glycine receptor subunits, especially the large cytoplasmic loop linking the TM3 and TM4 domains, include sites with specific interactions with protein kinases and which are dephosphorylated by phosphatases [138, 148]. Some studies have demonstrated decreases in glycine-activated currents on activation of protein kinase C [2, 153, 159]. Protein kinase C (PKC) has been shown to phosphorylate the S391 amino acid residue of the glycine receptor α1 subunit (Fig. 5) and to interact specifically with the surrounding amino acid residues [138]. Furthermore, intracellular perfusion of neurons with a phosphatase inhibitor evoked potentiation of responses to glycine [153]. However, other studies observed the opposite effect: activation of PKC evoked increases in glycine current amplitude [126, 144, 171]. These differences may be associated with analysis of different glycine receptor subtypes expressed in different parts of the brain and the use of different experimental models.

Similarly contradictory data have also been obtained for protein kinase A (PKA), activation of which in spinal cord neurons increased the amplitudes of glycine currents [149, 153, 159], thus increasing the probability of individual glycine-activated channels being in the open state [149]. Furthermore, the potentiating actions of PKC and PKA have

been shown to be mutually supplementary [56]. However, activation of PKA decreased the amplitudes of glycine-activated currents in substantia nigra neurons [77] and rapidly desensitizing currents in ventromedial hypothalamic neurons [1]. The latter study also demonstrated a PKA-induced decrease in the probability of activation of glycine receptors, which may be associated with acceleration of desensitization.

Phosphorylation can also lead to changes in the locations of glycine receptors. Thus, for example, activation of PKA and PKC in the retina decreased the amplitudes of glycine currents due to internalization of glycine receptors [160]. Furthermore, residue S403 was identified in the cytoplasmic domain of the β-subunit, phosphorylation of which by PKC decreases the affinity of the receptor for gephyrin. As gephyrin is responsible for retaining glycine receptors in the synapse, decreases in the affinity between them nay be the cause of lateral migration of receptors and decreases in the number of receptors in synapses [150]. More detailed studies are required to elucidate the role of phosphorylation in the functioning of glycine receptors and glycinergic synaptic transmission.

Thus, the actions of agonists, antagonists, and modulators on glycine receptors depend largely on receptor subunit composition. As receptors formed by different subunits have different functions and locations in the CNS, the search for specific modulator agents is of particular interest. This opens up the potential for more detailed study and treatment of diseases associated with impairments to the functions of particular glycine receptor subtypes.

Hyperekplexia – a Pathology Due to Impairments to the Functions of Glycine Receptors

Hyperekplexia is a disease in which an unexpected sound or light stimulus evokes convulsive grimaces, sharp retraction of the shoulders, hand gestures, jumping, and other uncontrollable movements. The unusual pathological "fright reactions" were first described in 1878 by the American neurologist George Berd (1839–1883), who observed a patient from the Franco-Canadian lumberjack community. He termed this "jumpers," or "jumping Frenchmen" [96]. Subsequent studies showed that hyperekplexia is an inherited disease phenotypically apparent as severely exaggerated reactions to unexpected acoustic and tactile stimuli [3]. The symptoms of hyperekplexia include hypertonia and muscle rigidity, which may lead to uncontrollable falling. Cases of death due to hyperekplexia-associated respiratory collapse have also been described [22, 122, 128]. Diseases with analogous symptoms have also been seen in a number of mammal species: mice, rats, and cows [60].

The causes of this rare inherited condition were not investigated until 1993, when a mutation in the glycine receptor α1 subunit provoking this type of pathology was found [147]. Genetic studies showed that patients with hyperekplexia have a characteristic mutation in the α1 subunit gene (*GLRA1*), located in chromosome region 5q33.1, leading to substitution of arginine R271 for L or Q [146, 147]. Other mutations in *GLRA1* triggering the development of hyperekplexia have been found in recent years: autosomal dominant, such as Y279C, K276E, Q266H, and P250T [60, 142], the main consequence of which is a decrease in receptor agonist sensitivity due to impairment to channel opening, and autosomal recessive mutations, such as I244N [134] and S231R [73], which hinder the processes of transcription/translation and insertion of the receptor into the cell membrane [29, 30] (Fig. 6).

Impairments to glycinergic transmission increasing the total level of arousal of spinal cord motoneurons may be both pre- and postsynaptic in nature [60, 106]. Mutations of several proteins involved in the process of glycinergic transmission may be the cause of the development of the hyperekplexic phenotype. The glycine receptor β subunit [136] and the GlyT2 glycine transporter, which are located in the presynaptic membrane [51], are the two proteins whose mutations are the most important (apart from the α1 subunit) in the development of hyperekplexia. The *SLC6A5* gene, which encodes GlyT2, has mutations altering its subcellular location, resulting in decreases in the activity of the glycine transporter in returning glycine from the synaptic cleft to the presynaptic ending [135]. Increased excitability may also result from changes in the amino acid sequence of the VIAAT transporter protein, which is responsible for pumping glycine and GABA into presynaptic vesicles [50, 139].

Mutations in the β subunit are third in the list of causes of hyperekplexia. In mice with this disease, a recessive mutation due to insertion of the retrotransposon LINE-1 into an intron produces a strong reduction in β subunit expression in neurons [84, 125]. Recent studies have demonstrated that the W310C mutation in β subunits impairs intramembrane packing of α-helixes, leading to significant difficulty in inserting heteromeric receptors into the postsynaptic membrane [78]. Furthermore, mutations in residues G229D and M177R, located in the immediate vicinity of the agonist binding site, lead to decreases in the sensitivity of the receptor for glycine, with the result that there is a decrease in the effectiveness of glycinergic synapse functioning [78].

Thus, three main types of genetic impairments to glycinergic transmission cause hyperekplexia in humans and similar diseases in animals. Firstly, mutations in the α and β subunits leading to decreases in the permeability of individual channels and decreases in glycine sensitivity; secondly, mutations leading to decreases in the expression of membrane glycinergic receptors and their localization in synaptic zones; and thirdly, mutations in neurotransmitter transporters leading to decreases in glycine accumulation in presynaptic vesicles [60].

Conclusions

Glycine receptors, which are members of the cys-loop ionotropic receptor family, are a key element in the motor neuron inhibitory control system providing for smooth movements with the required accuracy. However, because of the variety of subunit compositions and their wide distribu-

tion in the nervous system, glycine receptors also play an important role in the operation of other body systems: formation of visual images, sensations of pain, and neurogenesis. Glycine receptors are potential targets for the development of new pharmacological agents, including muscle relaxants, analgesics, and anti-inflammatories. All these points make studies of the characteristics of their molecular structure, functioning, and potential for modulation of particular interest in contemporary neurobiology.

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RESULTS

article II

Selective potentiation of alpha1 glycine receptors by ginkgolic acid

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Selective potentiation of alpha 1 glycine receptors by ginkgolic acid

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Glycine receptors (GlyRs) belong to the superfamily of pentameric *cys*-loop receptor-operated channels and are involved in numerous physiological functions, including movement, vision, and pain. In search for compounds performing subunit-specific modulation of GlyRs we studied action of ginkgolic acid, an abundant *Ginkgo biloba* product. Using patch-clamp recordings, we analyzed the effects of ginkgolic acid in concentrations from 30 nM to 25 μM on α1–α3 and α1/β, α2/β configurations of GlyR and on $GABA_A$Rs expressed in cultured CHO-K1 cells and mouse neuroblastoma (N2a) cells. Ginkgolic acid caused an increase in the amplitude of currents mediated by homomeric α1 and heteromeric α1/β GlyRs and provoked a left-shift of the concentration-dependent curves for glycine. Even at high concentrations (10–25 μM) ginkgolic acid was not able to augment ionic currents mediated by α2, α2/β, and α3 GlyRs, or by $GABA_A$R consisting of α1/β2/γ2 subunits. Mutation of three residues (T59A/A261G/A303S) in the α2 GlyR subunit to the corresponding ones from the α1 converted the action of ginkgolic acid to potentiation with a distinct decrease in EC_{50} for glycine, suggesting an important role for these residues in modulation by ginkgolic acid. Our results suggest that ginkgolic acid is a novel selective enhancer of α1 GlyRs.

Keywords: ligand-gated channels, glycine receptor, ion currents, whole-cell recording, patch clamp, CHO cells

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INTRODUCTION

Anion-selective GlyR channels provide the inhibitory drive in the vertebrate spinal cord, brainstem, retina and some other parts of central and peripheral nervous system (Malosio et al., 1991; Lynch, 2004; Betz and Laube, 2006). Together with cation-selective nicotinic acetylcholine receptors and serotonin type 3 receptors, as well as with anion-selective $GABA_A$ and $GABA_C$ receptors, they belong to the superfamily of pentameric *cys*-loop receptor-operated channels (Smart and Paoletti, 2012; Lynagh and Pless, 2014).

The family of GlyRs is relatively small. Molecular cloning has enabled identification of four alpha subunits (α1–α4) and one beta (β) subunit with several splice variants (Laube et al., 2002; Lynch, 2004; Oertel et al., 2007; Dutertre et al., 2012). Functional GlyRs can be either homomeric, formed from five α subunits, or heteromeric, formed from α and β subunits with still not definitively determined stoichiometry, suggesting either 3α/2β (Langosch et al., 1988, 1990; Burzomato et al., 2003; Durisic et al., 2012) or 2α/3β (Grudzinska et al., 2005; Yang et al., 2012)

Abbreviations: CB receptor, cannabinoid receptor; cDNA, complementary deoxyribonucleic acid; CHO cells, Chinese hamster ovary cells; DMSO, dimethyl sulfoxide; EC_{50}, half-maximal concentration; GABA, γ-aminobutyric acid; $GABA_A$R, γ-aminobutyric acid receptor, subtype A; GlyR, glycine receptor; I_{gly}, glycine-induced current; THC, Δ^9-tetrahydrocannabiol; TM, transmembrane domain; WT, wild type.

composition. Alpha subunits are highly homologous, with primary structures displaying 80–90% amino acid sequence identity (Lynch, 2004); however, they differ in their kinetic properties (Takahashi et al., 1992; Singer and Berger, 1999), temporal and regional expression (Malosio et al., 1991; Betz and Laube, 2006; Heinze et al., 2007; Dlugaiczyk et al., 2008; Aroeira et al., 2011) and physiological functions (Harvey et al., 2004; Villmann et al., 2009; Dutertre et al., 2012).

Due to their diverse distribution and functions, GlyRs are potential pharmacological targets for muscle relaxant, analgesic and anti-inflammatory drugs (Webb and Lynch, 2007; Zeilhofer et al., 2012), however, only a few compounds with preferable subunit specificity are known (Yang et al., 2008; Lynch, 2009; Balansa et al., 2013).

Several studies have demonstrated that ginkgolides, extracted from the leaves of the *Ginkgo biloba* tree, are specific and potent blockers of GlyR channels (Kondratskaya et al., 2002, 2004; Hawthorne et al., 2006). *Ginkgo biloba* extract contains three groups of active substances: (i) flavonoid glycosides including quercetin and rutin; (ii) terpene trilactones (ginkgolide A, B, C, J, and bilobalide) (Ude et al., 2013); and (iii) ginkgolic acids, which are predominantly contained in the nutshells and leaves (Jaggy and Koch, 1997; Fuzzati et al., 2003).

It has been shown that ginkgolide B displays subtype-selectivity, albeit weak, with about 5- and 3-fold preferences for α1 vs. α2 and α3 GlyR subunits, respectively (Kondratskaya et al., 2005). Moreover, the inhibitory ability of ginkgolide B was 5- to 100-fold higher on heteromeric than on homomeric GlyRs, i.e., incorporation of the β subunit substantially increased the antagonism of this compound (Kondratskaya et al., 2005). Similarly to that, the other terpen trialactones from *Ginkgo biloba* extract (ginkgolide A, C, and bilobalide) block GlyR channels though with weak subunit discrimination (Hawthorne et al., 2006; Lynch, 2009). Quercetin, belonging to the flavanoid group, also inhibits α1 GlyR activity (Lee et al., 2008) in a non-competitive manner, with an IC_{50} of about 45 μM (Raafat et al., 2010). This compound also inhibits $GABA_A$ and $GABA_C$ receptors (Kim et al., 2015), causing seizures in animal models (Nassiri-Asl et al., 2014).

The functional properties of ginkgolic acid have attracted much less attention. *Ginkgo biloba* extract, used in medicine, is cleared of ginkgolic acid because of the latter's possible side effects (Ahlemeyer et al., 2001; Hecker et al., 2002). However, it has been shown that as long as the carboxylic acid group is intact, either in free or in conjugated forms, no allergic manifestations are detected (Satyan et al., 1998). Moreover, it has been suggested that intact carboxylic acid groups are the bioactive components of the lipophilic extract of *Ginkgo biloba* leaves with antidepressant and antistress activities (Kalkunte et al., 2007). As, in contrast to ginkgolides, the effects of ginkgolic acid on the function of GlyRs and other receptor-operated channels have not been studied, we analyzed here the action of a specific compounds, a simple unsaturated (R = C15:1) ginkgolic acid (**Figure 1A**), on GlyRs and GABARs.

Using patch-clamp technique, we studied the effects of ginkgolic acid on ionic currents induced by activation of receptor-operated channels expressed in CHO and neuroblastoma cells. We have shown that ginkgolic acid causes specific potentiation of currents mediated by α1 GlyR subunits without strong modulation of α2, α3 GlyR, or $GABA_A$ receptors. Moreover, three aminoacids, mutation of which transformed the inhibitory effect of ginkgolic acid into potentiation, were identified in α2 GlyRs.

MATERIALS AND METHODS

Primary Culture and Transfection

The experiments were carried out on cultured Chinese hamster ovary (CHO-K1) cells obtained from the American Type Tissue Culture Collection (ATCC, Molsheim, France) and on mouse neuroblastoma cells (N2a) cells that were maintained in culture conditions as previously described (Waseem et al., 2010; Mukhtarov et al., 2013).

For electrophysiological analysis cells were transfected with cDNAs of different receptor-operated channels. One day before the transfection, cells were plated on the coverslips (12–14 mm in diameter), which were placed inside 35-mm cell culture dishes with 2 ml of medium. CHO-K1 cells were transfected with the following cDNAs encoding GlyR subunits: human α1 (1 μg/1 μl), α2 (2 μg/1 μl), α3-long (2 μg/1 μl), and β (in combination with α1 or α2 subunits with the ratio of cDNAs concentrations 1α:5β); or with a mixture of cDNAs encoding $GABA_A$ receptors: α1-GFP (1 μg/μl), β2 (1 μg/μl), γ2 (1 μg/μl) using the Lipofectamine 2000 transfection protocol (Life Technology, USA). To facilitate identification of expressing cells, in the case of GlyR, green fluorescent protein (GFP, 0.5 μg/μl) was added to the transfection medium. Visualization of $GABA_AR$ expression was achieved by using the α1-GFP construct (Bueno et al., 1998). Three hours after the initial exposure of the cells to the cDNAs, a fresh solution replaced the old one. To prevent spontaneous activation of GlyRs by the small amount of glycine present in culture medium, strychnine (1 μM) was added to cultures expressing all types of GlyR subunits. Electrophysiological recordings were performed on fluorescent cells 24–72 h after transfection.

Electrophysiological Recordings

Whole-cell recordings were performed at room temperature (20–25°C) using an EPC-9 amplifier (HEKA Elektronik, Germany). Cells were continuously superfused with external solution containing (mM): NaCl 140, $CaCl_2$ 2, KCl 2.8, $MgCl_2$ 4, HEPES 20, glucose 10; pH 7.4; 320–330 mOsm. Two intracellular solutions were used for filling recording patch pipettes. First, mainly used, 'CsCl solution' contained (mM): CsCl 140, $CaCl_2$ 6, $MgCl_2$ 2, MgATP 2, NaGTP 0.4, HEPES/CsOH 10, BAPTA/KOH 20; pH 7.3; 290 mOsm. In the experiments performed with CsCl intracellular solution, ionic currents were recorded at holding potential (V_h) −30 mV. In some experiments, 'Kgluconate solution' was used, in which CsCl 140 mM was replaced by KCl 20 mM + Kgluconate 120 mM. Recordings with this solutions were performed at V_h = 0 mV. Pipettes were pulled from borosilicate glass capillaries (Harvard Apparatus Ltd, USA) and had resistances of 5–10 MOhms. For rapid replacement of solutions, the fast application system was used in this study. Two

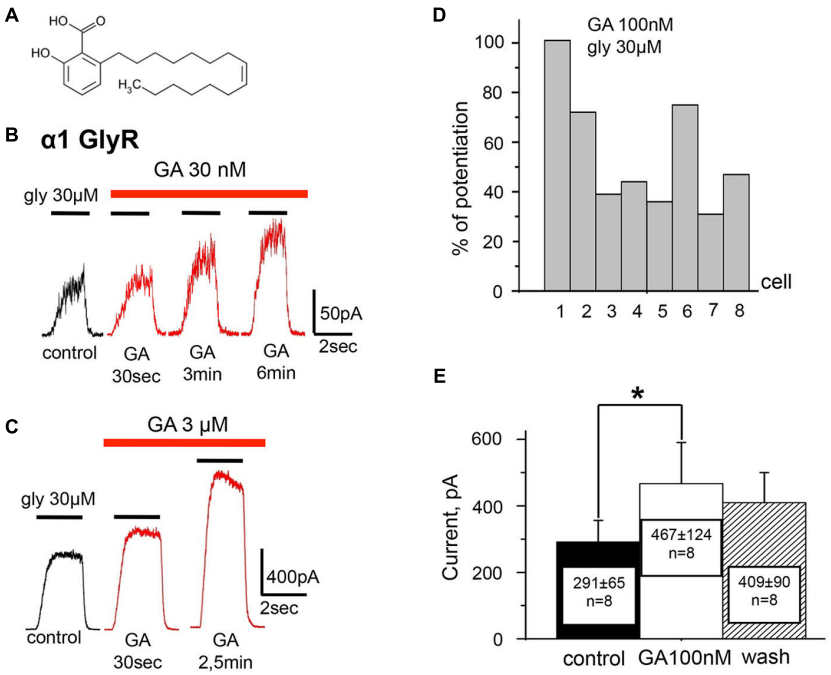

FIGURE 1 | Ginkgolic acid in nanomolar concentrations causes potentiation of I_{gly} mediated by homomeric α1 GlyRs expressed in CHO cells. **(A)** Structure of ginkgolic acids from *G.biloba* used in this study. **(B)** Whole-cell currents induced by 30 μM glycine in control (black trace) and at the different time points of treatment of cells with 30 nM of ginkgolic acid (red traces). For this and subsequent figures, black bars above the traces indicate the time of agonist application; red bars correspond to the duration of the ginkgolic acid application. Kgluconate pipette solution, holding potential (V_h) = 0 mV. **(C)** Examples of the whole-cell currents induced by 30 μM glycine in control (black trace) and during application of 3 μM ginkgolic acid (red traces). Note that at this concentration currents were already augmented 30 s after the beginning of ginkgolic acid application. Kgluconate pipette solution, V_h = 0 mV. **(D)** Percentage of potentiation by 100 nM ginkgolic acid (2 min of pre-application) of currents induced by 30 μM glycine for eight individual cells expressing α1 GlyRs. **(E)** Cumulative data on α1 GlyR-mediated current (glycine 30 μM) potentiation by 100 nM of ginkgolic acid. Mean current amplitude (pA) ± SEM from eight cells in control (black), after 2 min of ginkgolic acid (100 nM) application (white), and after washout (striped). Paired Student's *t*-test; asterisk (*) indicates significant difference, $p < 0.05$.

parallel rectangular tubes (100 μm × 100 μm) were positioned 40–50 μm above the recorded cell. The movement of the tubes was controlled by a computer-driven fast exchange system (SF 77A Perfusion Fast-Step, Warner, USA) allowing a 10–90% solution exchange in 3–5 ms, as measured by open electrode controls (1/10 external solution/water).

