Phosphorylation of the $\alpha 2$ glycine receptor induces an extracellular conformational change and slows the rise and decay rates of glycinergic synaptic currents

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Author contributions

S.I. – performed experiments, analysed and interpreted data

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Abstract

The $\alpha 2$ glycine receptor (GlyR) is a pentameric ligand-gated anion channel that plays a key role in cortical interneuron migration and in the differentiation of cortical progenitor cells into functional neurons. It also mediates tonic inhibitory chloride currents in adult forebrain neurons. Disruption of $\alpha 2$ GlyR gene expression or receptor function results in the aberrant functioning of neuronal circuits which contributes to the pathophysiology of schizophrenia, autism and epilepsy. This implicates the $\alpha 2$ GlyR as a possible therapeutic target for a range of neurological disorders. However, despite its therapeutic potential, little is known about the mechanisms by which $\alpha 2$ GlyRs are functionally modulated. To address this, we investigated whether the $\alpha 2$ GlyR is modulated by phosphorylation at a serine residue (S341) within the same PKA consensus sequence (R-E-S-R) that houses the $\alpha 3$ GlyR S346 residue that is known to be phosphorylated by PKA. Resolving this question might uncover a novel means of physiologically, pathologically or therapeutically modulating $\alpha 2$ GlyRs. We show using voltage-clamp fluorometry that forskolin-induced phosphorylation of S341 induces a conformational change in the glycine binding site. We also employed glycinergic artificial synapses to demonstrate that the S341E phospho-mimetic mutation slows the rise and decay rates of $\alpha 2$ -mediated glycinergic inhibitory postsynaptic currents. These results suggest that PKA phosphorylation alters the structural and functional properties of the $\alpha 2$ GlyR. This information may help to identify new mechanisms by which $\alpha 2$ GlyRs may be pathologically modified or therapeutically targeted for the treatment of neurological disorders.

Introduction

Ligand-gated ion channels (LGICs) allow cells to respond rapidly to changes in their external environment. Members of the pentameric ligand-gated ion channel (pLGIC) receptor family are composed of five protein subunits that form a pentameric arrangement around a central ion conducting pore. The major vertebrate members of the family include the cation permeable nicotinic acetylcholine receptor (nAChR) and serotonin type-3 receptor (5HT₃R), and the anion permeable GABA type-A (GABA_AR) and glycine receptor (GlyR). Each subunit consists of large N-terminal ligand-binding domain that incorporates orthosteric ligand-binding sites at the interface of adjacent domains. The ligand-binding domain is coupled to a transmembrane domain that comprises four transmembrane α -helices, termed M1-M4, that each span the full thickness of the cell membrane. Each subunit contributes an M2 domain to the lining of the axial water-filled pore. The M1, M3 and M4 domains are arranged concentrically around the M2 domain bundle and thereby help to isolate the water-filled pore from the non-polar lipid membrane. M1, M2 and M3 are connected by short intracellular and extracellular loops, respectively, whereas M3 and M4 are connected by a large intracellular domain that varies considerably in both length and amino acid sequence among different family members. The M3-M4 domain contains phosphorylation sites and binding sites for synaptic clustering and trafficking proteins.

GlyRs mediate fast inhibitory synaptic transmission in neurons of the spinal cord, brain stem, caudal brain and retina [1-3]. A total of five GlyR subunits are known: α 1- α 4 and β . However, the human α 4 subunit is a pseudogene due to a premature stop codon at the intracellular end of M4 that prevents the expression of full length transcripts [4]. All other α subunits function as homomeric receptors when recombinantly expressed, but the β subunit is functional only when incorporated with α subunits into $\alpha\beta$ heteromeric receptors. Synaptic GlyRs comprise heteromeric $\alpha\beta$ GlyRs, with the β subunit required for synaptic localization due to its ability to bind to the subsynaptic clustering protein, gephyrin [5-7].

The $\alpha 1\beta$ GlyR is the dominant isoform in the adult central nervous system. It is best known for mediating glycinergic inhibitory transmission in adult spinal cord motor reflex arcs [2]. Consistent with this role, hereditary mutations to either $\alpha 1$ or β genes result in a severe motor disorder termed hyperekplexia (or startle disease) that is characterised by an exaggerated reflex startle response to unexpected stimuli, which is often accompanied by temporary but complete muscular rigidity [8].

The $\alpha 3\beta$ GlyR mediates inhibitory neurotransmission in spinal nociceptive neurons in superficial laminae of the spinal cord dorsal horn [9-11]. Chronic inflammatory pain sensitization is caused by a prostaglandin E_2 (PGE_2)-mediated activation of protein kinase A (PKA), which in turn phosphorylates $\alpha 3$ GlyRs at S346, leading to a diminution of glycinergic inhibitory postsynaptic current (IPSC) magnitude [10]. This removal of inhibitory drive results in the disinhibition of spinal nociceptive sensory neurons which in turn induces chronic inflammatory pain sensitization. Because of their role in restraining nociceptive neuronal firing and their sparse distribution outside the spinal cord dorsal horn, $\alpha 3$ GlyRs have emerged as promising therapeutic targets for chronic pain, and indeed, agents that restore (i.e., potentiate) $\alpha 3$ GlyR function have been shown to exhibit analgesic efficacy in animal models of chronic inflammatory pain [11-17].