In all experiments, the duration of the pulses of agonist was 2 s. The duration of ginkgolic acid application varied from 20 s to 6 min. Cells with low input resistance (<150 MOhms) and a rapid run-down (>30% with repetitive application) were excluded from analysis.

Data Analysis and Statistics

All electrophysiological results were analyzed using PatchMaster (HEKA Electronik, Germany) software. Dose–response curves were constructed by fitting values obtained at different concentrations, after normalization. The responses to glycine concentration were fitted using the non-linear fitting routine of the Origin 7.5 software (OriginLabs, USA) with the Hill equation:

$$I = 1/(1 + (EC_{50}/[A])^{nH}),$$

where I is the normalized current amplitude induced by the agonist at concentration [A], n_H is the Hill coefficient and EC_{50} is the concentration at which a half-maximum response was induced.

Paired and unpaired Student's *t*-tests were used for statistical analysis. The data are expressed as the means ± SEM.

Drugs

Ginkgolic acid (C15:1, HWI Analytic GmbH, Germany) was initially dissolved in pure DMSO and then diluted with control medium to the maximal final concentration of DMSO 0.016% in experiments with using 25 μM ginkgolic acid. In test experiments, DMSO itself had no effects on the I_{gly} (data not shown; see also Mascia et al., 1996; Hall et al., 2004).

Other drugs were obtained from Tocris or Sigma–Aldrich (France).

RESULTS

Low Concentrations of Ginkgolic Acid Potentiate α1 GlyRs

To examine the effect of ginkgolic acid on the function of GlyRs, whole-cell currents in CHO cells expressing different receptor subunits were analyzed. We first investigated the effect

of the acid on homomeric α1 GlyRs. To cells expressing human α1 GlyR, pulses of glycine of different concentrations and 2-s duration were applied before, during and after addition of ginkgolic acid. In contrast to the previously described inhibitory action of ginkgolides (Kondratskaya et al., 2002, 2004; Hawthorne et al., 2006), ginkgolic acid at relatively low concentrations (30 nM–3 μM) strongly enhanced whole-cell currents induced by sub-saturating (EC_{10}–EC_{50}) glycine concentrations (**Figures 1** and **2**). In different cells the degree of potentiation induced by pre-application of 100 nM ginkgolic acid during 2 min varied from 30 to 100% (mean = 51 ± 10%, $n = 8$; **Figure 1D**) and the average I_{gly} increased from 291 ± 65 to 467 ± 124 ($n = 8$; **Figure 1E**).

The time course of the action of 100 nM ginkgolic acid on currents induced by repetitive application of sub-saturating glycine concentration is shown in **Figure 2A**. After obtaining the whole-cell configuration and stabilization of I_{gly} amplitude (first four pulses) the external solution was changed for the one containing 100 nM of ginkgolic acid. Following the first 40 s, I_{gly} was potentiated by 54% and reached a quasi-stable level after 2 min of ginkgolic acid application (see next three pulses). Then, after washing out for 3 min and partial recovery of I_{gly} amplitude, a second application of ginkgolic acid induced an even higher and more rapidly reversible potentiation (**Figure 2A**). Similar effects were observed in neuroblastoma cells expressing α1 GlyR (**Figure 2B**) and also in outside-out patches from CHO cells expressing homomeric α1 GlyRs (potentiation from 25 to 290%, $n = 5$, data not shown).

The kinetics of the potentiation depended on the concentration of ginkgolic acid; the effect of 30 nM ginkgolic acid was observed after 2–3 min of treatment (**Figure 1B**) whereas 3 μM ginkgolic acid caused an enhancement of I_{gly} by >30% after only 30 s (**Figure 1C**). Higher concentrations of ginkgolic acid caused even more rapid enhancement of I_{gly}. For instance, after 20–30 s of pre-treatment the mean potentiation induced by 25 μM ginkgolic acid was 153 ± 25% ($n = 12$), while 100 nM ginkgolic acid during the same time augmented I_{gly} by only 26 ± 8% ($n = 8$; data not shown).

Detailed analysis at different glycine concentrations revealed that ginkgolic acid potentiated currents induced by sub-saturating doses of the agonist, while amplitudes of currents induced by saturating concentrations of glycine (0.3–1 mM) were not affected but some acceleration of desensitization kinetics was observed (**Figure 3A**). Consequently, in the presence of ginkgolic acid, dose–response curves shifted to the left. **Figure 3B** represents an example of EC_{50} shift from 47 μM in control to 28 μM after application of 25 μM ginkgolic acid. On average, 25 μM ginkgolic acid caused a significant decrease in EC_{50} for glycine from 36 ± 3 μM ($n = 6$) to 22 ± 1.4 μM ($n = 6$; $p < 0.01$). Similar shift in EC50 was observed also at using lower concentration of ginkgolic acid. For instance, pretreatment of cells with 3 μM ginkgolic acid during 1–3 min caused a significant shift ($p < 0.01$) of EC_{50} from 36 ± 6 μM in control to 17 ± 2 μM ($n = 9$) after application of the acid (data not shown).

These observations demonstrate that ginkgolic acid, albeit with slow kinetics, is capable of causing strong potentiation of α1 GlyR even in the nanomolar range of concentrations.

Effect of Ginkgolic Acid on Heteromeric α1/β GlyRs

Some antagonists of GlyRs exhibit different abilities to change the activities of homomeric and heteromeric receptors. For instance, the plant alkaloid picrotoxin more effectively inhibits homomeric GlyR than heretomeric α/β receptors (Pribilla et al., 1992; Pistis et al., 1997), while ginkgolide B more effectively antagonizes heteromeric GlyRs (Kondratskaya et al., 2005). To clarify whether ginkgolic acid exhibits the homo/hetero subunit selectivity we studied its action on heteromeric α1/β receptors.

Similarly to homomeric α1 GlyR, whole-cell currents induced by glycine concentrations below EC_{50} (30 μM)

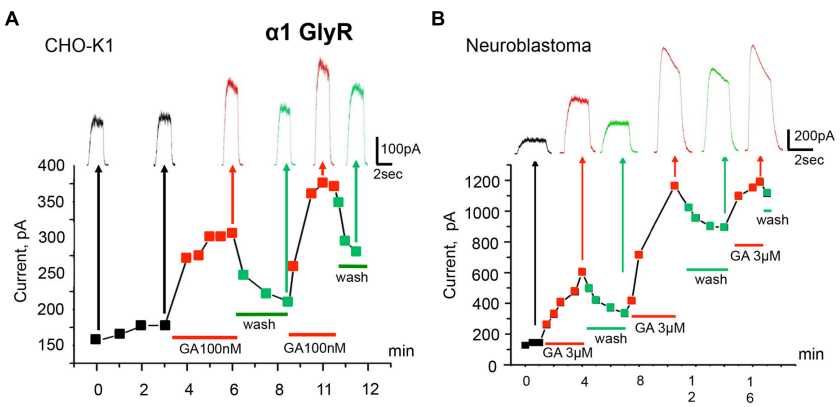

FIGURE 2 | Modulation of homomeric α1 GlyRs by ginkgolic acid. (A) Time course of the development of the effect of ginkgolic acid (100 nM) on whole-cell currents induced by 30 μM glycine. Squares on the graph and traces above indicate amplitudes of the currents in control (black), in the presence of 100 nM of ginkgolic acid (red) and during washout (green). Red and green bars below the graph correspond to the duration of ginkgolic acid application and washout. Kgluconate pipette solution, $V_h = 0$ mV. **(B)** Ginkgolic acid potentiates α1 GlyRs expressed in mouse neuroblastoma (N2a) cells. Time course of I_{gly} changes during several application of the ginkgolic acid (3 μM) to N2a cells expressing α1 GlyR. Notice more than twofold increase in and partial recovery of I_{gly} after the first two applications. Kgluconate pipette solution, $V_h = 0$ mV.

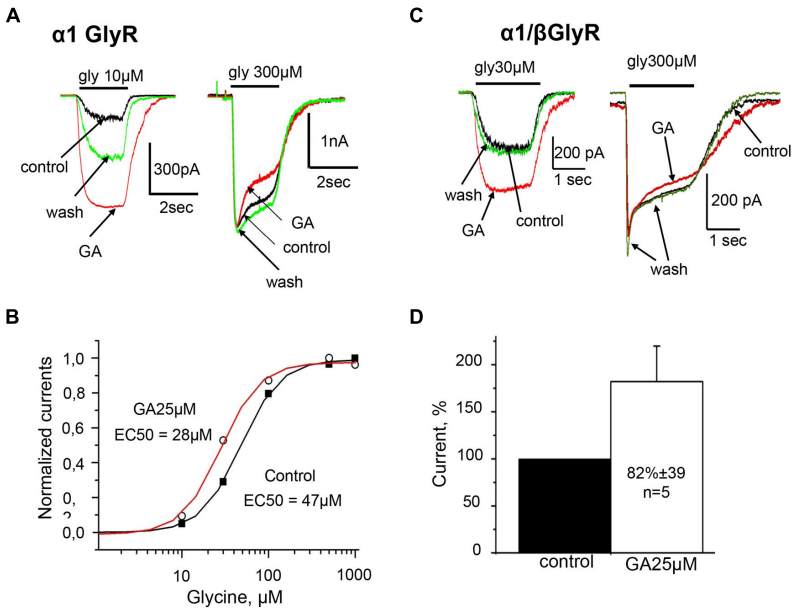

FIGURE 3 | Homomeric α1 and heteromeric α1/β GlyRs are similarly potentiated by ginkgolic acid. (A) Superimposed traces of whole-cell glycine-evoked currents induced by low (10 μM; left) and high (300 μM; right) concentrations of glycine, in control (black), after ginkgolic acid application (25 μM) (red) and after washout (green). Symmetrical CsCl pipette solution, V_h = –30 mV. **(B)** Ginkgolic acid causes reduction in EC_{50} for glycine. Representative dose–response curve for glycine in control (black squares) and during application of 25 μM ginkgolic acid (open circles). **(C)** Superimposed traces of whole-cell glycine-evoked currents induced by low (10 μM; *left*) and high (300 μM; *right*) concentrations of glycine, in control (black), after ginkgolic acid application (25 μM) (red) and after washout (green). Recording from the cell expressing heteromeric α1/β GlyRs. Symmetrical 'CsCl' pipette solution, V_h = –30 mV. **(D)** Cumulative data. Mean percentage of α1/β GlyR-mediated current potentiation after treatment with ginkgolic acid (25 μM).

were strongly potentiated by ginkgolic acid (**Figure 3C**, left). On average, currents induced by subsaturating glycine concentrations increased by 82 ± 39% (n = 5) in comparison with control (**Figure 3D**).

Similarly to homomeric α1 GlyRs, ginkgolic acid did not increase currents induced by application of saturated glycine concentrations (0.3–1 mM) to heteromeric α1/β receptors (**Figure 3C**, right).

Effect of Ginkgolic Acid on α2 GlyRs

Before the analysis of the action of ginkgolic acid on GlyRs formed of α2 subunits, we estimated its EC_{50} by obtaining dose–response curves. The EC_{50} to glycine varied from 24 to 69 μM with a mean value 42 ± 2 μM (n = 10; data not shown), i.e., slightly higher than for α1 GlyRs.

In contrast to the action on α1 GlyRs, low concentrations of ginkgolic acid (<10 μM) had no effect on the amplitude of I_{gly}. At ginkgolic acid concentrations of 10 μM or higher, a small inhibition of currents was observed. Thus, 10 and 25 μM of ginkgolic acid inhibited α2 GlyRs by about –10 ± 3% (n = 8) and –20 ± 5% (n = 11), respectively (**Figure 4A**). However, in many cells high doses of ginkgolic acid stimulated non-reversible run-down, which could be an additional reason for this small inhibition. At low concentrations (1 μM) the effect of ginkgolic acid was not detectable; with a long application (5–6 mins) even the tendency to weak elevation of I_{gly} was observed. This may result partially from a spontaneous run-up of responses during long-lasting whole cell recordings (data not shown, but see Fucile et al., 2000).

High concentrations of ginkgolic acid also caused a weak inhibition of heteromeric α2/β receptors (–14 ± 4%, n = 9). **Figure 4A** summarizes the action of high ginkgolic acid doses on α2 and α2β receptors.

Effect of Ginkgolic Acid on α3 GlyRs

The human α3 GlyR subunit exists in two splice variants, α3K (short) and α3L (long), the last one bears an additional segment of 15 amino acids within the cytoplasmic TM3-TM4 loop (Breitinger et al., 2002) To analyze the action of ginkgolic acid on α3 GlyRs we selected α3L splice variant as its TM3–TM4 insert is important for spatial structure stabilization of the cytoplasmic domain and it is involved in the regulation of GlyR channel gating (Breitinger et al., 2009).

Analysis of concentration dependencies showed that for GlyRs formed of this subunit the EC_{50} to glycine in control was 142 ± 9.8 μM (n = 11; data not shown), i.e., about threefold higher than for α1 and α2 GlyRs.

As illustrated in **Figures 4B,C**, the effect of ginkgolic acid (25 μM) on the amplitude of whole-cell currents recorded from CHO cells expressing α3 GlyR was negligible. In more detail, after 20–40 sec of pre-treatment with ginkgolic acid, the currents induced by a concentration of glycine 'below EC_{50}' (100 μM) slightly decreased (**Figure 4B**, left), on average by –9 ± 2%

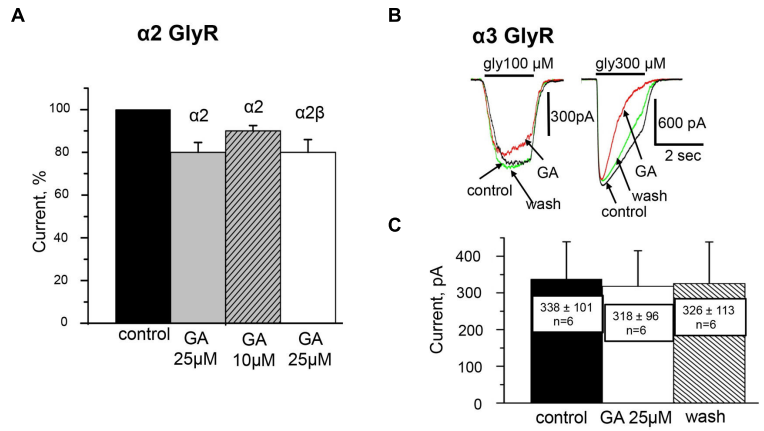

FIGURE 4 | Effect of ginkgolic acid on I_{gly} mediated by homomeric α2 and α3 GlyRs and heteromeric α2/β GlyRs. (A) Cumulative data. Mean percentage of the effect of 25 μM ginkgolic acid on homomeric α2 GlyRs (light gray), heteromeric α2/β GlyRs (white colomn), and 10 μM of ginkgolic acid on α2 GlyRs (striped). Glycine 30 μM was applied. Data from 7 to 12 cells for each case. **(B)** Superimposed traces of glycine-evoked currents induced by low for this subunit (100 μM; *left*) and high (300 μM; *right*) concentrations of glycine, in control (black), after ginkgolic acid application (red) and after washout (green). Symmetrical 'CsCl' pipette solution, $V_h = -30$ mV. **(C)** Summary of the data on the effect of ginkgolic acid on the α3-long subunit of GlyR. Mean amplitudes of currents (pA) ± SEM induced by 100 μM glycine from six cells in control (black), during ginkgolic acid application (white), and after washout (striped).

(from −4 to −11%; $n = 4$); two other cells showed no effect and in one cell a weak potentiation (+4%) was observed. For this concentration, mean currents in control, in the presence of ginkgolic acid and after washout were, respectively, 338 ± 101 pA, 318 ± 96 pA, and 326 ± 113 pA ($n = 6$; **Figure 4C**).

Similarly to its action on α1 and α2 subunits, ginkgolic acid accelerated the desensitization kinetics of currents induced by application of saturated glycine concentrations (≥300 μM; **Figure 4B**, right).

These data indicate that ginkgolic acid, even at high doses, is not capable of potentiating the function of α2 and α3 GlyRs.

Effect of Ginkgolic Acid on $GABA_A$Rs

We further analyzed the action of ginkgolic acid on GABA receptors expressed in CHO cells. Its effect was studied on the most widespread in mammalian brain $GABA_A$R combination – α1/β2/γ2 (Olsen and Sieghart, 2008). We assume that all cells that demonstrated GABA-evoked currents expressed on their surface α1/β2/γ2 receptors, as it was shown before that α1γ2, β2γ2 and homomeric receptors are retained within the endoplasmic reticulum (Connolly et al., 1996; Gorrie et al., 1997). Moreover, cells transfected only with β2 subunits do not produce ionic currents (Connolly et al., 1996) or surface staining (Taylor et al., 1999).

Analysis of concentration dependencies revealed that the EC_{50} of GABA for $GABA_A$Rs in control solution was 11 ± 1 μM (data not shown). The action of ginkgolic acid was tested on 16 cells at using concentration of GABA close to EC_{50} (10 μM) and on three cells using concentration of GABA close to EC_{10} (1 μM). After 20–40 s of treatment with ginkgolic acid (25 μM), we did not observe any changes in GABA-evoked currents, either with EC_{10} (**Figure 5A**) or with EC_{50} (**Figure 5B**) concentrations of GABA. For 10 μM GABA the mean currents in control, after ginkgolic acid application and after washout were 235 ± 65 pA, 228 ± 63 pA, and 232 ± 65 pA ($n = 16$), respectively (**Figure 5C**).

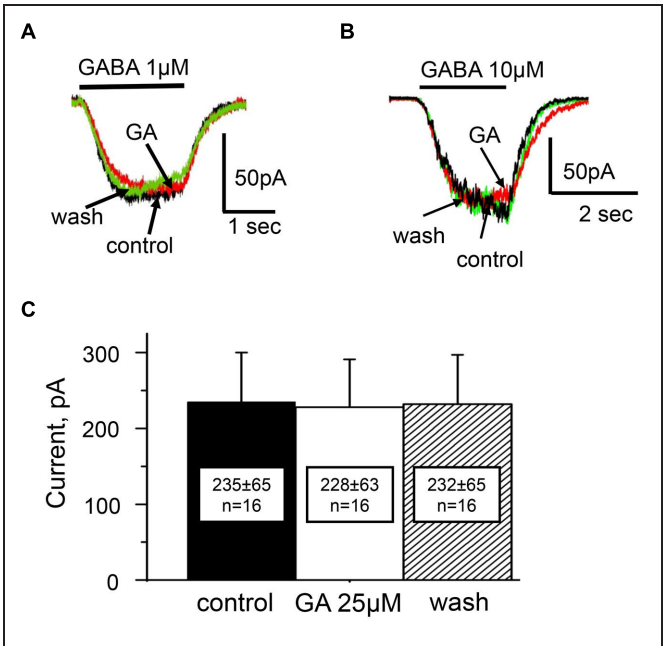

FIGURE 5 | Action of ginkgolic acid on GABA-evoked currents recorded in CHO cells, expressing of $GABA_A$ receptors. (A,B) Superimposed traces of currents evoked by GABA 1 μM **(A)** and 10 μM **(B)** in control (black), after ginkgolic acid application (red) and after washout (green). $V_h = -30$ mV. Symmetrical 'CsCl' pipette solution. **(C)** Summary of the data on the effect of ginkgolic acid on α1/β2/γ2 GABARs. Mean amplitudes of currents (pA) ± SEM induced by 10 μM GABA from 16 cells in control (black), during ginkgolic acid application (white) and after washout (striped).

Thus, similarly to the α3 GlyR, there was no significant difference between GABA-induced currents for the α1/β2/γ2

combination of $GABA_A$Rs before and after ginkgolic acid (25 μM) application.

Amino Acids Involved in the Modulation of GlyRs by Ginkgolic Acid

Finally, we searched for the residues responsible for the different actions of ginkgolic acid on α1 and α2 GlyR subunits. Recent studies have identified several residues involved in allosteric modulation of different GlyR subunits (Xiong et al., 2011; Yevenes and Zeilhofer, 2011a). Among them are the S296 residue in the third TM3, as well as alanine 52 in the extracellular region and glycine 254 in the TM2 domain.

As some parts of the chemical organization of endocannabinoids and ginkgolic acid show high similarity we investigated whether there are also similarities of functional effects. Indeed, both compounds produce potentiation of α1 GlyR subunits. Molecular sites for allosteric control of GlyRs by the endocannabinoid have been identified (Yevenes and Zeilhofer, 2011a). It has been shown that substitution in the α2 subunit of residues T59, A261, and A303 (**Figure 6A**) for corresponding residues from the α1 subunit (A52, G254, and S296) converts the effect of N-arachidonoyl–glycine from inhibition to potentiation (Yevenes and Zeilhofer, 2011a).

In order to check whether the same amino acid residues are indispensable for positive modulation of α1 GlyR by ginkgolic acid we performed T59A/A261G/A303S substitution in α2 subunit (**Figure 6A**) and studied the effect of ginkgolic acid on currents mediated by this α2 GlyR mutant. Ginkgolic acid applied to CHO cells expressing α2 T59A/A261G/A303S subunits caused potentiation of responses to glycine, similar to those observed for α1 GlyR (**Figure 6B**). After pre-application for 1–2 mins of 3 μM ginkgolic acid, currents induced by non-saturating glycine concentrations (30 μM) increased in different cells in the wide range from 25 to 300% (**Figure 6D**), with a mean value of 95 ± 21% ($n = 12$).

Analysis of concentration dependencies revealed that the sensitivity of the mutant GlyR to glycine was weaker than that of WT α2 GlyR. In control conditions, the glycine EC_{50}s for α2 mutant receptors varied from 56 to 238 μM. In the presence of 3 μM ginkgolic acid, dose–response curves showed a distinct left-shift (EC_{50} values varied from 31 to 118 μM). Thus, in the cell illustrated in **Figure 6C**, in control conditions EC_{50} was 157 μM and it became 69 μM in the presence of ginkgolic acid. On average, ginkgolic acid caused a significant ($p < 0.05$) decrease in EC_{50} from 119 ± 16 μM in control to 76 ± 9 μM ($n = 12$) in the presence of 3 μM ginkgolic acid.

Marked potentiation was observed following the application of ginkgolic acid in the nanomolar range of concentrations. As illustrated in **Figure 6E** the time-course and amplitude of I_{gly} potentiation induced by 100 nM ginkgolic acid were similar to those for α1 GlyR.

All together, these observations demonstrate that after mutation of three residues (T59A/A261G/A303S) in the α2

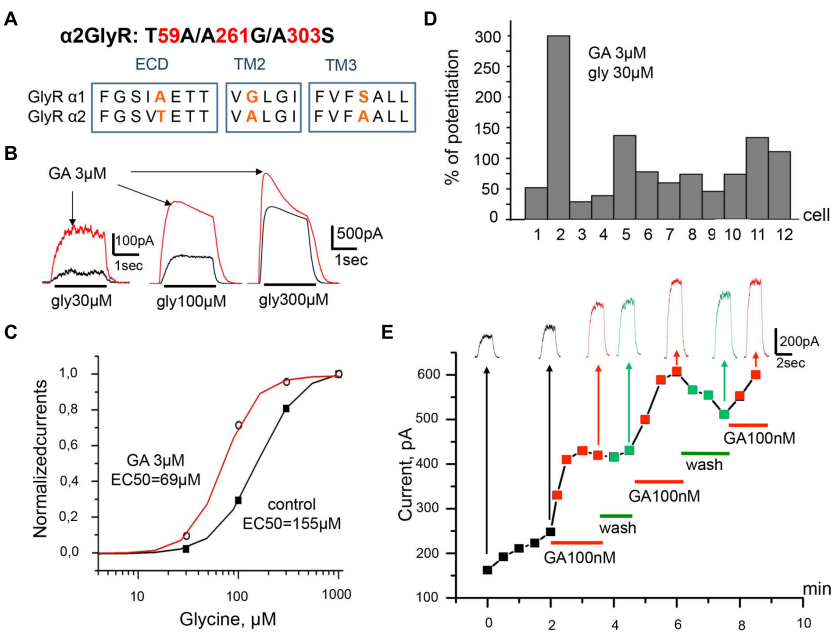

FIGURE 6 | Ginkgolic acid causes potentiation of I_{gly} on cells expressing the mutant α2 (T59A/A261G/A303S) GlyRs. (A) Primary sequence alignment of α1 and α2 GlyR subunits in the extracellular loop, TM2 and TM3 domains; substituted residues are in red. **(B)** Superimposed traces of glycine-evoked currents induced by different glycine concentrations (30, 100, 300 μM) in control (black) and after ginkgolic acid (3 μM) application (red). Kgluconate pipette solution, $V_h = 0$ mV. **(C)** Ginkgolic acid causes a reduction in EC_{50} for glycine. Normalized dose–response curves for glycine in control (black squares) and during application of 3 μM of ginkgolic acid (open circles). **(D)** Percentage of potentiation of glycine (30 μM) evoked currents after ginkgolic acid (3 μM) application for eight individual cells expressing α2 (T59A/A261G/A303S) GlyRs. **(E)** Example of the time course of the development of the ginkgolic acid effect (3 μM) on the amplitude of the ionic currents mediated by α2 (T59A/A261G/A303S) GlyRs; glycine 30 μM was applied. Kgluconate pipette solution, $V_h = 0$ mV.

subunit the effect of ginkgolic acid on the receptor became similar to that observed on α1 GlyR.

DISCUSSION

In this study we have shown that GlyRs are modulated by ginkgolic acid in a subunit-specific manner. After pre-application of ginkgolic acid (0.5–6 min), I_{gly} mediated by α1 GlyRs expressed in CHO and neuroblastoma cells were strongly potentiated. This effect was observed at nanomolar ginkgolic acid concentration (30–100 nM). High doses of ginkgolic acid (25 μM) caused a small inhibition of α2 GlyRs, while there was no detectible effect of ginkgolic acid on amplitudes of currents mediated by α3 GlyRs or on $GABA_A$Rs composed of α1/β1/γ2 subunits. These observations indicate that low concentrations of ginkgolic acid highly specifically potentiate α1 GlyRs.

The effects of ginkgolic acid on α1 GlyRs exhibit the following features. Firstly, potentiation is accompanied by significant left-shifts of dose–response curves and a decrease in EC_{50} values, suggesting modulation of gating properties of α1 GlyR channels. Analysis of dose–response curves demonstrated that 25 and 3 μM of ginkgolic acid caused a similar shift in EC_{50}: respectively, from 36 to 22 μM and form 36 to 17 μM. This allows to suggest that the maximal potentiation of α1 GlyR can be achieved even at relatively low doses of the acid.

Secondly, potentiation develops slowly, on a time scale of minutes, and the strength of the effect depends on the concentration of ginkgolic acid. During application of 30 nM ginkgolic acid the onset of potentiation was observed only after 2–3 min, while high concentrations (25 μM) caused potentiation by more than 150% after only 30 s of ginkgolic acid presence in the external solution.

Recovery of glycine-evoked currents after potentiation of α1 GlyRs by ginkgolic acid, developed slowly, usually not being complete, in the time range of minutes. This could occur for two main reasons. First, the slow unbinding rate of ginkgolic acid from the potentiating site of the receptor situated in a hydrophobic membrane environment. Very low concentrations of the drug could accumulate at binding sites and produce long-lasting enhancement, similar to the inhibitory effects of lipophilic blockers of GlyR (Islam and Lynch, 2012). Second, spontaneous increase in I_{gly}, as it has been previously demonstrated that during long-lasting whole-cell recordings the EC_{50} of GlyRs for glycine spontaneously increases (Fucile et al., 2000). While this spontaneous enhancement of currents was clearly distinguishable from effects of ginkgolic acid (**Supplementary Figure S1**), it could contribute to the irreversible increase.

Ginkgolic acid at very high doses (>10 μM) caused a weak inhibition of whole-cell currents mediated by receptors formed of α2 subunits, without modulating the function of α3 GlyRs and $GABA_A$R. Moreover, at 1 μM ginkgolic acid was not able to modulate α2 GlyRs, confirming its selectivity to α1 GlyRs.

In order to further investigate this subunit-specific effect of ginkgolic acid we have focused on possible interaction sites for this compound inside different GlyR domains. In previous studies it has been shown that most of the residues that are responsible for GlyR modulation by ions, cannabinoids, alcohols, and anesthetics are located in the extracellular domain, in the TM2 and TM3 domains (Mihic et al., 1997; Lynch et al., 1998; Maksay et al., 2009; Yevenes and Zeilhofer, 2011b).

It has been demonstrated that extracellularly localized amino acid 52 of the α subunit is responsible for the differences in the ethanol sensitivity of GlyRs composed of homomeric α1 and α2 subunits (Mascia et al., 1996). Specifically, α1 GlyRs were more sensitive to the action of the ethanol than were α2 GlyRs or the mutant α1 (A52S) receptors. Situated in TM3 domain, residue S296 was found to be crucial for GlyR potentiation by THC, the major psychoactive component of marijuana (Xiong et al., 2011). THC more effectively potentiated currents mediated by GlyR α1 subunits than the currents mediated by α2 subunits. Mutants of α1 subunits in which serine 296 was substituted for alanine showed a decrease in the potentiation magnitude (Xiong et al., 2012).

Subunit-specific modulation of GlyRs has also been demonstrated for the endocannabinoid *N*-arachidonoyl-glycine and synthetic CB1 and/or CB2 receptor ligands (HU-210, WIN 55,212-2), which potentiate α1 GlyR and inhibit α2 GlyR (Yang et al., 2008). Searching for sites involved in positive modulation of GlyR by endocannabinoids it was revealed that substitution of three amino acids in α2 subunits for corresponding amino acids from α1 subunits T59A/A261G/A303S can convert the inhibitory effect of NA-Gly into potentiation (Yevenes and Zeilhofer, 2011a). Based on the similarity of NA-Gly and ginkgolic acid in the GlyR modulation profile we have suggested that the same amino acid residues could be responsible for α1 GlyR potentiation by ginkgolic acid.

Indeed, application of ginkgolic acid to cells expressing α2 T59A/A261G/A303S subunits resulted in (i) an increase in responses to low concentrations of glycine; (ii) a slow development of the effect, similarly as for α1 GlyR. This augmentation effect was observed at as low as 100 nM of ginkgolic acid. Our results reinforce the important role of these amino acids for specific modulation of α1 GlyR. The molecular mechanisms underlying the interaction of drugs with these residues and processes determining specificity of their action needs further analysis.

In contrast to selective potentiation of alpha 1 GlyR, ginkgolic acid caused similar acceleration of desensitization of all GlyR subunits (see, for instance, **Figures 3** and **4**). In line with previous observations (Breitinger et al., 2002) it suggests that regulation of ion channel activation and desensitization can involve different domains. The molecular determinants of desensitization may involve extracellular and TMs or interface between them (Bouzat et al., 2008; Wang and Lynch, 2011), as well as TM1–TM2 (Breitinger et al., 2001) and TM2–TM3 (Nikolic et al., 1998; Breitinger et al., 2002; Meiselbach et al., 2014) cytoplasmic domains. A recent study presented compelling experimental and modeling analysis of this phenomenon demonstrating that the internal end of TM3 and TM1–TM2 linker control desensitization (Gielen et al., 2015). As these parts of molecular sequences are identical for all GlyR subunits, in a view of the study by Gielen et al. (2015), one can suggest that regulation of desensitization by ginkgolic acid may be developed at this level.

Being lipophilic ginkgolic acid can penetrate plasma membrane and interact with various intracellular targets (Fukuda et al., 2009; Lu et al., 2012; Ma et al., 2015) causing regulation of receptor functioning through the intracellular pathways. Thus, activation of protein phosphatase 2C (PP2C) by ginkgolic acid and, consequently, stimulation of neuronal death in cell cultures has been previously demonstrated (Ahlemeyer et al., 2001). However, ginkgolic acid caused effects on PP2C at very high concentrations (>100 μM) (Ahlemeyer et al., 2001), while the effects in our experiments effects were observed at 100 nM, i.e. 1000x less concentration.

We also performed testing of ginkgolic acid action on outside-out patches from cells expressing α1 GlyRs. This configuration should accelerate washing out of intracellular components and eliminate potentiation. However, modulation was very similar to that seen during whole-cell recordings. In addition, ginkgolic acid potentiated mutant α2 GlyRs suggesting its interaction with receptor proteins.

Although these observations reduce the assumption of regulation through the intracellular pathways, this possibility is not excluded. Careful analysis in a separate study using the inside-out configuration and other approaches is necessary to clarify this question.

Several previous studies have demonstrated that cannabinoids and endocannabinois cause modulation of GyR function (Lozovaya et al., 2011). The most effective is a natural component of marijuana, THC, which at nanomolar concentrations (beginning from 30 nM) caused potentiation of α1 and α3 GlyR subunits with weak augmentation of α2 GlyR-mediated currents (Xiong et al., 2011). However, effects of other compounds from this family are complicated, as they cause direct modulation of voltage-gated and receptor-operated ion channels (see reviews, Oz, 2006). While the action of gingkolic acid on other receptors, ion channels, and synaptic networks needs future analysis, the observations presented here suggest that this compound acts as a specific enhancer of α1 GlyR subunits, with the threshold of potentiation in the range of 30 nM.

A large variety of evidence indicates that GlyR subtypes are differentially distributed in the nervous system. GlyR functions depend on subunit composition, subsynaptic localization, and activation mode, they are involved in the control of many motor and sensory pathways, including those necessary for audition, vision, respiration and nociception (Kirsch, 2006; Harvey et al., 2009; Dutertre et al., 2012; Zeilhofer et al., 2012). Thus, α1 GlyRs are primary localized in adult spinal cord, being responsible for movement and muscle tone control (Kneussel and Betz, 2000; Lynch, 2004); α2 GlyRs are important for embryonic brain development (Kneussel and Betz, 2000) and visual perception (Haverkamp et al., 2004). These receptors are dominantly expressed in prenatal brain, but their number dramatically decreases between birth and the third postnatal week (Sato et al., 1992). At the same period, the level of α1 GlyRs increases and they become widely distributed in spinal cord, retina, and brainstem nuclei (Malosio et al., 1991; Sato et al., 1992; Greferath et al., 1994; Zeilhofer et al., 2012). GlyRs are differentially expressed in hippocampus and their subcellular localization and subunit composition change over development (Aroeira et al., 2011). Thus, our observations on subunit-specific modulation of GlyRs by ginkgolic acid might be relevant for specific regulation of the physiological functions mediated by GlyRs in pathological conditions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnmol.2015.00064

Figure S1 | Differences in the time course of elevation of current amplitudes induced by glycine (30 μM) at spontaneous run up and during application of 100 nM ginkgolic acid.

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article III

Voltage-dependent inhibition of glycine receptor channels by niflumic acid

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The action of niflumic acid (NFA), a commonly used blocker of Ca^{2+}-dependent voltage-gated Cl^- channels, on homomeric α1, α2 and α3 and heteromeric α1β, α2β glycine receptors (GlyRs) expressed in CHO cells was studied using the whole-cell configuration of patch-clamp recordings. Analysis of the current/voltage relations has shown that NFA inhibits glycine-induced currents in a voltage-dependent manner. Its effect noticeably increases with depolarization. By applying varying concentrations of NFA we were able to determine NFA IC_{50} at different holding potentials. We have demonstrated that NFA inhibitory strength varies among different subunits of GlyRs, being higher at α2 and α3 subunits in comparison to α1. For instance, at +80 mV NFA IC_{50}s for α1, α2 and α3 GlyRs were, respectively, 197±18μM (n=10), 9±2μM (n=8) and 16±6μM (n=7). Efficiency of NFA action on α1 GlyRs enhanced with the elevation of the agonist concentration and became 90±8μM (n=10) at +80 mV. Mutation G254A in the pore-forming domain of α1 subunit increased its sensitivity to NFA. Incorporation of β subunit did not have any significant impact on α1 sensitivity to NFA, but slightly decreased voltage dependence of NFA interaction with α2. Our results evidence that NFA acts as a pore-blocker of GlyRs.