GlyR $\alpha 2$ transcripts predominate in the neonatal and embryonic central nervous system, and are replaced postnatally by those of $\alpha 1$ and, to a lesser extent, $\alpha 3$ GlyRs, by adulthood [18]. Embryonic cortical interneurons express $\alpha 2$ GlyRs at high levels during their initial migratory phase, and knockout of these receptors results in neuronal migration defects [19]. Genetic ablation of the $\alpha 2$ GlyR gene also impairs the production of mature neurons from cortical progenitor cells which reduces the number of excitatory projection neurons in deep and upper layers of the cerebral cortex, thereby resulting in reduced brain size [20]. The $\alpha 2$ GlyR also mediates tonic inhibitory chloride currents in adult forebrain neurons [21], and disruptions to its function would alter the excitability of forebrain neuronal circuits. All these effects work in concert to disrupt neuronal circuit development and function [22] which in turn contribute to the pathophysiology of schizophrenia, autism and epilepsy [23]. Consistent with this, hereditary mutations in the human $\alpha 2$ GlyR gene are associated with autism spectrum disorder [24-27]. Together, this information implicates the $\alpha 2$ GlyR as a potential

therapeutic target for a range of neurodevelopmental disorders. To date, however, there has been little attempt to identify unique features of druggable binding sites in this receptor that could be exploited in designing drugs specific for $\alpha 2$ over the highly homologous $\alpha 1$ and $\alpha 3$ GlyR isoforms.

Moreover, despite their importance in neurodevelopment, little is known about the physiological or pathological mechanisms of $\alpha 2$ GlyR modulation. For example, it is as yet unclear whether the $\alpha 2$ GlyR is modulated by phosphorylation. It is known, however, that the $\alpha 2$ GlyR incorporates a serine residue (S341) within the same PKA consensus sequence (R-E-S-R) that houses the $\alpha 3$ GlyR S346 residue that is known to be phosphorylated by PKA [10, 28]. Resolving this question might uncover a possible alternate means of physiologically, pathologically or therapeutically modulating $\alpha 2$ GlyRs.

The aim of the present study was to determine whether $\alpha 2$ GlyRs are phosphorylated by PKA, and if so, whether this induced a conformational change in the glycine binding site, as has previously been shown for the homologous phosphorylation site in the $\alpha 3$ GlyR [28]. To achieve this, we employed voltage-clamp fluorometry (VCF) [29] to directly monitor phosphorylation-mediated conformational changes in the extracellular glycine binding site. We also employed glycinergic artificial synapses [30, 31] to determine whether phosphorylation may alter the functional properties of the inhibitory postsynaptic currents (IPSCs) mediated by $\alpha 2$ GlyRs.

Materials and Methods

Chemicals

Methanethiosulfonate-rhodamine (MTSR) and 2-((5(6)-tetramethylrhodamine)carboxylamino)ethyl methanethiosulfonate (MTS-TAMRA) were obtained from Toronto Research Chemicals. Glycine, β -alanine, taurine, strychnine, forskolin, were all obtained from Sigma. Glycine, β -alanine, taurine and strychnine were dissolved in water. All other drugs were prepared as 20-100 mM stocks in dimethylsulfoxide and kept frozen at -20 °C. From these stocks, solutions for experiments were prepared on the day of recording.

Molecular biology

For *Xenopus* oocyte recordings, the plasmid DNA encoding the human α 1 and α 2 GlyRs were subcloned into pGEMHE, a plasmid vector optimized for *Xenopus* oocyte expression. Site directed mutagenesis was performed using the QuikChange mutagenesis kit (Stratagene). Successful incorporation of the mutations was confirmed through automated sequencing of the entire cDNA coding region. Chimera-1 was constructed using a multiple-template-based sequential PCR protocol as previously described [32]. The join site between the α 1 and α 2 sequences used to create chimera-1 was located between α 1 Y223/L224 and α 2 Y223/L224 at the N terminal end of M1. Chimera-1 also included the α 1-N203C mutation.

Ten micrograms of each cDNA was linearized by NheI or PstI and then purified by PCR-purification kit (Qiagen). The capped RNAs were transcribed from cDNA using the Ambion T7 mMessage mMachine kit, purified by RNAMinikit (Qiagen) eluted with DNA/RNAase free water and diluted to 200 ng/ μ l for oocyte injection. For HEK293 cell transfections, we employed plasmid DNA encoding the human α 2 GlyR (in pCIS2), the mouse neuroligin 2A splice variant (in pNice) and empty pEGFP plasmid. Site-directed mutagenesis was performed using the QuikChange mutagenesis kit, and the successful incorporation of mutation was confirmed by DNA sequencing.