Introduction

Niflumic acid (NFA) is a member of the fenamate class of nonsteroidal anti-inflammatory drugs originally developed for the treatment of rheumatic disorders. This drug and its derivatives are used worldwide clinically for the relief of chronic and acute pain conditions (Vincent et al., 1999; Kang et al., 2008; Cremonesi and Cavalieri, 2015). As the compound with anti-inflammatory, antipyretic, and analgesic therapeutic efficacy, NFA has been successfully used in clinical trials in adults (Sauvage et al., 1990; Mero et al., 2013) and children (Manach and Ditisheim, 1990; Lantz et al., 1994; Sturkenboom et al., 2005). The primary mechanism of NFA action is the inhibition of enzymes involved in the synthesis of proinflammatory prostaglandins (Smith, 1992; McCarberg and Gibofsky, 2012): cyclooxygenase (prostaglandin synthase) (Barnett et al., 1995; Johnson et al., 1995) and phospholipase

A2 (PLA_2) (Jabeen et al., 2005). Importantly, the structure of the complex of PLA_2 with NFA has been determined at 2.5 A° resolution and allowed to identify the interactive residues in the substrate-binding hydrophobic channel of the enzyme (Jabeen et al., 2005).

This compound is known also as a modulator, mainly inhibitor, of different types of Cl^--selective channels. However, mechanism of its action on these proteins remains unclear. It was demonstrated that NFA blocks voltage-gated chloride channels, ClC-1 (Liantonio et al., 2007), as well as Ca^{2+}-activated Cl^- -channels (CaCC) (White and Aylwin 1990, Yang et al., 2008; Huanosta-Gutierrez et al., 2014). Inhibition of CaCC channels by NFA was voltage-independent and was produced during application of NFA from inside as well as from outside of the membrane (Qu et al., 2001). In the recent study voltage-independence of NFA action on TMEM16A-encoded CaCC channels was confirmed, but obtained data did not clarify the site of its interaction with the receptor, suggesting both pore-blocking and allosteric mechanisms (Ni et al., 2014).

Distinct branch of the ClC protein family, ClC-K kidney Cl^--channels, which are important for renal and inner ear transepithelial Cl^- -transport (Zifarelli and Pusch, 2007), are modulated by NFA in a biphasic way: it activates ClC-K at low concentrations, but blocks the channels at high concentrations, above ~1 mM (Zifarelli et al., 2010).

Similar biphasic way of NFA action has been demonstrated on the ligand-gated Cl^--selective $GABA_A$ receptors formed by α1/β2/γ2 subunits, the main receptor combination in the brain (Sinkkonen et al., 2003). The other configurations of $GABA_A$ receptors formed by α1/β2 or α6/β1/γ1 subunits were inhibited by sub mM concentrations of NFA without potentiation. These observations suggest that NFA modulate these receptors also via two pathways: allosteric potentiation and open channel blocking (Sinkkonen et al., 2003).

$GABA_A$ receptors belong to the superfamily of pentameric cys-loop receptor-operated channels, together with cation-selective nicotinic acetylcholine and serotonin type3 receptors, as well as anion-selective glycine receptor channels (GlyRs) (Betz,

1990; Miller and Smart, 2010). Cl^--permeable $GABA_A$Rs and GlyRs provide the main inhibitory drive in mammalian CNS decreasing cellular excitability (Sigel and Steinmann, 2012; Lynagh and Pless, 2014).

GlyRs are localized in a spinal cord (Young and Snyder, 1973), in brain stem (Probst et al., 1986; Frostholm and Rotter 1985), retina (Haverkamp et al., 2003) and higher brain regions (Bristow et al., 1986). Functionally they participate in the movement control, perception of visual and acoustic signals, and pain sensation (Harvey, 2004; Betz and Laube, 2006). Dysfunction of these receptors associated with hyperekplexia and temporal lobe seizures accompanied by memory deficits (Lynch, 2009; Schaefer et al., 2012; Eichler et al., 2008; Zuliani et al., 2014). In the nervous system of vertebrates molecular cloning identified four genes encoding alpha (α1-α4) subunits and single gene encoding beta GlyR subunits (Grenningloh et al. 1987; 1990; rev. Dutertre et al., 2012). They are able to form homomeric (composed only of α subunits) and heteromeric (α and β subunits) receptors (Lynch, 2004).

Using a synthetic peptide corresponding to TM2 domain of GlyR, that has been proven to form functional ionic channels upon incorporation into the lipid bilayer, it was shown that NFA could block the pore of GlyRs (Reddy et al. 1993). But electrophysiological profile and subunit specificity of this interaction was not studied.

To clarify molecular mechanism of NFA action on GlyRs, we expressed them in different subunits combinations in CHO cells and recorded modulation of glycine-induced ionic currents under NFA application using patch-clamp technique. Analysing functioning of homomeric (formed by α1-α3 subunits) and heteromeric (formed by α1β of α2β subunits) GlyRs at different NFA/glycine concentrations and membrane potentials, we have demonstrated that the NFA apparent affinity and voltage-dependence of its action strongly varies between different subunits of GlyRs.

Methods

Cell culture and transfection

Experiments were carried out on cultured Chinese hamster ovary (CHO) cells obtained from the American Type Tissue Culture Collection (ATCC, Molsheim,

France) that were maintained in culture conditions as previously described (Mukhtarov et al., 2013; Maleeva et al., 2015).

For electrophysiological analysis cells were transfected with cDNAs of different subunits of glycine receptor (α1, α2, α3, α1 G254A, β). One day before transfection, cells were plated on the coverslips (12 mm in diameter) and placed inside 35-mm cell culture dishes with 2 ml of medium. Transfection was performed using the Lipofectamine 3000 protocol (Life Technology, USA). To facilitate identification of transfected cells green fluorescent protein (GFP) was added to the transfection mixture. For expression of functional heteromeric receptors cells were simultaneously transfected with cDNAs of α and β subunits in the ratio 1:10. Three hours after the initial exposure of cells to cDNAs the culture medium was replaced for the one that contained strychnine (1μM), which prevents spontaneous activation of GlyRs. Electrophysiological recordings were performed on fluorescent cells 24-72 hours after transfection.

Electrophysiological recordings

Whole-cell recordings were performed at room temperature (20-25^{o} C) using an EPC-9 amplifier (HEKA Elektronik, Germany). Cells were continuously superfused with external solution containing (mM): NaCl 140, $CaCl_2$ 2, KCl 2.8, $MgCl_2$ 4, HEPES 20, glucose 10; pH 7.4; 320-330 mOsm. Intracellular solution used for filling recording patch pipettes contained (mM): CsCl 140, $CaCl_2$ 6, $MgCl_2$ 2, MgATP 2, NaGTP 0.4, HEPES/CsOH 10, BAPTA (tetrapotassium salt) 2; pH 7.3; 290 mOsm. Recordings were performed at -30 and +30mV or using the ramp protocol that allowed changing the membrane potential (MP) from -80 to +80mV during 1 sec. Recording pipettes were pulled from borosilicate glass capillaries (Harvard Apparatus Ltd, USA) and had resistances of 5-10 MOhms. For the rapid replacement of solutions, a fast application system was used. Three parallel rectangular tubes (100x100μm) were positioned 40-50 μm above the recorded cell. The movement of the tubes was controlled by a computer-driven fast exchange system (SF 77A Perfusion Fast-Step, Warner, USA) allowing a 10%–90% solution exchange in 3–5 ms, as measured by

open electrode controls (1/10 external solution/water). Cells with a low input resistance (<150 MOhms) and a rapid run-down (>30% with repetitive application) were excluded from analysis.

Drugs

All drugs were obtained from Tocris or Sigma–Aldrich (France).

Niflumic acid (100mM) and picrotoxin (50mM) were first dissolved in DMSO and then diluted with extracellular solution to the final concentrations. In the test experiments DMSO itself had no effect on I_{gly} (data not shown; see also Hall et al., 2004). Stock solution of glycine (1M) was prepared using MilliQ water.

Data analysis and statistics

Electrophysiological recordings were performed using PatchMaster (HEKA Electronic, Germany) software. To construct concentration-response curves responses to different concentrations of glycine and NFA were fitted using non-linear fitting routine of the Origin 7.5 software (OriginLabs, USA) with the Hill equation:

$I = Imax/(1+(EC_{50} \text{ or } IC_{50}/[A])^{nH})$,

where I is the current amplitude induced by the agonist at concentration [A]; $Imax$ is a maximal current at each cell; n_H is the Hill coefficient; EC_{50} is the concentration of agonist at which a half-maximum response was induced; IC_{50} is the concentration of NFA that induced a half-maximum inhibition.

For statistical analysis paired and unpaired t-tests were used. Data are represented as means ± SEM.

Results

Action of niflumic acid on α1 GlyRs

Ability of niflumic acid (NFA) to modulate activity of different subtypes of glycine receptors was determined using a whole-cell configuration of patch-clamp technique. GlyRs composed of different subunits were transiently expressed in the CHO cell line.

First, we have examined the effect of varying concentrations of NFA on homomeric α1 GlyRs at constant holding membrane potentials (V_{hold}) of +30 and -30mV using a "long" protocol of solutions application. Ionic currents were evoked by 30μM of glycine alone or mixed with different concentrations of NFA. As fig. 2, A illustrates inhibition was more pronounced at positive membrane potentials. Thus, at V_{hold} -30mV, NFA in concentrations 30, 100 and 300μM caused inhibition of GlyR α1-mediated currents by 3±4, 16±6 and 48±9% (n=7) respectively, while at V_{hold} +30mV, currents were inhibited by 16±7, 43±11 and 75±4% (n=7) respectively.

To examine in details voltage dependence of glycine receptor block by NFA we have used a "ramp" protocol that allowed a fast change of the membrane potential (MP) from -80 to +80 mV (fig. 2, B). Fig. 2, C demonstrates representative current/voltage dependence curves recorded during application of glycine alone or mixed with different concentrations of NFA. Near EC_{50} concentration of glycine (30μM) produced an outwardly rectifying current, due to the higher probability of α1GlyR channels open state at positive potentials (Fucile et al., 1999). While NFA exhibited rather low affinity to α1 GlyRs, especially at negative potentials, we have revealed significant ($p<0.01$) voltage dependence of inhibition. This summarized in the fig. 2, E, which shows that at -80mV NFA IC_{50} comprised 315±30μM, while at +80mV NFA IC_{50} was 197±18μM (n=10). The voltage dependence of inhibition suggested that NFA acts as an open channel blocker of α1 GlyRs.

To test this hypothesis we performed the same experiment with higher, near saturating, concentration of glycine (100μM) that causes an increase in the mean open time of GlyR channels. The efficiency of channel block by NFA increased, particularly at positive potentials ($p<0.001$). NFA IC_{50} at -80mV was 270±26μM, while at +80mV IC_{50} = 90±8μM (n=10) (Fig. 2, D, E).

These results demonstrate that NFA inhibits α1 GlyRs currents operating, presumably, as an open channel blocker.

Action of niflumic acid on α2 GlyRs

Analysis of NFA action on α2 GlyRs revealed two important differences. Firstly, the inhibition was much stronger in comparison with α1 GlyRs, secondly, voltage-dependence of the inhibition was more remarkable.

Using a "long application" protocol we have demonstrated that already 10μM of NFA inhibited currents by about 50% at MP +30 mV (fig. 3, A). Voltage dependence of α2 inhibition by NFA (10μM) was prominent: at -80mV during application of NFA glycine-evoked currents comprised 89±4% from the control, while at +80mV only 43±5% (n=5) (fig. 3, B).

To study in details voltage dependence of α2 subunit block by NFA we have used the same "ramp" protocol as for α1 receptors. Contrary to α1 receptors activation of α2 GlyRs by non-saturating agonist concentration (30μM) produced inwardly rectifying currents, suggesting that the open probability of α2 receptors channel is higher at negative potentials. Another distinctive feature of NFA interaction with α2 GlyRs was a strong voltage dependence of inhibition (Fig. 3, C). Thus, at -80mV NFA IC_{50} was 166±28μM, while at +80mV it decreased to 9±2μM (n=8). Contrary to α1 the efficiency of NFA action did not increase further with augmentation of the agonist concentration (Fig. 3, D, $p>0.05$). Currents induced by 100μM of glycine were inhibited by NFA with IC_{50} of 133±20μM at -80mV and 9±2μM at +80mV (n=7).

Strong voltage dependence of NFA inhibitory action suggests that this compound operates as an open channel blocker interacting with residues deeply in the pore.

Action of niflumic acid on α3 GlyRs

As two different subunits of GlyRs have shown distinct profiles of interaction with NFA we thought it was necessary to verify the effect of NFA on α3 subunit, expecting that it will help to determine the critical amino acids that define strength of interaction between NFA and glycine receptor. Notably, the TM2 domain of α3 is the same as of α2, having alanine at 2' position but not glycine as α1. This would suggest a strong effect of NFA on α3 GlyRs.

Indeed, during “long applications” currents induced by 100μM of glycine were blocked by NFA with the higher efficacy than currents mediated by α1 GlyRs (Fig. 4, A). In concentration 30μM NFA inhibited α3-mediated currents by 32±4% at -30mV and by 62±5% at +30mV, while 300μM NFA at -30mV inhibited ionic currents by 86±5% and by 90±2% at +30mV (n=7). As it was reported before (Nikolic et al., 1998) α3 GlyRs desensitize rapidly, which complicated accurate estimation of the strength of NFA block. Thus, percentage of inhibition was measured as ratio between the current amplitude at the point of max inhibition and at the washout of NFA, where glycine-induced current reaches a quasi-stationary level (fig. 4, A).

Interestingly, current/voltage dependence curves recorded during application of 100μM of glycine demonstrated outward rectification, similarly to what was observed on α1 GlyRs. Like for both previously examined subunits, the efficiency of α3 GlyRs block by NFA was higher at positive potentials (fig. 4 B, C). At MP -80mV NFA IC_{50} was 86±14μM, while at MP +80mV it comprised 16±6μM (n=7).

Thus, sensitivity of α3 GlyRs to NFA is higher than sensitivity of α1 subunits and, while weaker, the efficiency of their interaction is closer to the one registered at α2 GlyRs.

Action of niflumic acid on α1 G254A mutant GlyRs

Taking into the account voltage dependence and presumably pore blocking mode of NFA action we suggested that amino acids crucial for interaction of NFA with glycine receptor are located in its ion pore formed by TM2 domains. TM2 domain is highly conservative between different subunits of GlyR, only one amino acid substitution differ them: at 2’ position of the TM2 domain, α1 subunit has glycine, while α2 and α3 – alanine. We suggested that this difference is the main determinant of the distinct profiles of inhibition of different GlyR subunits by NFA.

To test this hypothesis we performed a single amino acid mutation at position 254 of α1 subunit exchanging glycine for alanine. We expected to convert α1 low NFA sensitivity phenotype to the high affinity one that characterizes α2 and α3 subunits.

Analysis of dose/response relationships showed that the sensitivity of homomeric α1 G254A receptors to glycine is similar to the wild type α1 GlyRs: the EC_{50} comprised 34±6μM (n=6, data not shown), which is in the agreement with previously obtained results (Shan et al., 2001).

As figure 5, A illustrates, at "long application" protocol, NFA relatively weakly inhibited currents activated by 30μM glycine, but with slightly higher efficacy than at wild type α1 GlyRs. At V_{hold} -30mV, NFA concentrations 30, 100 and 300μM caused inhibition of α1 G254A-mediated currents by 22±4, 34±6 and 52±11% (n=6) respectively, while at V_{hold} +30mV, currents were inhibited by 23±7, 43±10 and 76±4% (n=7) respectively.

This effect became more pronounced at higher potentials, which was demonstrated using “ramp” protocol. Notably, at +80mV currents induced by 30μM of glycine were inhibited by NFA with IC_{50} of 64±10μM (n=13), which was significantly lower ($p<0.001$) than for α1 wt receptors (192±23μM, n=8). While at negative potentials sensitivity of mutant G254A α1 receptors was close to WT α1 GlyR and comprised 257±25μM (n=13). With the increase of agonist concentration strength of the block did not increase significantly, it comprised 58±8μM (n=8) at +80mV.

It is important to mention that NFA blocking efficacy at α1 G254A receptors was lower in comparison with α2 and α3 GlyRs. This suggests that NFA might interact with several sites on GlyRs.

Action of niflumic acid on α1β and α2β GlyRs

The predominant subtype of GlyRs in the adult CNS of vertebrates is heteromeric α1β receptor (Lynch 2004). Several inhibitory molecules, for instance, picrotoxin (Pribilla et al., 1992) and ginkgolides (Kondratskaya et al., 2005) demonstrate different affinity to heteromeric and homomeric receptors Thus, we decided to study the interaction of niflumic acid with α1β and α2β GlyRs, expecting that it might also help to investigate further its binding site.

To prove formation of functional heteromeric receptors we implemented a widely used picrotoxin (PTX) test – αXβ receptors are blocked by picrotoxin with

much lower potency, than homomeric αX GlyRs (Pribilla et al., 1992; Shan et al., 2001). In our preparations, under application of 20μM of PTX amplitudes of α1 and α2-mediated currents comprised respectively 27±3% (n=12) and 3±1% (n=9) from control. Heteromeric receptors were inhibited to much lower extent: α1β receptors only to 75±2% (n=15) and α2β to 41±4% (n=7) from control (fig. 6, B, C).

Estimation of NFA IC_{50}s at different membrane potentials have shown that incorporation of β subunit does not change significantly ($p>0.05$) the sensitivity of α1 subtype to NFA – at +80 mV NFA IC_{50} comprised 150±14μM (n=5) (fig. 7, A). Analysis of I/V curves revealed that similarly to α1 homomeric GlyRs, currents mediated by heteromeric α1β receptors (glycine 30μM) were outwardly rectifying (data not shown).