Oocyte preparation, injection and labeling

All *Xenopus laevis* handling procedures were approved by the University of Queensland Animal Ethics Committee (approval numbers: QBI/059/13/ARC/NHMRC and QBI/AIBN/087/16/NHMRC/ARC). Female *Xenopus* frogs (Xenopus Express) were anaesthetized with 5 mM MS-222 (Sigma Aldrich) and stage VI oocytes were removed from ovaries and washed thoroughly in OR-2 (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES, pH 7.4). The oocytes were then incubated in collagenase (Sigma Aldrich) in OR-2 for 2 hr at room temperature, rinsed and stored in OR-2 at 18 °C.

All oocytes were injected with 10 ng of mRNA into the cytosol. To achieve the high levels of expression required for the detection of the fluorescent signal over the background (due to oocyte autofluorescence and non-specific binding of the dye) the oocytes were incubated at 18 °C for 3-10 days after injection. The incubation solution contained 96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, 0.6 mM theophylline, 2.5 mM pyruvic acid, 50 µg/ml gentamycin (Cambrex Corporation) and 5 % horse serum (Hycell), at pH 7.4. On the day of recording, the oocytes were transferred into ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, pH 7.4) and stored on ice. To label with either MTSR or MTS-TAMRA, oocytes were transferred into the labeling solution containing 10 µM of either compound in ND96 for 25 s. The oocytes were then washed and stored in ND96 for up to 6 hr before recording. All labeling steps were performed on ice.

VCF

We employed an inverted microscope (Ti-S, Nikon Instruments) equipped with a DAPI filter set (49000, Chroma Technology) and a CFI Fluor 40X water immersion objective (N.A. 0.80, MRF07420, Nikon Instruments). A Lambda LS 175 W xenon arc lamp served as a light source and was coupled to the microscope via a liquid light guide (Sutter Instruments). Fluorescence was detected using a H7360-03 photomultiplier (Hamamatsu Photonics) coupled to a PMT400R photomultiplier subsystem (Ionoptix). Oocytes were placed securely in the bath and were continually perfused with ND96 solution. Agonists were dissolved in the same solution and were applied to the oocytes via a gravity-fed perfusion system. Currents were recorded via two-electrode voltage clamp using 0.2-2 MΩ

resistance glass pipettes filled with 3 M KCl. Oocytes were voltage-clamped at -40 mV and currents were recorded using a Gene Clamp 500B amplifier (Molecular Devices). ΔI and ΔF signals were digitized at 2 kHz via a Digidata 1322A interface and pCLAMP 9.2 software (Molecular Devices).

Primary culture of spinal neurons

Spinal neurons were prepared using methods as previously described [30]. Briefly, E15 timed-pregnant rats were euthanized via CO₂ inhalation in accordance with procedures approved by the University of Queensland Animal Ethics Committee (QBI/142/16/NHMRC/ARC). The spinal cords were rapidly removed, triturated and plated onto poly-D-lysine-coated coverslips in a 4-well plate at a density of $8\text{--}10 \times 10^4$ cells/well, and cultured for 3-4 wk until spontaneous inhibitory postsynaptic currents (IPSCs) could be detected. The cells were initially cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% foetal bovine serum (DMEM-FBS). After 24 h the entire DMEM-FBS medium was replaced with Neurobasal medium including 2% B27 and 1% GlutaMAX supplements. A second (and final) feed 1 wk later replaced half of this medium with fresh Neurobasal medium. Neurons were used in co-culture experiments between 1-4 wk later.

HEK293 cell culture, transfection and artificial synapse formation

HEK293 cells were cultured in DMEM-FBS until $\sim 90\%$ confluent. One day prior to transfection, they were trypsinized and plated onto glass coverslips in 35 mm culture dishes at a density of 5×10^3 cells/dish. For artificial synapses, we transfected plasmid DNA encoding the $\alpha 1$ or $\alpha 2$ GlyR, EGFP and neuroligin 2A in equal ratios. Transfection was performed via a Ca²⁺ phosphate-DNA co-precipitation method for 15-20 h in a 3% CO₂ incubator and terminated by washing cells twice with divalent cation-free phosphate buffered saline. For artificial synapse experiments, transfected cells were trypsinized the next day, centrifuged and re-suspended in Neurobasal medium (including 2% B27 and 1% GlutaMAX supplements) then seeded onto the neurons. One 35 mm dish of HEK293 cells was typically sufficient to seed four coverslips of neurons. Once seeded with HEK293 cells, the co-cultures were returned to the incubator overnight to allow artificial synapses to

form between neurons and transfected HEK293 cells. Cells were used for patch-clamp recording over the following 1-3 d.

Patch clamp electrophysiology

Cells were viewed using an inverted microscope and currents were recorded by whole-cell patch-clamp recording. Cells were perfused by an extracellular solution containing (in mM): 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES/ NaOH and 10 glucose (pH 7.4 adjusted with NaOH). Patch pipettes had a tip resistance of 1- 4 MΩ when filled with the standard intracellular solution consisting of (mM): 145 CsCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES and 10 EGTA (pH 7.4 adjusted with CsOH). In experiments where forskolin was applied to HEK293 cells in an attempt to phosphorylate α₂ GlyRs, the intracellular solution consisted of (mM): 145 CsCl, 2 CaCl₂, 2 Mg-ATP, 0.2 Na-GTP, 10 HEPES and 10 EGTA (pH 7.4 adjusted with CsOH).

For isolated HEK293 cell whole-cell recordings, cells were voltage clamped at -40 mV and membrane currents were recorded using Multiclamp 700B, a Digidata 1440A and pClamp 10 software (Molecular Devices). Currents were filtered at 1 kHz and digitized at 2 KHz. Solutions were applied to cells via gravity forced perfusion and parallel microtubules and manual control of this system was achieved via a micromanipulator with a solution exchange time < 250 ms. For artificial synapse recordings, cells were voltage clamped at -70 mV and membrane currents were recorded using Multiclamp 700B, a Digidata 1440A and pClamp 10 software (Molecular Devices). Currents were filtered at 4 kHz and digitized at 10 KHz. Experiments were conducted at room temperature (19-22 °C). Recordings with series resistance above 20 MΩ were discarded. Capacitance of the HEK293 cells was typically 20 pF, resulting in a typical corner frequency of 398 Hz. Because this was satisfactory for our experiments, series resistance compensation was not applied. Sliding templates in Axograph X were used to identify well-isolated IPSCs. 10-90% rise times were recorded and mono-exponential simplex fits applied to the decay period of individual events, which were then averaged for each cell.

Data analysis

EC₅₀ and n_H values for glycine-induced activation of current (ΔI) and fluorescence (ΔF) signals were obtained using the empirical Hill equation, fitted with a non-linear least squares algorithm (SigmaPlot 12.0, Systat Software). All results are expressed as mean $\pm$ SEM. Data sets were first tested for normal distribution prior to using ANOVA tests to determine statistical significance, with $p < 0.05$ as the significant threshold (SigmaPlot 12.0).

Results

VCF experiments reveal extracellular structural differences between $\alpha 1$ and $\alpha 2$ GlyRs

VCF involves introducing a cysteine into a location of interest in an otherwise cysteine-free protein [29]. The cysteine is then reacted with an thiol-reactive fluorophore, typically containing a methanethiosulfonate (MTS) or maleimide moiety. A key requirement is that the spectral emission properties of the fluorophore varies according to the hydrophobicity of their environment. This means that protein conformational changes occurring in the vicinity of the fluorophore can be reported in real time as fluorescence changes. Here we employed VCF to compare glycine-induced conformational changes in the N-terminal domains of $\alpha 1$ and $\alpha 2$ GlyRs in the absence and presence of phosphorylation. For this purpose we selected two sites (N203C and R271C using $\alpha 1$ GlyR numbering), both of which have been successfully labelled by rhodamine derivatives in previous VCF studies on $\alpha 1$ and $\alpha 3$ GlyRs [28, 33, 34]. Fig 1A shows a structural model of an $\alpha 1$ GlyR subunit displaying the location of both sites. The $\alpha 1$ GlyR N203 residue lies at the tip of the loop C binding domain which closes around the bound glycine molecule to initiate channel activation [35]. The corresponding $\alpha 2$ GlyR residue is N210. We also investigated the $\alpha 1$ GlyR R271 residue (which corresponds to R278 in the $\alpha 2$ GlyR) as it lies distant from the glycine binding site in a region critically involved in the channel gating mechanism [35, 36] and thus reports conformational changes associated with channel gating but not binding.

Fig 1. Comparison of ΔI and ΔF activation properties of WT and indicated mutant $\alpha 1$ and $\alpha 2$ GlyRs. In this and subsequent figures, ΔI and ΔF recordings are shown in black and red, respectively.

A. Structural model of an $\alpha 1$ GlyR subunit showing the locations of N203 (at the tip of C domain) and R271 (at the top of M2 domain). The corresponding $\alpha 2$ GlyR residue numbering is also shown. **B.** Example of a $\alpha 1$ -WT GlyR glycine ΔI dose-response relationship. **C.** Example of a $\alpha 2$ -WT GlyR glycine ΔI dose-response relationship. **D.** Averaged ΔI and ΔF dose-response relations for the $\alpha 1$ -WT and $\alpha 2$ -WT GlyRs. Mean parameters of best fit to individual dose-response relations are summarized in Table 1. **E, F.** Examples of glycine ΔI and ΔF dose-response relationships recorded from oocytes that expressed MTS-TAMRA-labeled $\alpha 1$ -N203C and $\alpha 2$ -N210C GlyRs, respectively. **G.** Averaged ΔI and ΔF dose-response relations for MTS-TAMRA-labeled $\alpha 1$ -N203C and $\alpha 2$ -N210C GlyRs. Mean parameters of best fit to individual dose-response relations are summarized in Table 1. **H.** Averaged ΔI and ΔF dose-response relations for MTSR-labeled $\alpha 1$ -R271C and $\alpha 2$ -R278C GlyRs. Mean parameters of best fit to individual dose-response relations are summarized in Table 1.