Surprisingly, α2β receptors also demonstrated outwardly rectifying currents, contrary to α2 homomers that had inward rectification (fig. 7, B, C). NFA caused strong inhibition of heteromeric α2β receptors with weaker voltage dependence of the block than at homomeric: at -80 mV, IC_{50} was 107±37μM, and at +80 mV - 20±8μM (n=5, fig. 7, D).

Thus, β subunit does not have a strong impact on the interaction of NFA with GlyRs.

Discussion

Using electrophysiological approach we have investigated the effect of NFA, on Cl^--selective GlyR channels. While this compound is widely used for inhibition of some types of Cl^--selective channels mechanism of its action remains elusive.

We have demonstrated that NFA inhibits currents mediated by GlyRs of different subunit composition. Homomeric α1, α2 and α3 GlyRs have different sensitivity to NFA: α1 receptors demonstrated the lowest affinity to NFA – at +80mV NFA IC_{50} comprised 197±18μM (for EC_{50} glycine concentration), while for α2 and α3 GlyRs the mean NFA IC_{50} significantly decreased - till 9±2μM and 16±6μM, respectively. Inhibition of all three subunits by NFA was voltage dependent with more prominent efficacy at positive potentials. This effect was especially pronounced for α2

GlyRs. Voltage dependence of NFA action allowed us to suggest that the site of NFA interaction with GlyR lay in the pore of the channel.

The action of NFA on α1 receptors was studied at different concentrations of glycine – 30μM (EC_{50}) and at 100μM (saturating concentration). NFA ability to block α1 GlyRs at positive potentials was significantly dependent on the concentration of glycine. With an augmentation of glycine concentration to 100μM the efficiency of α1 inhibition by NFA increased – IC_{50} at +80mV comprised 90±8μM. Contrary to α1 GlyRs, at α2 GlyRs we have not observed an augmentation of the sensitivity to NFA while increasing concentration of the agonist. We suggest that this effect originates form the difference in the kinetics of channel's open state between α1 and α2 GlyRs. Analysis of single channel properties of different GlyR subunits demonstrated that the mean open time of alpha2 GlyR channels excide the mean open time of alpha1 channels almost in 100 time (alpha1 – 2.38 ms; alpha2 – 174ms) (Takahashi et al., 1992). Presumably, the difference in the sensitivity of α1 currents induced by 30 and 100μM of glycine to NFA caused by the increase of the channel's mean open time. Augmentation of the agonist concentration did not have an impact on α2 block by NFA, because at this GlyR subtype the mean open time even at low concentration of glycine was long enough for the development of NFA maximum effect.

The ion channel of glycine receptors is composed by TM2 domains of each of the five subunits that form functional receptor. Suggesting that this domain plays a crucial role in determination of NFA inhibitory efficiency we have analyzed amino acid sequences of TM2 domains of α1, α2 and α3 subunits of glycine receptor using protein sequences data bank UniProt. Proteins alignment has revealed that TM2 domains of different alpha subunits of glycine receptor differ only at 2' position (counting from the intracellular side of the pore): α1 receptors contain GLY, while alpha2 and 3 subunits - ALA at that position. Based on the pore-blocking mode of NFA action we suggested that difference in subunits sensitivity to NFA originates from their difference in the amino acid that occupies 2' position of the TM2 domain.

In order to verify this we have performed a single mutation in α1 subunit exchanging GLY254 for ALA. The α1 G254A mutant receptor was more sensitive to

NFA than α1 wt receptor and similarly to α2 GlyRs activity of NFA did not depend on the concentration of the agonist. However, this mutation did not convert completely the profile of α1 GlyR interaction with NFA to the one of α2, suggesting that amino acids in other domains are also involved.

In our study we have confirmed the importance of 2' residue of TM2 domain for the GlyRs interaction with pore-blocking molecules. Previously it was shown that cyanotryphenilborate blocks more effectively α1 GlyRs than α2, while mutation G254A in α1 subunit makes it less sensitive to CTB (Rundstrom et al., 1994).

Activity of heteromeric α1β and α2β receptors was as well inhibited by application of NFA. In the case of α1 receptors incorporation of β subunit did not change significantly its sensitivity to NFA, while α2β receptors demonstrated a lesser voltage dependence of inhibition in comparison with α2 receptors. The amino acid sequence of β subunit is highly different from other subunits, which complicates a prediction of the amino acids responsible for the shifting voltage-dependence of α2β inhibition. It was shown that F258 plays important role in determination of α1β sensitivity to PTX, its substitution for T significantly increases affinity of heteromeric receptors to PTX (Shan et al., 2001). However, this mutation strongly influences gating of the channel, making difficult interpretation of its role in interaction with modulatory compounds.

Thus, several results of our study evidence in favor of the pore-blocking mode of NFA action on glycine receptors: (i) voltage dependence of the block; (ii) increase of the α1 GlyR block efficacy with an elevation of glycine concentration; (iii) mutation G254A in the TM2 domain of α1 subunit resulted in the increase of the receptors sensitivity to NFA.

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Figure Legends

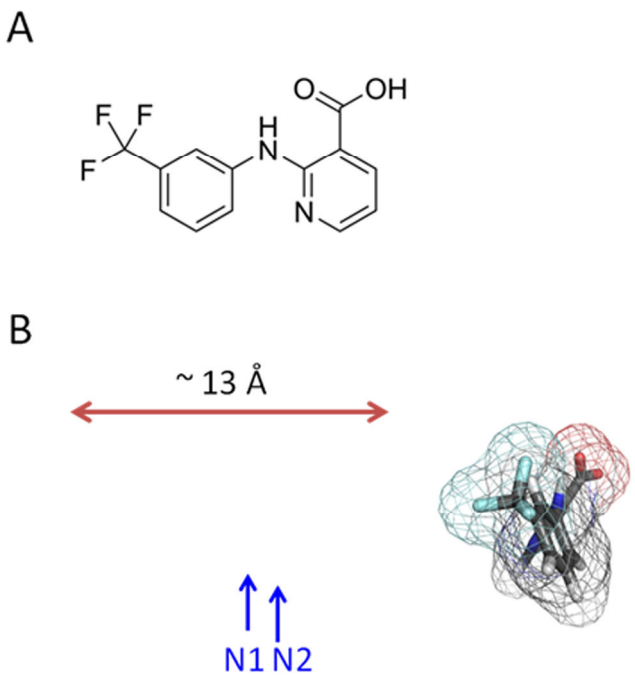

Figure 1. Niflumic acid: **A.** Structural formula. **B.** Orthogonal views at the 3D structure. The distance between most remote atoms is 10.6 Å and the maximal distance between van der Waals surfaces of these atoms (the maximal profile) is ~ 13 Å.

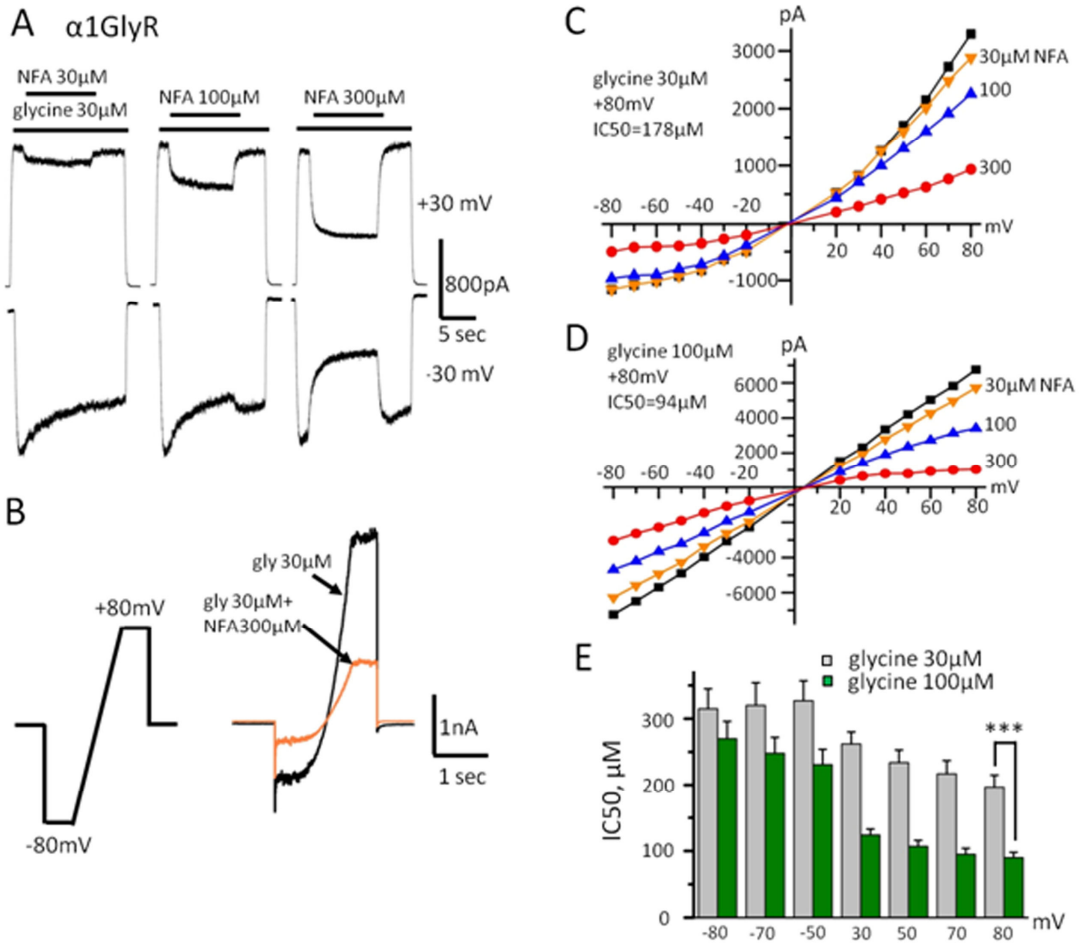

Figure 2. Effect of NFA on homomeric GlyRs formed by α1 subunits. A. Inhibition of glycine-evoked currents (30μM) by different concentrations of NFA (30, 100, 300μM). Glycine was applied for 2 and 5 sec at the beginning and at the end of the trace respectively, in the middle of the trace mixture of glycine with NFA was applied (10 sec), durations of applications are indicated by bars above the traces. Recordings were performed at V_{hold} +30mV (upper traces) and -30mV (bottom traces). **B.** Scheme of the "ramp" protocol and representative traces obtained using this protocol in control (30μM glycine, black) and while applying a mixture of glycine 30μM and NFA 300μM (orange). **C.** Representative current-voltage relationships obtained during application of 30μM of glycine alone (black) or mixed with different concentrations of NFA (30μM-yellow, 100-blue, 300-red). **D**. Representative current-voltage relationships recorded in the presence of 100μM of glycine alone or mixed with different concentrations of NFA (30, 100, 300μM). **E.** NFA IC_{50} at different holding potentials, currents were evoked by application of 30μM (gray columns) and 100μM (green columns) of glycine, recordings were performed using "ramp" protocol.

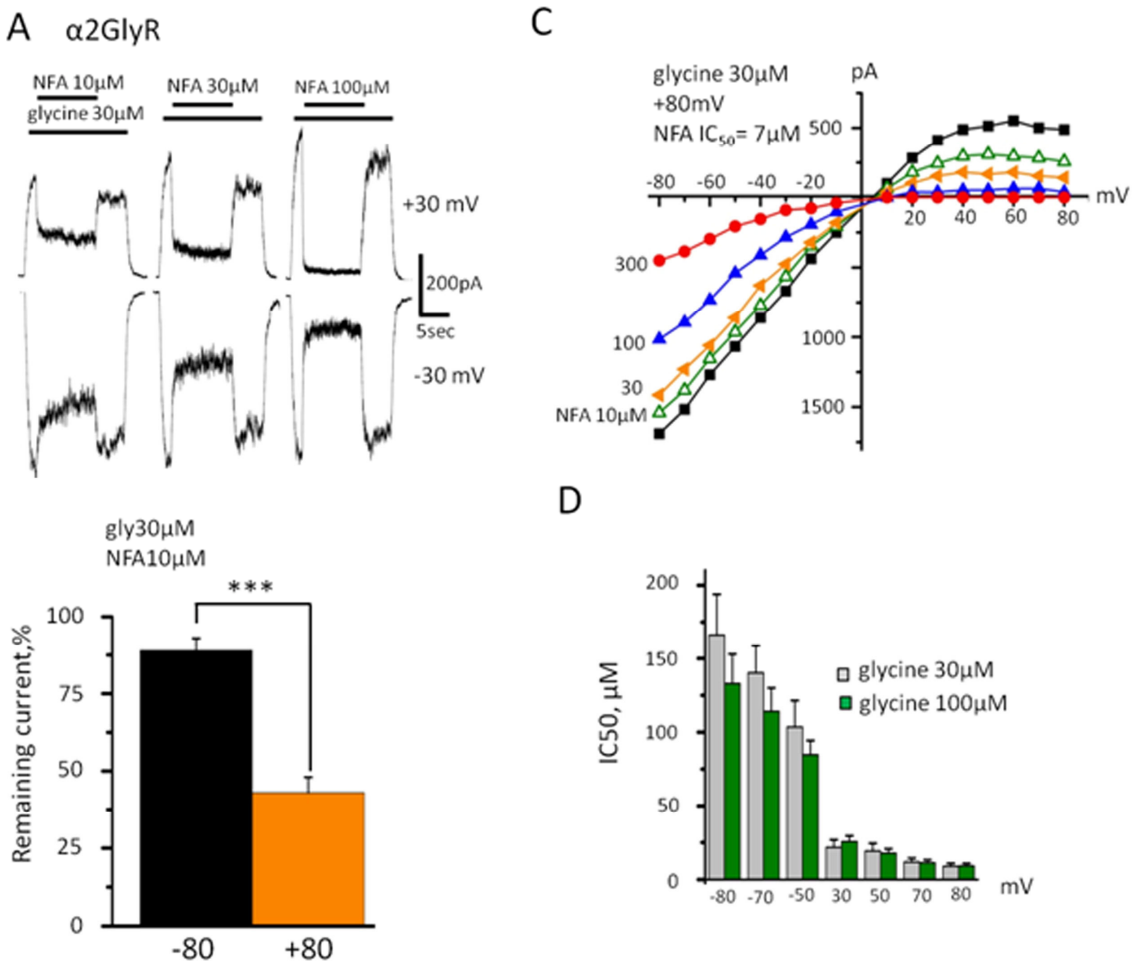

Figure 3. **Action of NFA on GlyRs formed by α2 subunits. A.** Inhibition of glycine-induced currents (30μM) by different concentrations of NFA (10, 30 and 100μM), V_{hold} +30mV (upper traces) and -30mV (bottom traces). **B.** Percentage of the current from control that remained under application of 10μM of NFA at +80 and -80mV. **C.** Representative current/voltage dependence curves obtained in the presence of glycine 30μM alone or mixed with different concentrations of NFA (10μM-green, 30-yellow, 100-blue, 300-red). **D.** NFA IC_{50} at different potentials, channels were activated by 30μM (gray) or 100μM (green) of glycine.

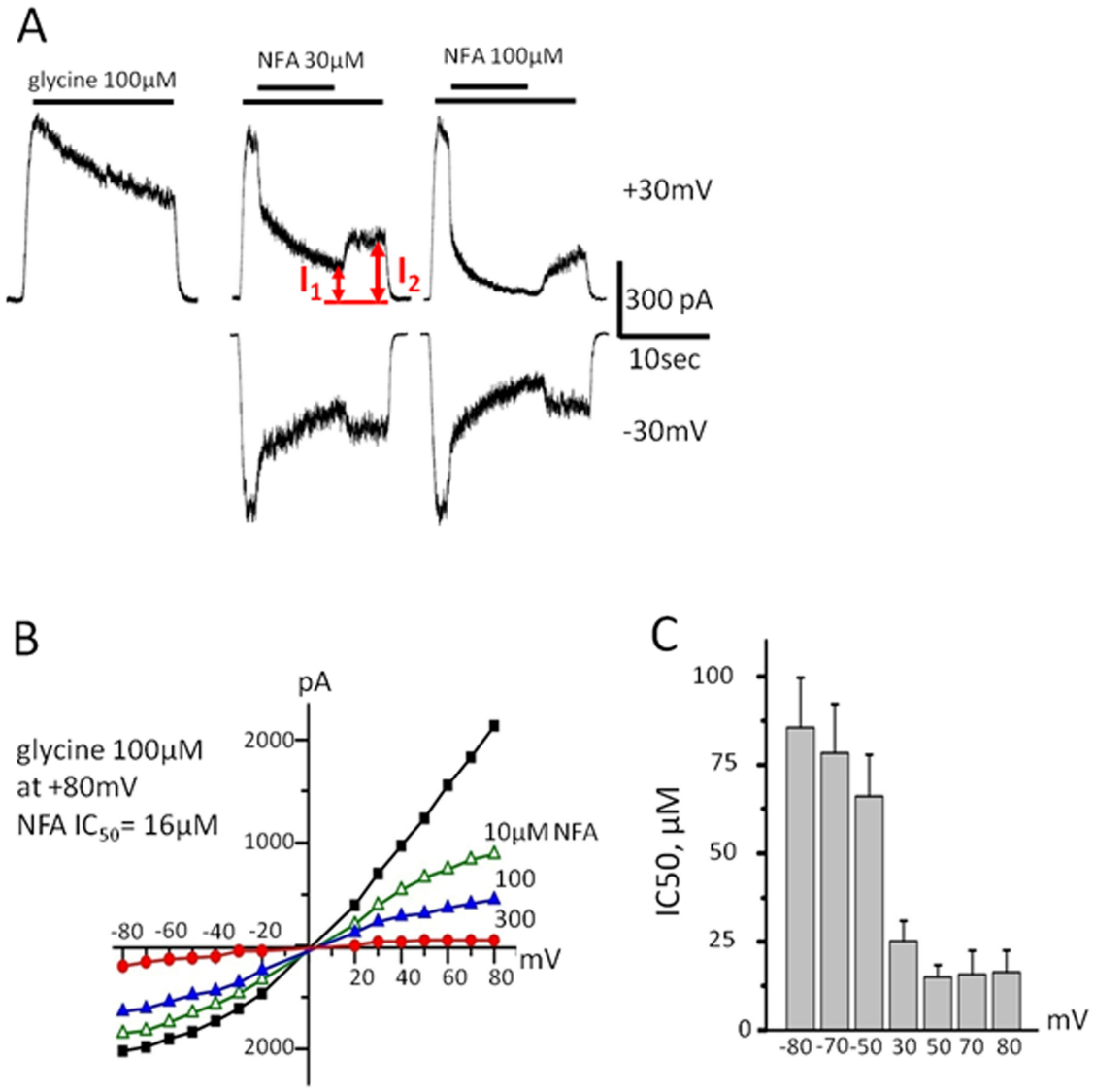

Figure 4. Action of NFA on α3 GlyRs. A. Control glycine–induced current (100µM) and its inhibition by different concentrations of NFA (30 and 100µM), Vhold +30mV (upper traces) and -30mV (bottom traces). **B.** Representative current/voltage relationships recorded during application of glycine (100µM) alone or in the presence of different concentrations of NFA. **C.** NFA IC_{50} at different potentials, currents were induced by application of 100µM of glycine.

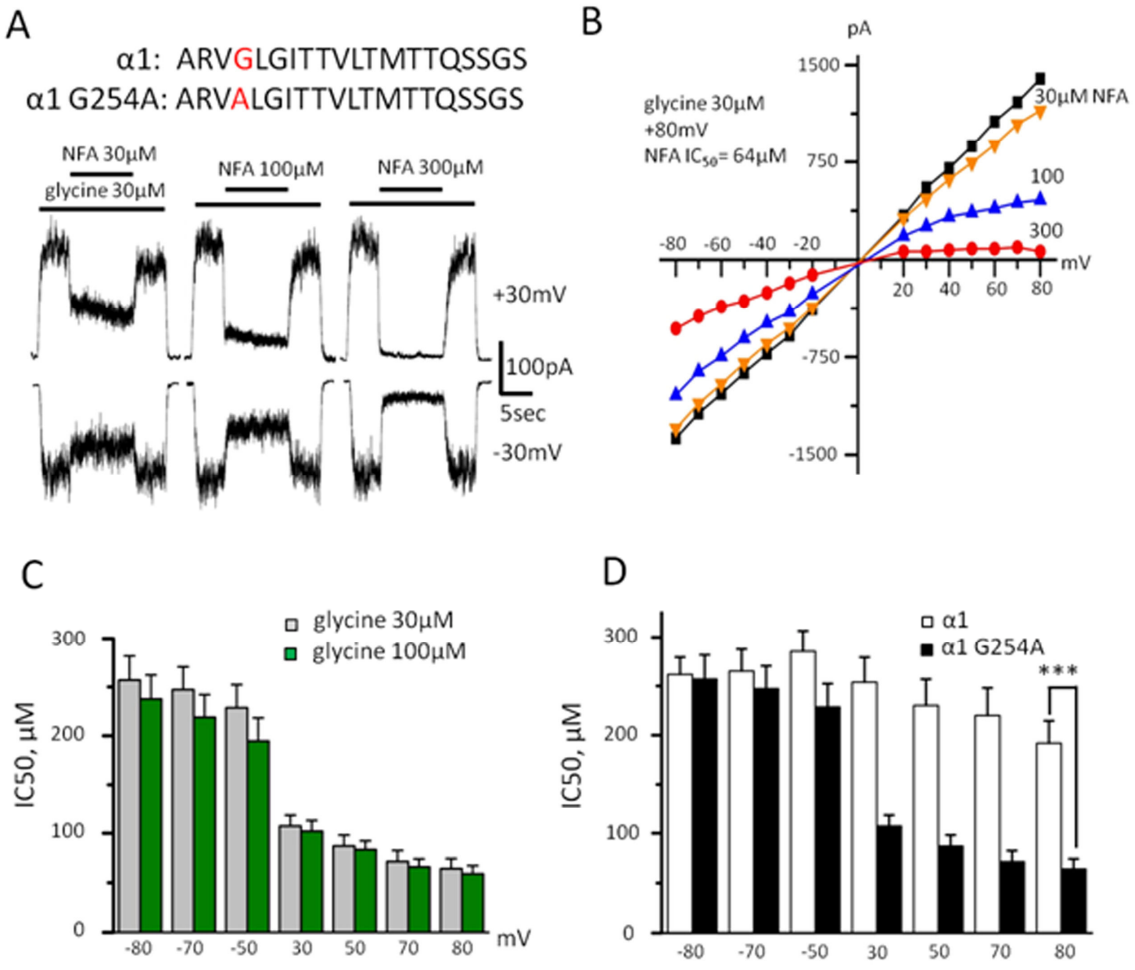

Figure 5. Action of NFA on α1 G254A receptors. A. Amino acid sequences of TM2 domain of α1 wt and mutant subunits (mutated residue highlighted by red) and recordings of inhibition of glycine-induced currents (30μM) by different concentrations of NFA (30, 100 and 300μM), Vhold +30mV (upper traces) and -30mV (bottom traces). **B.** Representative current-voltage relationships recorded during application of glycine (30μM) alone or in the presence of different concentrations of NFA. **C.** NFA IC_{50} at different potentials, currents were induced by application of 30μM (gray) and 100μM (green) of glycine. **D.** Comparison of NFA sensitivities of α1 wt (white) and α1 G254A (black) receptors (currents induced by 30μM of glycine).

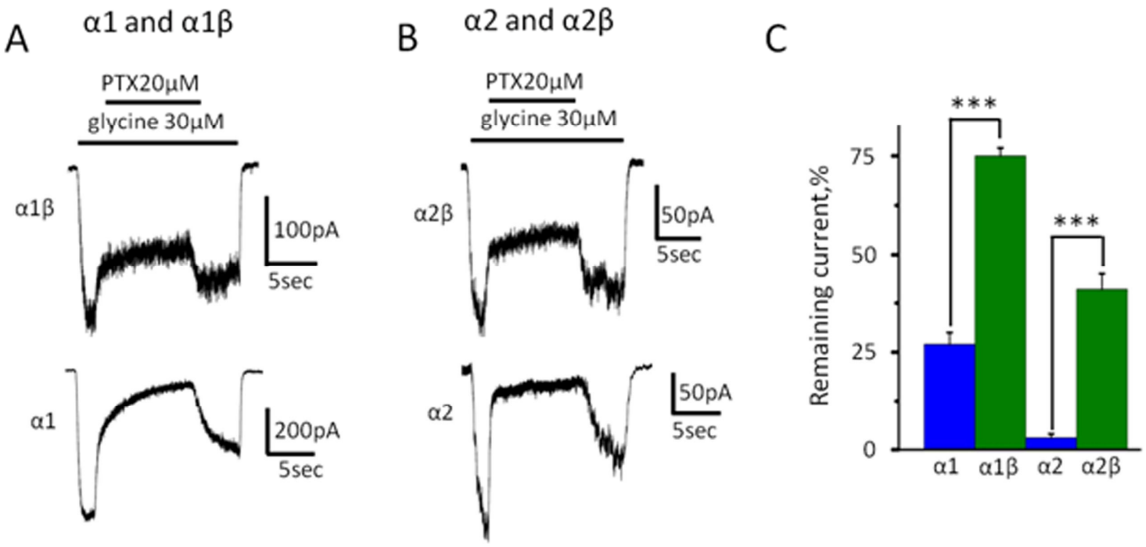

Figure 6. Inhibition of homomeric and heteromeric receptors by picrotoxin. **A.** Action of PTX (20μM) on currents mediated by α1β (upper trace) and α1 GlyRs (bottom trace); glycine 30μM, V_{hold} -30mV. **B.** Action of PTX (20μM) on currents mediated by α2β (upper trace) and α2 GlyRs (bottom trace); glycine 30μM, V_{hold} -30mV. **C.** Percentage of the current from control under application of PTX (20μM) for α1, α1β, α2 and α2β receptors (homomeric receptors – blue, heteromeric - green).

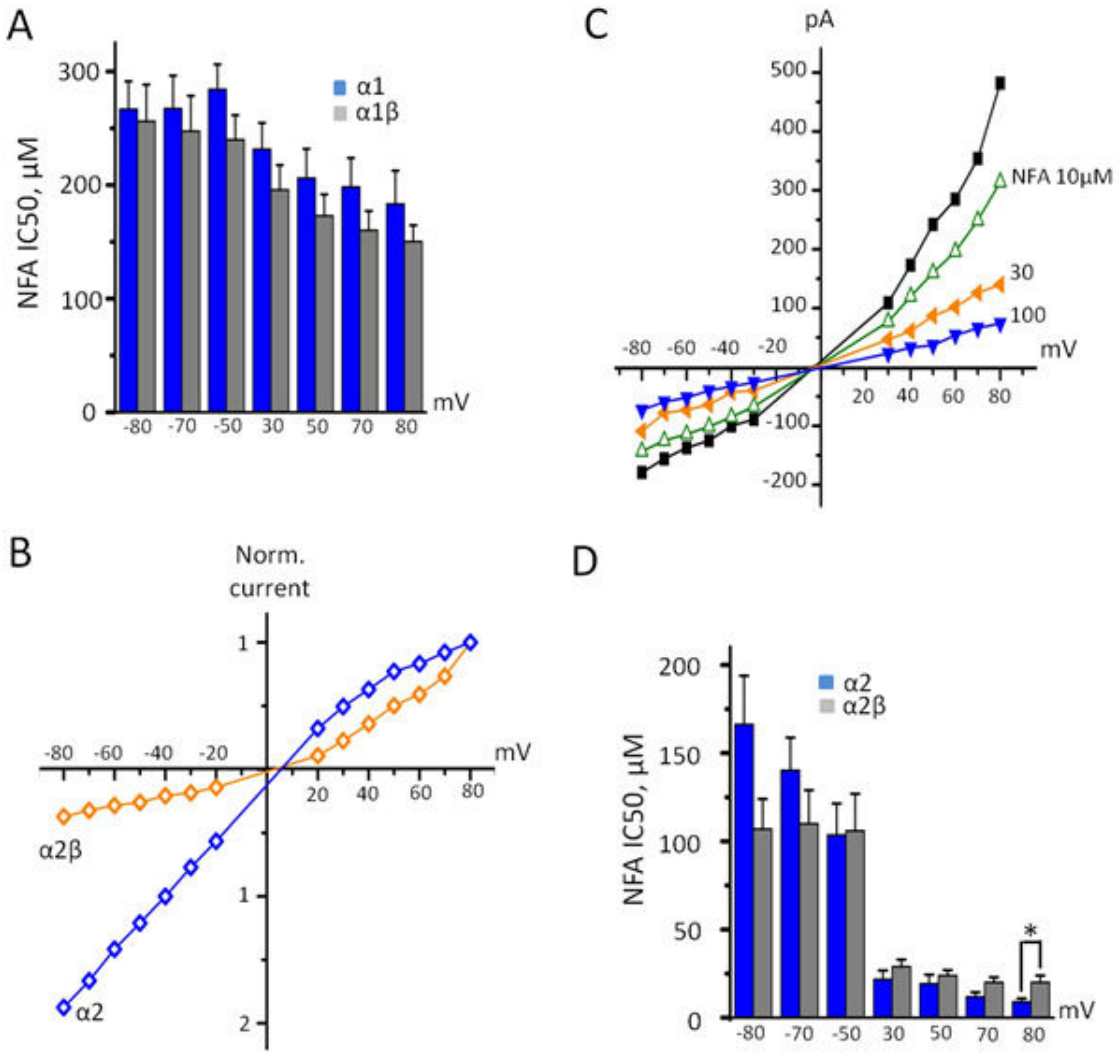

Figure. 7. Action of NFA on heteromeric α1β and α2β receptors. A. Comparison of NFA IC_{50} for α1 (blue) and α1β (grey) receptors at different potentials, currents were induced by application of 30µM glycine. **B.** Representative current/voltage relationship curves for α2 (blue) and α2β (orange) receptors (glycine 30µM). **C.** Representative I/V curves recorded at α2β receptors during application of 30µM of glycine alone or in the presence of different concentrations of NFA. **D.** Comparison of NFA IC_{50} for α2 (blue) and α2β (gray) receptors at different potentials, currents were induced by application of 30µM of glycine.

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article IV

Efficient and cost-effective generation of mature neurons from human induced pluripotent stem cells

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Induced pluripotent stem cells (IPSCs) are resulting from reprogramming of somatic cells and subsequently they can be differentiated into cells of each of three germ layers – endoderm, ectoderm or mesoderm (Takahashi and Yamanaka, 2006). Technology of generation of IPSCs opens new possibilities for modeling and investigation of different human pathologies on cellular level as well as for development of the cell therapy approaches. Particular interest these cells represent for studying CNS, as access to human neuronal cells can be obtained only in the case of medically required surgery or post mortem. Thus, elaboration of new efficient methods for generation of IPSCs and their differentiation into neurons is of great importance for CNS pathologies research.

For the first time neurons were obtained from IPSCs by Wernig and co-authors in 2008. The protocol that they have used implies: (i) a use of feeder cells – fibroblasts of embryos (to stimulate proliferation of IPSCs); (ii) use of the medium that contains fibroblasts growth factor (*FGF2*) – for the formation of neuronal progenitors; (iii) adding of factors *sonic hedgehog* and *FGF8* – for the final differentiation into β-III-tubulin positive cells with neuronal morphology (Wernig et al., 2008).

Transplantation of neurons generated from IPSCs in future can be used for therapy of many diseases, such as Parkinson disease (Kriks et al., 2011), spinal cord injuries (Nori et al., 2011; Tsuji et al., 2010), macular degeneration (Okamoto and Takahashi, 2011). Cells obtained from patients can be used to model different pathologies, study molecular mechanisms of their development, drugs screening and elaboration of individual treatment strategies. It is important to mention that IPSCs give a possibility to reproduce not only monogenic diseases phenotypes but also multifactor pathologies: Parkinson disease (Devine et al., 2011), Alzheimer disease (Israel et al., 2012; Yagi et al., 2011) and schizophrenia (Brennand et al., 2001).

Taking into the account relatively short period during which technologies of IPSCs generation develop results of their differentiation require comprehensive investigation not only in the pathological condition but also in control. In the case of neuronal differentiation a characterization of ion channels is of great importance, as they are involved in determination of functional state of neuronal cells and circuits. Besides that, a great number of CNS pathologies are provoked by dysfunction of ion channels.

It was shown that action potentials generated by neurons obtained from IPSCs do not differ from action potentials registered from neurons obtained from embryonic stem cells. Both cell types possessed TTX-sensitive inward sodium currents and TEA-, 4-AP-sensitive outward potassium currents (Zeng et al., 2010). In another study it was shown that activation of Na^+ TTX-sensitive channels occurred at -40mV, max current

was observed at -10, -20mV, while K^+ channels sensitive to TEA and 4-AP were activated at -30mV. In addition to that, currents induced by application of GABA were registered (Haythornthwaite et al. 2012).

Recently, AMPA and NMDA receptors expressed by IPSCs neurons were characterized. Using a method of Ca^{2+} transients monitoring researchers have demonstrated AMPA-induced currents blocked by specific AMPA antagonist GYKI-53784. PCR analysis has revealed a high level of expression of GluA1 and GluA2 subunits of AMPA receptor and GluN1 and GluN2 subunits of NMDA receptor. Analysis of voltage-activated calcium channels with a use of special blockers and PCR analysis has demonstrated that expression of Cav1.2, Cav2.2 and Cav2.3 channels (Dage et al., 2014).

Thus, neurons obtained from IPSCs have main features of functional neuronal cells, express on their surface voltage- and ligand-gated ionotropic channels and represent a promising model for studying functioning of CNS. However, ion channels of IPSCs neurons not completely characterized and require further investigations.

STEM CELLS TRANSLATIONAL MEDICINE®

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Efficient and Cost-Effective Generation of Mature Neurons From Human Induced Pluripotent Stem Cells

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Key Words. Neuronal progenitors • Neural induction • Neural differentiation • Human induced pluripotent cells • Patch clamp • Dopaminergic neuron • Voltage-gated currents • GABA and glycine receptors

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ABSTRACT

For years, our ability to study pathological changes in neurological diseases has been hampered by the lack of relevant models until the recent groundbreaking work from Yamanaka's group showing that it is feasible to generate induced pluripotent stem cells (iPSCs) from human somatic cells and to redirect the fate of these iPSCs into differentiated cells. In particular, much interest has focused on the ability to differentiate human iPSCs into neuronal progenitors and functional neurons for relevance to a large number of pathologies including mental retardation and behavioral or degenerative syndromes. Current differentiation protocols are time-consuming and generate limited amounts of cells, hindering use on a large scale. We describe a feeder-free method relying on the use of a chemically defined medium that overcomes the need for embryoid body formation and neuronal rosette isolation for neuronal precursors and terminally differentiated neuron production. Four days after induction, expression of markers of the neurectoderm lineage is detectable. Between 4 and 7 days, neuronal precursors can be expanded, frozen, and thawed without loss of proliferation and differentiation capacities or further differentiated. Terminal differentiation into the different subtypes of mature neurons found in the human brain were observed. At 6–35 days after induction, cells express typical voltage-gated and ionotrophic receptors for GABA, glycine, and acetylcholine. This specific and efficient single-step strategy in a chemically defined medium allows the production of mature neurons in 20–40 days with multiple applications, especially for modeling human pathologies. STEM CELLS TRANSLATIONAL MEDICINE *2014;3:1467–1472*

INTRODUCTION

The recent development of induced pluripotent stem cells (iPSCs) has shown promise for understanding and modeling a number of human pathologies. These cells, obtained after reprogramming of somatic cells, are characterized by their capacity to proliferate and differentiate into the three embryonic layers—endoderm, mesoderm, or ectoderm—and subsequent derivatives [1, 2].

The advent of iPSCs has transformed the prospects for disease modeling and our capacity to study pathological processes, especially in the central nervous system [3–5]. A growing number of reports describe models of constitutive disorders that are based on human iPSCs (hiPSCs). This approach remarkably enhances our understanding of the pathological mechanisms of these different diseases and allows their use in drug discovery, opening new grounds for testing new therapeutics such as pharmacological treatments or regenerative cell-based therapies [3, 6, 7]. Although the generation of disease-specific iPSCs has become routine, the potential of iPSCs in tissue modeling poses challenges on multiple fronts, including directing the fate of iPSCs into relevant cell populations.

Protocols are established for some lineage commitment, but in some cases, experimental development is required or needs to be optimized to reduce the cost of the process and obtain large amounts of well-characterized differentiated cells.

MATERIALS AND METHODS

Materials and methods are included in the supplemental online data.