Table 1. Glycine-dependent activation properties of the GlyR constructs employed in VCF experiments. Black and red text denote ΔI and ΔF responses, respectively.

Construct	EC ₅₀ (μM)	η _H	I _{max} (μA)	ΔF _{max} (%)	n
α1-WT	39 ± 3	2.0 ± 0.3	28.3 ± 1.8		5
α1-N203C labeled	79 ± 3	2.2 ± 0.2	16.6 ± 4.0		10
α1-N203C labeled ΔF	256 ± 9	0.7 ± 0.1		30 ± 8	10
α1-R271C labeled	363 ± 49	1.0 ± 0.1	21.7 ± 7.2		5
α1-R271C labeled ΔF	360 ± 52	1.0 ± 0.1		10 ± 1	4
α2-WT	230 ± 10	2.2 ± 0.2	10.9 ± 3.8		8
α2-N210C labeled	1353 ± 103	1.2 ± 0.1	5.3 ± 0.2		5
α2-N210C labeled ΔF	No ΔF detected				5
chimera-1 unlabeled	238 ± 12	1.6 ± 0.2	18.6 ± 5.7		8
chimera-1 labeled	229 ± 18	1.5 ± 0.2	12.5 ± 2.6		11

chimera-1 labeled ΔF	426 ± 35	1.3 ± 0.1		20 ± 2	11
chimera-2 unlabeled	179 ± 32	1.6 ± 0.4	8.2 ± 1.7		4
chimera-2 labeled	377 ± 15	1.5 ± 0.1	7.2 ± 0.9		4
chimera-2 labeled ΔF	369 ± 32	1.5 ± 0.2		5.4 ± 0.4	4

Examples of glycine-activated current (ΔI) dose-response relationships for wild type (WT) $\alpha 1$ and $\alpha 2$ GlyRs are shown in Fig 1B and C with mean parameters of best fit to individual dose-response relationships summarized in Fig. 1D and Table 1. The $\alpha 2$ -WT GlyR exhibits a dramatically higher EC_{50} value than the $\alpha 1$ -WT GlyR (230 ± 11 versus 39 ± 1 μM , $n = 5$ each, $p < 0.001$), consistent with previous studies [37] [38].

We have previously shown that when the $\alpha 1$ -N203C or $\alpha 3$ -N203C GlyRs are labelled by MTS-TAMRA, glycine application elicits large, reversible increases in ΔF [28, 34]. An example of a glycine ΔI and ΔF dose-response relationship recorded in an oocyte expressing the MTS-TAMRA-labelled $\alpha 1$ -N203C GlyR is shown in Fig. 1E with the mean dose-response relationships plotted in Fig. 1G. The corresponding experiment for the MTS-TAMRA-labelled $\alpha 2$ -N210C GlyR also yielded robust ΔI responses but no detectable ΔF (Fig. 1F, G). Mean parameters of best fit to all dose-response relationships are summarized in Table 1.

Similarly, we have previously shown that the $\alpha 1$ -R271C and $\alpha 3$ -R271C GlyRs are productively labelled by MTSR [28, 34]. Mean ΔI and ΔF dose-response relationships for the MTSR-labeled $\alpha 1$ -R271C and $\alpha 2$ -R278C GlyRs are shown in Fig. 1H with parameters of best fit summarized in Table 1. Again, whereas $\alpha 1$ -R271C GlyRs yielded robust ΔF responses, $\alpha 2$ -R278C GlyRs did not. Together, these results strongly suggest, despite their high amino acid sequence identities, significant structural differences exist in the N-terminal domains of $\alpha 1$ -WT and $\alpha 2$ -WT GlyRs.

To narrow down the receptor region responsible for the differential ΔF response, we generated a chimera (termed chimera-1) that comprised the N-terminal ligand-binding domain of the $\alpha 1$ -N203C GlyR and the transmembrane domains and C-terminal tail of the $\alpha 2$ -WT GlyR (Fig. 2A). A sample

glycine ΔI dose-response of the unlabeled chimera-1 is shown in Fig. 2B. As seen in the mean dose-response curves presented in Fig. 2C, the glycine ΔI dose-response relationships for the $\alpha 2$ -WT GlyR and chimera-1 were not significantly different. Following MTS-TAMRA labelling, the glycine ΔI EC_{50} was unchanged, suggesting minimal structural impairment, although the glycine ΔF dose-response now yielded a robust ΔF_{max} of $20 \pm 2\%$ ($n = 11$, Table 1). However, we reasoned chimera-1 was too structurally distinct from the $\alpha 2$ -WT GlyR to confidently infer the effects of phosphorylation on the $\alpha 2$ -WT GlyR glycine binding site. Nevertheless the result indicated that the $\alpha 2$ -WT GlyR N-terminus houses the domain responsible for the structural change that eliminates ΔF responses in MTS-TAMRA-labelled $\alpha 2$ -N210C mutant GlyRs.