RESULTS

We developed a simple procedure to induce differentiation of human pluripotent cells (supplemental online Fig. 1) into neurons (Fig. 1; supplemental online Fig. 2). The first step requires predifferentiation and expansion of precursor cells in a defined medium on plates

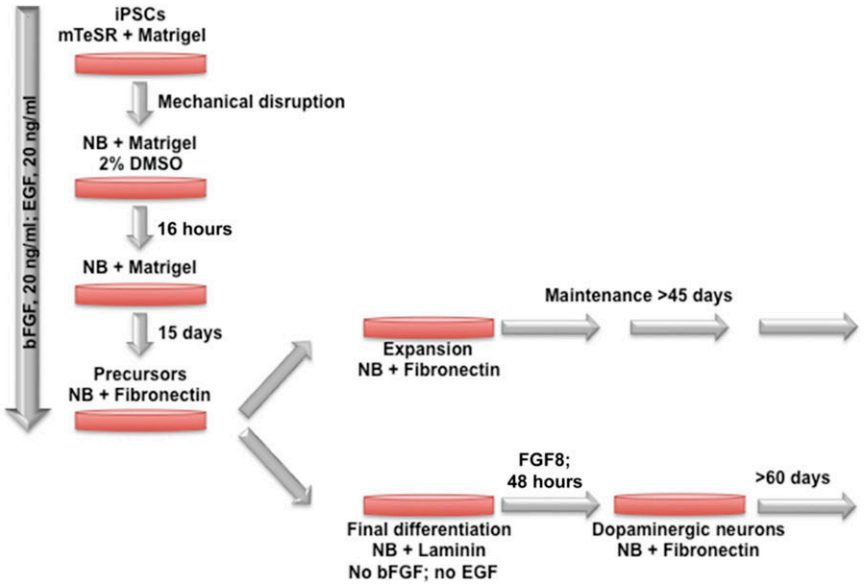

Figure 1. Schematic representation of the differentiation procedure. Days 1–15: differentiation of human iPSCs (hiPSCs) into human neuronal stem cells (hNSCs). The hiPSCs were expanded, and mature hiPSCs cultured in mTeSR on Matrigel-coated plates were mechanically disrupted in 30–50 small clumps using a 23-gauge needle and plated onto a Matrigel-coated 35-mm culture dish in differentiation medium (DM) supplemented with 20 ng/ml bFGF, 20 ng/ml EGF (DM+). Optimal results were obtained with 2% (v/v) DMSO for 16 hours. After overnight incubation, medium was replaced with DM+. Differentiated cells progressively emerge as a monolayer in the periphery of the hiPSC colony and can be maintained and expanded for up to 15 days with medium replacement every day. After 10–15 days of differentiation, cells at 90%–100% confluence are dissociated with Dispase. Small clumps of hNSCs were plated onto fibronectin-coated 35-mm culture dishes, and 90% of cells adhered within a few minutes after plating. hNSCs can be maintained for several passages or expanded after splitting with Accutase or a cell scraper and replating at a density of 1×10^5 cells per 35-mm culture dish. For final differentiation, cells were mechanically separated with a 23-gauge needle and plated onto laminin-coated 6-well plates in DM without bFGF and EGF. Medium is replaced every day. Neurons develop in 5–7 days after plating. An example of final differentiation into dopaminergic neurons is presented after addition of specific cytokines, such as FGF8 and SHH. Abbreviations: bFGF, basic fibroblast growth factor; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; iPSC, induced pluripotent stem cell; NB, Neurobasal medium.

coated with Matrigel. The second step requires plating on fibronectin and then on laminin-coated dishes for final differentiation into mature neurons (Fig. 1). Alternatively, the production of specialized neurons can be achieved in the presence of specific factors.

Following induction in the presence of dimethyl sulfoxide, hiPSC morphology is progressively modified (Fig. 2A, 2B). Immunofluorescence staining at different time points confirmed the progressive loss of the OCT4 pluripotency marker in flat spindle-like cells and the acquisition of a neuroepithelial phenotype, with expression of the *NESTIN* neural stem cell marker indicating a switch toward the neuronal lineage [8] as early as 4 days after-induction (Fig. 2B). Expression of pluripotency markers becomes almost undetectable after 15 days (Fig. 2B). After plating on laminin, these neuronal progenitors self-renew, maintain their potency, and can be expanded for several weeks, as shown by the high percentage of cells expressing *NESTIN*, *PAX6*, or *SOX1* at passages 1 and 6 (supplemental online Fig. 2). In the different population of neuronal progenitors, we also observed decreased expression of the *NANOG* and *OCT4* pluripotency markers and an increase in *PAX6* mRNA level but the absence of expression of the *BRACHYURY* or *SOX17* mesodermal and endodermal markers (supplemental online Fig. 3A). Furthermore, this population of progenitors can be frozen and thawed without loss of capacity. This strategy is highly reproducible, and similar yields were obtained from different clones derived from different healthy human donor dermal fibroblasts (supplemental online Fig. 1).

After induction of the neuronal lineage, NESTIN-positive cells (Fig. 3B) can either be maintained and expanded in differentiation medium on fibronectin-coated plates or differentiated into mature neurons on laminin-coated plates after removal of basic fibroblast growth factor and epidermal growth factor and without additional factors. Final differentiation characterized by βIII-tubulin and NeuN staining, two markers of postmitotic neurons (Fig. 3C), and choline acetyltransferase (supplemental online Fig. 3B) is then achieved in 5–7 days after plating on laminin. In order to determine whether this protocol allows the production of specialized neurons, we tested differentiation toward the dopaminergic lineage by inducing mature neurons with FGF8 and SHH for 48 hours [9] (Fig. 3C). Functionality of dopaminergic neurons was assessed by immunofluorescence staining (expression of tyrosine hydroxylase [*TH*], a marker of functional dopaminergic neurons) (Fig. 3C) and quantitative reverse transcription polymerase chain reaction (expression of *DDC*) (supplemental online Fig. 3C). At 15 days after induction, we obtained high enrichment in functional dopaminergic neurons expressing tyrosine hydroxylase. Based on counting of TH-positive cells after immunofluorescence, we estimate that more than 80% of cells are positive for TH (Fig. 3C).

To confirm the neuronal profile of hiPSC-derived neurons, we investigated their membrane properties, such as presence of neuronal voltage-gated and receptor-operated channels, by performing whole-cell patch clamp recordings [10] after maintenance for 7–35 days on laminin-coated 11-mm cover slips (Fig. 4). Cells had resting membrane potentials that ranged from −20 to

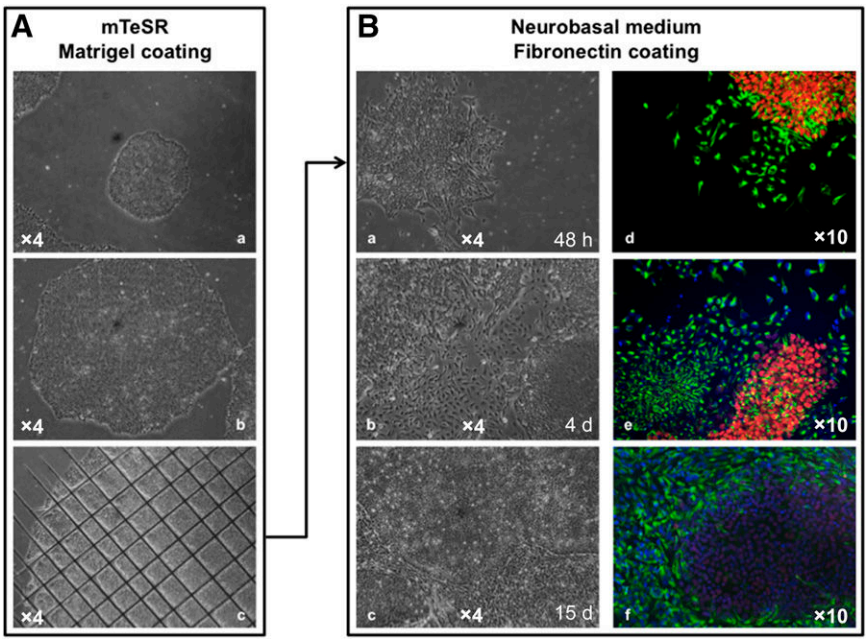

Figure 2. Differentiation of human induced pluripotent stem cells (hiPSCs) into neuronal progenitors. **(A):** Bright-field images of small immature **(Aa)** and mature **(Ab)** hiPSC colonies grown on Matrigel-coated plates in mTeSR before mechanical disruption **(Ac)**. **(B):** After mechanical disruption using a 23-gauge needle, clumps of cells were plated on Matrigel and grown in differentiation medium. Left, bright-field images of neuronal differentiation at 48 hours **(Ba)**, 4 days **(Bb)**, or 15 days **(Bc)** after induction; **(Bd)** at 48 hours after induction, differentiated cells expressing the Nestin neuronal marker (green) migrate out of the OCT4-positive hiPSC colony (red); **(Be)** neuronal precursors express *NESTIN* (green) and hiPSCs express *OCT4* at day 4 after induction; after 15 days **(Bf)**, OCT4 expression is barely detectable, and Nestin-positive neuronal precursors reach 90%–100% confluence. Abbreviations: d, days; h, hours.

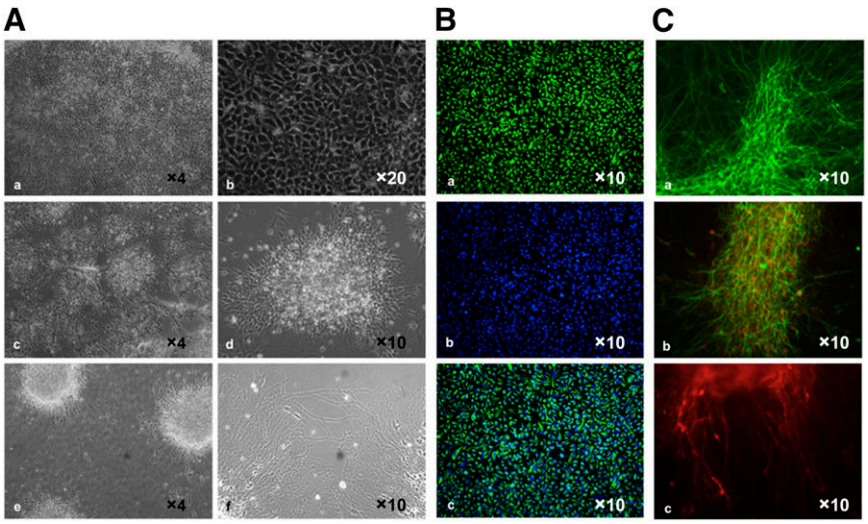

Figure 3. Production of mature neurons. **(A):** Illustration of the different steps of neuronal maturation. Neuronal progenitors can be expanded on solid-coated plates to 90%–100% confluence (magnification ×5 **[Aa]** and ×10 **[Ab]**) or dissociated and plated at a lower density (magnification ×5 **[Ac]** and ×10 **[Ad]**) for further differentiation (magnification ×5 **[Ae]** and ×10 **[Af]**). **(B):** Immunofluoresence staining 2 days after plating of neuronal progenitors on laminin in Neurobasal medium but without basic fibroblast growth factor and epidermal growth factor. The majority of cells express Nestin **(Ba)**; cells were counterstained with 4′,6-diamidino-2-phenylindole **(Bb)** and merged **(Bc)**. **(C):** At 20–30 days, mature neurons derived from hiPSCs express βIII-tubulin (green **[Ca, Cb]**) and the marker of mature neurons, NeuN (red **[Cb]**). Dopaminergic differentiation was induced by addition of SHH and FGF8, as described. The production and functionality of dopaminergic neurons were assessed by immunofluorescence staining with antibodies against tyrosine hydroxylase 15 days after induction (red **[Cc]**).

−60 mV (mean ± SEM: −39 ± 3 mV; $n = 17$) and input resistance of 634 ± 136 MΩ ($n = 19$). To detect the presence of voltage-gated channels, cells were held at −70 mV and then at 10-mV incremental voltage from −80 mV to +30 mV with prepulse to −120 mV (Fig. 4B, insert). The vast majority of cells (32 of 37 tested) displayed voltage-gated currents. On depolarizing steps, increasing amplitude outward currents, typical for neuronal K^+ ones, were recorded (Fig. 4A, 4B) with a mean current of 453 pA ($n = 32$) at 30 mV. At depolarization to +30-mV amplitude, currents increased in cells maintained in culture from 272 ± 96 pA

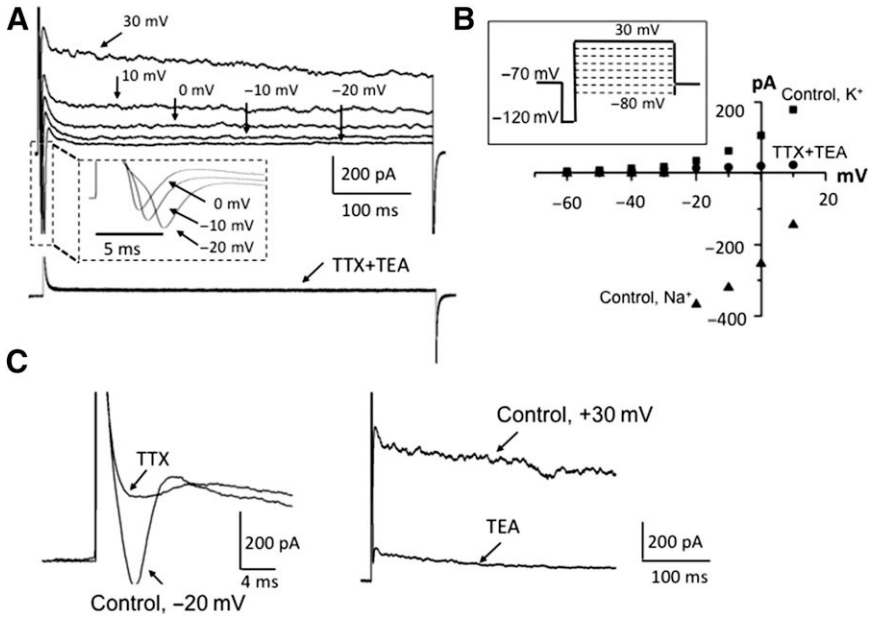

Figure 4. Electrophysiological properties of human induced pluripotent stem cell (hiPSC) differentiated neurons. **(A):** Representative example (upper panel) of currents evoked by depolarizing voltage steps indicated above the traces (see scheme of the protocol in 4B, insert); expanded from dashed lines (insert), rectangular traces of fast-activating, fast-inactivating inward currents evoked by depolarizing voltage steps, as indicated; example (bottom traces) of currents evoked by the same set of depolarizing voltage steps as indicated above after 1-minute preapplication of TTX (1 μM) plus TEA (20 mM). Note the strong inhibition of both inward and outward components. **(B):** Current-voltage relations of outward K^+ (■) and inward Na^+ (▲) in control conditions and after 1-minute application of 1 μM TTX + 20 mM TEA (●); scheme of depolarizing protocol (insert) for recording current-voltage relations. In the different tests, pulse protocol was the same. **(C):** Examples of single traces illustrating separate inhibition of voltage-gated Na^+ currents by 1 μM TTX (left) and K currents by 20 mM TEA (right). Note different time scales. Abbreviations: TEA, tetraethylammonium; TTX, tetrodotoxin.

(n = 5 at day 7 of differentiation) to 694 ± 52 (n = 3 at day 35 of differentiation). These currents were reversibly blocked by 20-mM tetraethylammonium (TEA) (Fig. 4A–4C), which confirmed its K^+ nature.

Fast-inactivating inward components, typical of neuronal Na^+ currents were also observed in response to depolarizing pulses (Fig. 4A). Following a 100-ms hyperpolarizing prepulse to −120 mV to relieve Na^+ channel inactivation, voltage steps ranging from −80 to +30 mV with 10-mV intervals (scheme of stimulation shown in Fig. 4B, insert) evoked inward currents; the threshold of activation was about −30 mV, and maximal inward amplitude was reached at about −20 mV (Fig. 4A, 4B). These currents were reversibly blocked by the specific antagonist of voltage-gated Na^+ channels, tetrodotoxin (TTX) (Fig. 4A–4C). Simultaneous application of TTX and TEA suppressed both components of voltage-gated currents (Fig. 4A), indicating that these cells exhibit neuronal properties and are able to generate and propagate action potentials.

We investigated whether these cells express receptor-operated channels. Three agonists—glycine, acetylcholine (ACh), and GABA—were applied using a fast perfusion system. GABA- and glycine-evoked currents were recorded 7 days after differentiation (Fig. 5C, 5D). With 20-mM Cl^- in the intracellular solution, reversal potential obtained from current voltage relations of GABA-induced currents was about −50 mV (Fig. 4B), which is close to the reversal potential for Cl^- in similar conditions. Half maximal effective concentration (EC_{50}) for GABA-induced currents was determined from dose-response relationships (Fig. 5C). It varied from 6 μM to 65 μM with a mean value of 22 ± 4 μM (n = 18). Sensitivity of GABA receptors to agonist varies strongly depending on subunit composition, localization, and developmental stage [11–14]. In our experiments, a tendency toward decreasing sensitivity with age in culture was observed. At the eighth day after induction of differentiation, for example, EC_{50} for GABA was 19 ± 4 (n = 6), whereas at the 21st day, we observed EC_{50} of 35 ± 19 (n = 3). This coincides with the developmental profile observed in thalamic nucleus neurons [13].

As described previously, neuronal ionotropic GABA and glycine receptors exhibit similar Cl^- selectivity [15]. In our study, from 25 cells on which GABA and glycine were simultaneously tested, 21 cells showed responses to GABA and glycine, whereas on 4 other cells, responses to both neurotransmitters were absent. First responses for ACh application were observed after 21 days following differentiation (Fig. 5A). At this stage, expression of ACh receptors coincided with those for glycine and GABA for all examined cells (n = 10). Reversal potential for acetylcholine-induced currents was about 0 mV (data not shown), indicating cation selectivity of these channels, as described previously [16]. Altogether, 82% of cells replied to GABA (37 of 45 cells), 71% responded to glycine (27 of 38 cells), and 25% responded to acetylcholine (10 of 40 cells).

Discussion

These data indicate that after plating on laminin, neuronal progenitors are differentiated into mature neurons that can be maintained for up to 35 days without loss of membrane property, as indicated by patch clamp recording. In addition, these cells can be differentiated toward specialized neurons (e.g., dopaminergic) in the presence of specific cytokines.

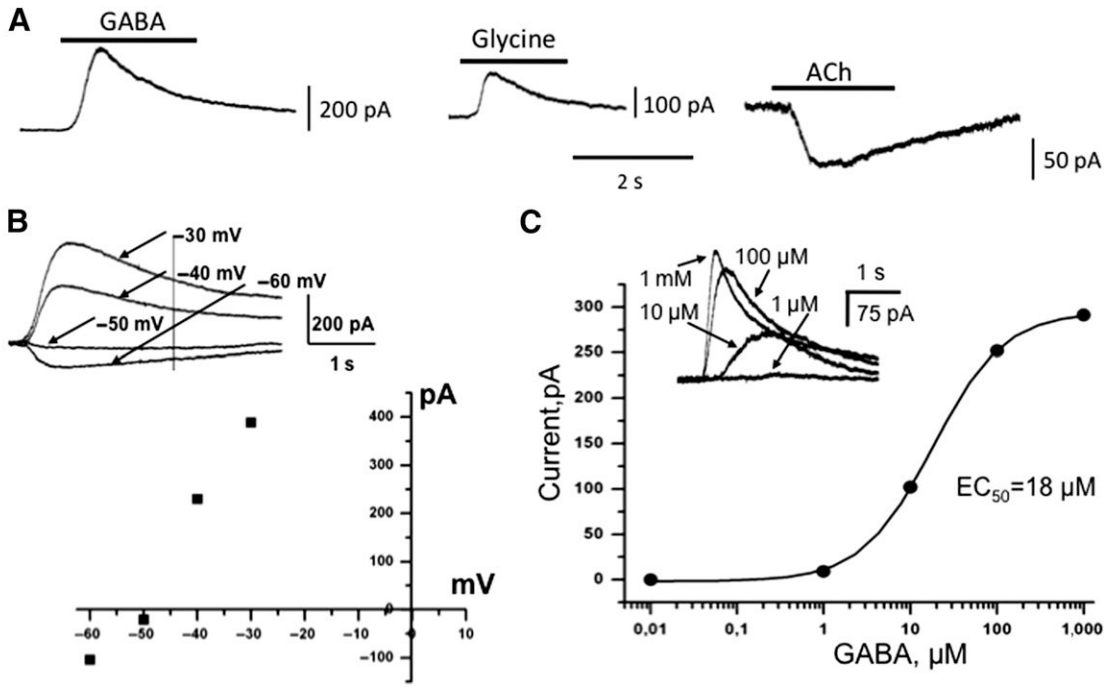

Figure 5. Expression of neurotransmitter-activated channels in human induced pluripotent stem cell (hiPSC) differentiated neurons. **(A):** Representative examples of ionic currents induced by application of 1 mM GABA, 1 mM glycine, or 100 μM ACh in differentiated hiPSCs. Whole-cell recordings with intracellular solution containing 20 mM Cl^- at holding potential 0 mV (for GABA and glycine) and at −40 mV for ACh. Bars above traces indicate the time of agonist application. **(B):** Current-voltage relationship of the GABA-evoked inward current. Example (top) of superimposed traces of GABA-evoked currents at different potentials. Reversal potential was approximately −50 mV, as predicted from Nernst equation for intracellular solution containing 20 mM KCl. **(C):** Dose-response dependency and superimposed traces (insert) of whole-cell currents induced by rapid application of different concentration of GABA. Recording at 0 mV. Abbreviations: ACh, acetylcholine; EC_{50}, half maximal effective concentration; s, seconds.

Self-renewing human embryonic stem cells and hiPSCs have the potential to differentiate into any cell type, representing an invaluable source of biological material, particularly for regenerative medicine. In numerous cases, however, the use of these cells in translational medicine is hampered by the efficiency and the low scale of the differentiation process.

We have described a novel and efficient protocol for the differentiation of hiPSCs into neuronal cells. Our protocol requires no feeder layers. Furthermore, compared with other published protocols, our procedure does not necessitate embryoid bodies followed by rosette (primitive neuroepithelial cells) and neurosphere formation [17–21], which might modify purity of the cell population; drug addition, which might perturb cellular homeostasis [20–24]; or cell sorting, limiting the quantity of differentiated cells available. Moreover, we believe that the method described reduces the overall cost of the differentiation process because our protocol does not require BDNF, Noggin, NT3, or GDNF, as described [18, 20, 21, 25], but only two cytokines at initial differentiation steps. Furthermore, it is not necessary to perform cell sorting, and our method yields large quantities of neuronal progenitors, which can be maintained and regularly expanded or further differentiated in only 10–15 days, limiting the quantity of reagents necessary for the production of neuronal progenitors or mature neurons.

These neuronal cells express different neuronal markers together with the Na^+ and K^+ voltage-operated channels and are able to generate and propagate action potentials. Moreover, these cells expressed different types of receptor-operated channels such as the Cl^--selective GABA receptors, responsible for the main inhibitory synaptic drive in the central nervous system.

Conclusion

A growing number of reports describe hiPSC-based models of constitutive disorders for understanding the pathological mechanisms of these different diseases and allowing their use in drug discovery and, potentially, cell therapy. Our method can be used to produce large amounts of mature neurons, including dopaminergic neurons. Our strategy provides a valuable tool for studying neuronal differentiation pathways and synaptic and postsynaptic responses and for testing pharmacological treatments. This approach could open the way to understanding a large number of pathologies including neurodevelopmental and neurodegenerative diseases.

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Author Contributions

C.B.: conception and design, collection and assembly of the data, data analysis and interpretation; G.M.: collection and assembly of the data, data analysis and interpretation; C.E.-Y., E.B., and P.T.: provision of study materials; M.L.: collection and assembly of the data; B.B.: financial support, final approval of the manuscript; P.B.: conception and design, collection and assembly of the data, data analysis and interpretation, manuscript writing; F.M.: conception and design, collection and assembly of the data, data analysis and interpretation, manuscript writing, financial support.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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DISCUSSION

Functioning of CNS is determined by interaction of two main synaptic signals – excitation and inhibition. Glycine receptor is an important component of the system of fast inhibition in CNS. They are mainly localized in spinal cord and brain stem, where they play a crucial role in regulation of motoneurons activity, respiration and pain perception (Schmid et al., 1991; Malosio et al., 1991; Harvey et al., 2004). Glycine receptors also expressed in different zones of forebrain and participate in the control of neuronal networks excitability (Chattipakorn and McMahon 2002; Brackmann et al., 2004).

Pharmacological modulation of glycine receptors

Ginkgolic acid – a novel positive modulator of glycine receptors

It has been shown that glycine receptors are potentiated by several pharmacological agents, such as ivermectine, ethanol, anesthetics, and canabinoids (Webb and Lynch, 2007; Yevenes and Zeilhofer, 2011). However, these molecules have a low specificity, as they interact with other ion channels of CNS (Lynagh and Lynch, 2012; Yevenes and Zeilhofer, 2011). Thus, the search for specific modulators of glycine receptors is of the great importance.

We have shown that ginkgolic acid is a highly efficient potentiator of α1 glycine receptors, being active in nanomolar concentrations (30-100 nM). High concentration of ginkgolic acid (25μM) provoked small inhibition of α2 glycine receptors, while the amplitude of currents, mediated by α3 and $GABA_A$ receptors was not influenced. Action of ginkgolic acid on heteromeric α1β and α2β receptor subtypes was similar to the one demonstrated on homomeric variants: potentiation of α1β currents and small inhibition of α2β. Thus, our data provide evidence for the selective potentiation of α1 glycine receptors by ginkgolic acid.

Detailed investigation of ginkgolic acid interaction with α1 subunits of glycine receptor allowed to determine some of its features: (i) potentiation was accompanied by the shift of concentration dependency curves to the left and by the decrease of EC_{50} for glycine, which suggests the ability of ginkgolic acid to increase the mean duration of channel open-time; (ii) effect of potentiation developed slowly and it depended on the concentration of the acid: upon application of 30 nM of ginkgolic acid, the potentiation was observed after 2-3 min, while the high concentration of ginkgolic acid provoked the current increase on 150% already after 30 sec of application.

Recovery of the glycine-evoked currents amplitude during the wash-out of the acid was slow and usually not complete. There are several possible reasons of this

phenomenon: (i) the effect could be produced due to the slow detachment of the acid from its binding site on the receptor and (ii) due to the accumulation of the acid in its binding site.

In order to determine the interaction site of ginkgolic acid with α1 glycine receptor we have investigated amino acid sequences of different receptor subunits, using a protein sequences data base UniProt, and also analyzed previous works that were focused on the determination of amino acid residues involved in the modulation of glycine receptors.

Amino acid residue A52, that is located in the extracellular domain of glycine receptor, participates in interaction of α1 subunits with ethanol. It has been shown previously that ethanol potentiates α1 receptors to much higher extent than α2 receptors. Amino acid substitution in α1 subunit (when alanine was substituted for characteristic for α2 serine) leads to the decrease of the efficiency of ethanol binding (Mascia et al., 1996). It allowed suggesting that A52 is involved in forming of the site of receptor interaction with ethanol. Later it was demonstrated that residues located in TM2 and TM3 domains, S267 and A288 respectively, are also implicated in the ethanol effect, probably by forming another site (Mihic et al., 1997).

TM2 and TM3 domains of different subunits of glycine receptors are highly conservative. TM2 domains of each subunit participate in forming of the channel pore, while TM3 domains are also in contact with bilayer membrane. Amino acid sequences of α1 and α2 subunits differ only by two positions: by one residue in TM2 and one residue in TM3 domains. It seems that these two positions are responsible for different sensitivity of α1 and α2 receptors to several modulators.

Receptors formed by α1 and α2 subunits have different sensitivity to pore blocker CTB – it can efficiently inhibit α1 receptors but it weakly interacts with α2 subunits (Rundstrom et al., 1994). Substitution of amino acid G254 for A in TM2 greatly decrease the affinity of α1 receptors to CTB. Besides that, the amino acid residue situated at the position 254 determines the main conductance state of the channel. Substitution of glycine for alanine in α1 receptors provokes appearance of additional conductance (Bormann et al., 1993).

Cannabinoid Δ^9 –tetrahydrocannabinol, a main psychoactive component of marijuana, can directly modulate the activity of glycine receptors, by increasing the amplitude of currents mediated by α1 and α3 subunits, but not by α2 subunit. Xiong and co-workers (2011) have shown that TM3 domain plays an important role in subunit selectivity of Δ^9 –tetrahydrocannabinol. In particular, amino acid residues S296 (α1) and S307 (α3) are implicated in formation of hydroxyl bond with OH group

of Δ^9 –tetrahydrocannabinol, providing a structural basis for positive modulation of α1 and α3 receptors (Xiong et al., 2011).

In the present work we have shown that difference in α1 and α2 receptors responses to application of ginkgolic acid is determined by tree amino acid residues different in these two subunits of glycine receptor. Mutation T59A/A261G/A303S – substitution of three amino acids in α2 subunit for corresponding ones from α1 imparted α2 receptors with sensitivity to ginkgolic acid.

Our investigation has demonstrated, for the first time, the role of amino acid residues A52, G254 and S296 in potentiation of α1 receptors by ginkgolic acid and confirmed their importance for the formation of sites of glycine receptor interaction with modulators.

Niflumic acid – inhibitor of glycine receptors

Searching for new modulators we have determined that niflumic acid, a well known blocker of Cl-selective voltage-gated channels, can inhibit the activity of glycine receptors. Our results suggest the pore-blocking voltage-dependent mechanism of niflumic acid action on glycine receptors.

Homomeric α1, α2 and α3 glycine receptors have different sensitivity to niflumic acid: α1 receptors demonstrated the highest IC_{50} = 197±18 μM (n=10) at +80mV, while for α2 and α3 glycine receptors it comprised 9±2 μM (n=8) and 16±6 μM (n=7) respectively. Inhibition of all three subunits was dependent on the membrane potential – its efficiency was much higher at positive potentials. This effect was especially prominent for α2 subunits. Based on voltage-dependence of NFA inhibitory action we have suggested that site of its interaction with the receptor is situated in the channel pore, formed by TM2 domains of each subunit.

Studying interaction of niflumic acid with α1 glycine receptors we have noticed that efficiency of their interaction depends on the concentration of the agonist – with the increase of glycine concentration IC_{50} for niflumic acid was decreasing, especially at positive potentials. Increase of glycine concentration till 100μM provoked decrease of NFA IC_{50} till 90±8 μM (n=10), which is significantly lower than IC_{50} obtained at co-application of NFA with 30μM of glycine.

This effect was not characteristic for the α2 receptors – increase of glycine concentration did not provoke decrease of NFA IC_{50}. We suggest that it might be caused by the difference in the kinetics of channel opening for α1 and α2 subunits. The

study of single channel kinetics of α1 and α2 receptors demonstrated that mean duration of channel open time for α2 homometic glycine receptors is nearly 100 times longer than for α1 glycine receptor (α1 – 2.38 msec; α2 – 174 msec) (Takahashi et al., 1992). Presumably, in the case of α1 receptors, with increase of the agonist concentration mean duration of the open state of the channel increases, which facilitates the access of the blocker to the pore. This effect was not observed for α2 receptors – an increase of the agonist concentration did not influenced efficiency of the block. We suggest that at α2 receptors even low concentration of glycine activate channels for the mean open duration sufficient for the development of NFA maximum effect.

Ionic channel of glycine receptor is formed by TM2 domains of each of 5 subunits that compose a receptor (Lynch, 2004). Base on the pore-blocking mode of NFA action we suggested that this domain plays a crucial role in the interaction of niflumic acid with glycine receptor. Analysis of amino acid sequences of TM2 domains of α1, α2 and α3 subunits demonstrated that they differ only by 2' position of the pore (254 in α1 subunit). At that position α1 receptors have glycine, while α2 and α3 – alanine. Presumably, this position determines the difference in the sensitivity of different receptor subunits to niflumic acid.

To check this hypothesis we have performed a point mutation in α1 subunit, exchanging G254 for A (mutation G254A). Electrophysiological analysis of niflumic acid action on mutant α1 receptors have demonstrated that (i) their interaction was voltage-dependent; (ii) α1 G254A receptors are more sensitive to niflumic acid than α1 WT receptors; (ii) similarly to α2 receptors IC_{50} of niflumic acid did not depend on the concentration of glycine. However, this mutation did not entirely increase the sensitivity of α1 receptors to the one for α2 subunit. Accordingly, we suggest that glycine receptor has several sites of interaction with niflumic acid.

Studying interaction of niflumic acid with heteromeric (αxβ) we have shown that incorporation of β subunit does not have a prominent effect on the sensitivity of α1 and α2 receptors to niflumic acid.

In the current research we have demonstrated that niflumic acid inhibits glycine receptors of different subunit composition. We have succeeded to identify one of the sites of interaction of the receptor with niflumic acid and to confirm an important role of 2' amino acid of TM2 domain for the channel blockers activity.

Earlier it was shown that this amino acid participates in interaction of glycine receptors with other modulators. In particular, mutation G254A imparted α1 receptors with higher sensitivity to an inhibitor tutine (Fuentealba et al. 2011). Moreover,

difference in main conductance states of channels formed by α1 (86pS) and α2 subunits (111pS) also determined by this amino acid (Bormann et al., 1993). Point mutation G254A in α1 receptor resulted in appearance of new sub-conductive state and increasing of main single channel conductance to 107pS.

Thus, several of our results suggest the pore-blocking mode of niflumic acid action on glycine receptors. In particular, (i) voltage-dependence of niflumic acid action; (ii) for α1 receptors the augmentation of the blocking efficiency upon increase of the glycine concentration was observed; (iii) mutation G254A in TM2 domain of α1 subunit provoked an increase in its sensitivity to niflumic acid.

Neurons generated from human fibroblasts are promising system for studying glycine receptors

In the current work we have investigated a possibility to use neurons generated from induced pluripotent stem cells, obtained from human fibroblasts, for studying the glycine receptors. We have shown that induced neurons, generated using the protocol developed by Badja et al., 2014, are functional and capable to generate action potentials. They express on their surface voltage-gated sodium and potassium channels, as well as ionotropic ligand-gated receptors: glycine-, nicotinic acetylcholine- and GABA-receptors.

In the course of our work new simplified and highly efficient protocol of differentiation of neurons from IPSCs was developed. A possibility to obtain functional neurons from human fibroblasts that possess all genetic characteristics of a specific organism opens new opportunities for investigation of molecular mechanisms of neurological diseases and their treatment. In particular, this method can be used for studying hereditary disease hyperekplexia caused by dysfunction of glycine receptors.

Hyperekplexia – is a neurological disease caused by mutations of genes that code α1 and β subunits of glycine receptor. Characteristic symptom of hyperekplexia is a long lasting non controlled contraction of muscles in response to unexpected stimuli that can be accompanied by unprotected fall. The reason for hyperekplexia is the inability of mutant glycine receptors to provide efficient inhibitory neurotransmission in the spinal cord (Shiang et al., 1993). Mostly hyperekplexia mutations are localized in TM-2 domain or in linkers connecting trans-membrane domains. Amino acid substitutions in TM2 domain influence ion conductance and disturb the process of channel opening, decreasing amplitudes of currents mediated by glycine receptors (Laube et al., 2002).

Nowadays, the study of the mechanisms of hyperekplexia origination involves: determination of glycine receptor mutations from patients and electorphysiological analysis of mutant receptors, using heterologous expression system. A use of heterologous systems complicates interpretation of obtained results and does not allow studying disturbances in expression and traffic of the receptor.

Thus, a use of neurons generated from fibroblasts of patients that carry mutations of glycine receptors will allow characterization of molecular mechanisms of hyperekplexia in native system. It will as well facilitate a search for the development of individual therapy.

CONCLUSIONS

In the current work using methods of mammalian cells culture and electrophysiological analysis we have studied pharmacological modulation of glycine receptors and functional expression of ion channels by neurons generated from human fibroblasts.

Based on the results of our investigation we have made following conclusions:

1. Ginkgolic acid, a component of *Ginkgo biloba* extract, in nanomolar concentrations selectively potentiates currents mediated by α1 glycine receptors, without affecting other subunits of the receptor. Amino acid residues A52, G254 and S296 play a key role in the interaction of α1 glycine receptors with ginkgolic acid.
2. Niflumic acid is a voltage-dependent blocker of the glycine receptor channel. Its affinity is higher to α2 and α3 subunits of the receptor in comparison with α1. Amino acid substitution G254A increases sensitivity of α1 receptors to niflumic acid.
3. Neurons generated from human fibroblasts express on their surface voltage-gated and ligand-gated channels, in particular glycine receptor. This suggests the possibility to use neurons generated from human fibroblasts to study dysfunctions of glycinergic neurotransmission.

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