Fig 2. ΔI and ΔF activation properties of chimera-1 expressed in oocytes. **A.** Schematic model of chimera-1. Orange and gray coloring denote segments from the $\alpha 1$ and $\alpha 2$ GlyR sequences, respectively. **B.** Example of a chimera-1 glycine ΔI dose-response relationship. **C.** Averaged ΔI dose-response relations for the $\alpha 2$ -WT GlyR and chimera-1. Mean parameters of best fit to individual dose-response relations are summarized in Table 1. **D.** Examples of glycine ΔI and ΔF dose-response relationships recorded from an oocyte that expressed MTS-TAMRA-labeled chimera-1. **E.** Averaged ΔI and ΔF dose-response relations for MTS-TAMRA-labeled chimera-1. Mean parameters of best fit to individual dose-response relations are summarized in Table 1.

The N-terminal domains of the $\alpha 1$ -WT and $\alpha 2$ -WT GlyRs are highly conserved. The region of lowest sequence identity is contained within binding domain loop F, also known as the $\beta 8$ - $\beta 9$ loop. As part of the glycine binding site [35, 39], loop F is positioned at the extracellular subunit interface where it could interact directly with a label attached to binding domain loop C of the neighboring subunit (Fig. 1A). We thus hypothesized that non-conserved loop F residues were responsible for eliminating the ΔF response of $\alpha 2$ -N210C GlyRs. To test this, we generated a new chimera, termed chimera-2, that comprised the $\alpha 2$ -N210C GlyR with its loop F replaced by that of the $\alpha 1$ -WT GlyR

(Fig. 3A). This resulted in a total of five non-conserved residues between the $\alpha 2$ -N210C GlyR and chimera-2 (Fig. 3A, inset). A sample glycine ΔI dose-response of the unlabeled chimera-2 is shown in Fig. 2B. As seen in the averaged data presented in Fig. 2C, the $\alpha 2$ -WT GlyR and chimera-2 glycine ΔI dose-response relationships overlapped, implying minimal impact of the loop F structural changes on glycine binding interactions. Following MTS-TAMRA labelling the glycine ΔI dose-response of chimera-2 was unchanged, again implying minimal structural disruption, although now the ΔF dose-response yielded a $\Delta F_{\max}$ of $5.4 \pm 0.4 \%$ ($n = 4$) (Fig. 3D, E; Table 1). As with the correspondingly labelled $\alpha 1$ -N203C and $\alpha 3$ -N203C GlyRs [28, 34], the MTS-TAMRA-labelled chimera-2 was also found to elicit a potent $\Delta F_{\max}$ response ($3.3 \pm 0.7 \%$; $n = 6$) to a saturating ($10 \mu\text{M}$) concentration of strychnine. Together, these results suggest that MTS-TAMRA labelled chimera-2 may be useful for probing phosphorylation-induced structural changes in the $\alpha 2$ -WT GlyR glycine binding site.

Fig 3. ΔI and ΔF activation properties of chimera-2 and the effect of phosphorylation when expressed in oocytes. **A.** Schematic model of chimera-2. Orange and gray coloring denote $\alpha 1$ and $\alpha 2$ GlyR segments, respectively. Two versions of chimera-2 were generated: with and without the S341A mutation. **B.** Example of a chimera-2 glycine ΔI dose-response relationship. **C.** Averaged ΔI dose-response relations for the $\alpha 2$ -WT GlyR and chimera-2. Mean parameters of best fit to individual dose-response relations are summarized in Table 1. **D.** Examples of glycine ΔI and ΔF dose-response relationships recorded from an oocyte that expressed MTS-TAMRA-labeled chimera-2. **E.** Averaged ΔI and ΔF dose-response relations for MTS-TAMRA-labeled chimera-2. Mean parameters of best fit to individual dose-response relations are summarized in Table 1. **F.** The top panel shows examples of ΔI and ΔF responses mediated by MTS-TAMRA-labeled chimera-2 in response to saturating applications of glycine (10 mM) and strychnine ($10 \mu\text{M}$) before and after a 15 min forskolin (FSK) treatment and after a 15 min wash ($n = 5$ oocytes). The center panel shows averaged data for the experiment shown in the top panel ($n = 5$ oocytes), with all data points normalized to the respective control values recorded prior to FSK application. The bottom panel shows the effects of a control 15 min dimethyl sulfoxide (DMSO) treatment in place of FSK ($n = 4$ oocytes). *** $p < 0.001$ compared

to control using paired t-test. **G.** Identical experiment to F except that the chimera-2 phosphorylation site has been eliminated by the S341A mutation (n = 6 oocytes).

Phosphorylation of chimera-2 as detected by VCF

Based on the results of our previous study on the MTS-TAMRA-labelled α 3-N203C GlyR [28], we hypothesized that phosphorylation of S341 may alter the magnitude of the glycine-induced $\Delta F_{\max}$ of the MTS-TAMRA-labelled chimera-2. We tested this hypothesis directly by exposing oocytes expressing MTS-TAMRA-labelled chimera-2 to 20 μ M forskolin for 15 min to in an attempt to phosphorylate S341. As shown in the sample recording in Fig. 3F (top and centre panels), it significantly reduced the glycine-mediated $\Delta F_{\max}$ without affecting the strychnine-mediated $\Delta F_{\max}$ (n = 5 oocytes). A control dimethyl sulfoxide application to the same labelled receptors had no effect on $\Delta I_{\max}$ or $\Delta F_{\max}$ values (n = 4 oocytes), thus ruling out solvent-dependent effects (Fig. 3F, bottom panel). A second control experiment revealed that forskolin produced no significant change in $\Delta F_{\max}$ magnitude in phosphorylation-deficient (S341A) mutant chimera-2 receptors (Fig. 3G), ruling out the possibility of non-specific forskolin effects on $\Delta F_{\max}$ responses (n = 6 oocytes). As the glycine $\Delta I_{\max}$ magnitudes remained constant throughout these experiments (Fig. 3F-G), we can also rule out an effect of phosphorylation on GlyR surface expression levels. Together, these results indicate that phosphorylation of S341 induced a conformational change in the immediate vicinity of the label attached to N203C in chimera-2.

Phosphorylation of α 2 GlyRs expressed in HEK293 cells

Given that S346 phosphorylation inhibits α 3 GlyRs when expressed in mammalian HEK293 cells but not when expressed in *Xenopus* oocytes [28], it is evident that this effect can be expression system-specific. It was thus important to determine the effects of PKA-dependent phosphorylation on the functional properties of α 2-WT GlyRs expressed in HEK293 cells. Note that it is not feasible to perform VCF on receptors expressed in HEK293 cells due to the high level of non-specific

fluorophore labelling in HEK293 cell membranes. Our first experiment involved applying 20 μ M forskolin for 15 min to α 2-WT GlyRs that were subject to brief (2 s) applications of EC₁₀₀ (3 mM) and EC₅₀ (100 μ M) glycine concentrations every 60 s. In a total of 16 cells we observed no significant effect of forskolin on current magnitudes activated by either EC₁₀₀ or EC₅₀ glycine concentrations. Although we supplemented the pipette solution with 2 mM Mg-ATP and 0.5 mM Na-GTP (see Methods) in these experiments, it remains possible that the lack of forskolin effect may have been due to insufficient levels of essential second messenger components in our HEK293 cell strain.

We thus adopted an alternate approach whereby we compared the functional properties of α 2-WT GlyRs with those of phosphorylation-deficient α 2-S341A GlyRs, and phosphorylation-mimicking α 2-S341E GlyRs. Sample glycine dose-response experiments for the three receptors are shown in Fig. 4A. As shown in Fig. 4B and C and summarised in Table 2, the averaged glycine EC₅₀ values, n_H values and I_{max} values were not significantly different from each other. Furthermore, when we applied a saturating (3 mM) glycine concentration for 25 s, the macroscopic decay rates of currents mediated by all three constructs were not significantly different from each other (Fig. 4D, E). However, we acknowledge that subtle changes in current activation and deactivation rates are not readily observable with standard whole-cell recording.

Fig 4. Functional analysis of α 2-WT, α 2-S341A and α 2-S341E GlyRs in HEK293 cells. A.

Glycine dose-response sample traces for the indicated GlyRs. **B.** Normalized, averaged glycine dose-response results for the indicated human GlyRs. Parameters of best fit to the Hill equation are summarized in Table 2. **C.** Mean I_{max} values for the three receptors. **D.** Comparison of macroscopic desensitisation rates of currents activated by saturating (3 mM) glycine. Mono-exponential curve fits to the current decay phases are indicated together with the fitted time constants. **E.** Mean decay time constants for the experiment shown in panel D.

Table 2. Glycine-dependent activation properties of WT and mutant $\alpha 2$ GlyRs expressed in HEK293 cells

Construct	EC ₅₀ (μ M)	η_H	I _{max} (nA)	decay time constant (s)	n
$\alpha 2$ -WT	153 $\pm$ 20	2.4 $\pm$ 0.2	5119 $\pm$ 501	4.85 $\pm$ 0.42	10
$\alpha 2$ -S341A	142 $\pm$ 19	2.6 $\pm$ 0.2	6083 $\pm$ 1029	4.69 $\pm$ 0.46	10
$\alpha 2$ -S341E	142 $\pm$ 15	2.4 $\pm$ 0.2	8543 $\pm$ 535	4.59 $\pm$ 0.48	12

Accordingly, we next investigated the properties of inhibitory postsynaptic currents (IPSCs) mediated by $\alpha 2$ -WT, $\alpha 2$ -S341A and $\alpha 2$ -S341E GlyRs in artificial synapses. Whole-cell recordings from transfected HEK293 cells in co-culture with spinal neurons regularly exhibited robust, spontaneous IPSCs mediated by each of the three constructs (Fig. 5A). The advantage of this approach over transfected native neurons is that we can be sure that IPSCs are mediated by a pure population of $\alpha 2$ GlyRs. Fig. 5B shows digitally averaged and normalised IPSCs from single HEK293 cells expressing the WT and each mutant GlyR. Mean IPSC 10-90% rise times, amplitudes and decay time constants are presented in Figs. 5C-E. The respective parameters for the $\alpha 2$ -WT GlyR correspond closely to those reported previously [31]. Although IPSC amplitudes did not vary significantly among the three receptors, the phosphorylation-mimicking $\alpha 2$ -S341E GlyRs exhibited significantly slower IPSC rise and decay times relative to $\alpha 2$ -WT and $\alpha 2$ -S341A GlyRs (Fig. 5C, E).

Fig 5. Properties of spontaneous IPSCs recorded from artificial synapses incorporating $\alpha 2$ -WT, $\alpha 2$ -S341A or $\alpha 2$ -S341E GlyRs. **A.** Representative recordings of IPSCs from HEK293 cells expressing each isoform at two temporal scales. **B.** Averaged (from 50 - 100 events), normalized IPSCs from individual cells expressing each isoform. **C-E.** Mean IPSC 10 - 90% rise times,

amplitudes and decay time constants. Significance was assessed using one way ANOVA, * $p < 0.05$,
 ** $p < 0.01$, *** $p < 0.001$.

Discussion

We conclude that PKA-dependent phosphorylation of the $\alpha 2$ GlyR elicits two separable effects. The first effect, observed in artificial synapses, is a slowing in the IPSC rise and decay rates. As this was inferred from phospho-mimetic mutagenesis, it remains to be confirmed in native $\alpha 2$ GlyRs in neurons. The second effect of phosphorylation, observed in the *Xenopus* oocyte expression system, is a conformational change in or near the glycine-binding site. The main line of evidence in support of this is a forskolin-mediated change in the micro-environment of a fluorophore attached to loop C of the glycine-binding site, that is not observed when the phosphorylation site is eliminated. Note that phosphorylation modulated the glycine-induced $\Delta F_{\max}$ but not the strychnine-induced $\Delta F_{\max}$. Such a differential effect is not surprising given that the two ligands bind in different orientations and induce distinct conformational changes in the glycine binding site [33, 35].

We found that forskolin elicited no apparent effect on $\alpha 2$ GlyRs when recombinantly expressed in HEK293 cells. One possibility is that HEK293 cells did not have appropriate levels of expression of one or more intracellular signaling molecules essential for the phosphorylation process. Another possibility is that a phosphorylation-mediated binding site conformational change did occur but elicited no change in glycine sensitivity or current magnitude. Given that S341 may not have actually been phosphorylated by forskolin in our HEK293 cell experiments, we adopted the alternate approach of investigating the effects of phosphorylation-deficient ($\alpha 2$ -S341A) and phosphorylation-mimicking ($\alpha 2$ -S341E) mutations. Although we observed no effect of the mutations on glycine EC_{50} , $\Delta I_{\max}$ or macroscopic decay rates in conventional whole-cell recordings (Fig. 4), we found that homomeric $\alpha 2$ -S341E GlyRs mediated IPSCs with significantly slower rise and decay times in artificial synapses (Fig. 5).

The $\alpha 2$ GlyR is initially expressed as a homomer in embryonic neurons [40] and its expression in these neurons has long been known to be important for synaptogenesis [41-43], cortical development [44] and retinal photoreceptor development [45]. Because the expression of the potassium-chloride cotransporter type-2 (KCC2) is low in embryonic neurons [46], the intracellular chloride concentration is high and as a result, $\alpha 2$ GlyR activation is excitatory [47]. In embryonic migrating cortical interneurons, activation of $\alpha 2$ GlyRs thus leads to neuronal depolarisation, the subsequent activation of a voltage-gated calcium influx and to the modulation of spontaneous calcium oscillations [19]. These in turn modulate myosin activity that alters interneuron nucleokinesis and increases interneuron migration speed [19]. Functional $\alpha 2$ GlyRs also contribute to the differentiation of cortical neuroprogenitors into functioning neurons[20]. Thus, $\alpha 2$ GlyRs are essential for the development of correctly functioning cortical circuits. Conversely, loss or dysfunction of $\alpha 2$ GlyRs is associated with neurodevelopmental disorders including schizophrenia, autism and epilepsy [25] [26, 27, 48, 49]. [50] [51].

Although it undergoes a decline in expression levels during development[40], the $\alpha 2$ GlyR remains widely distributed throughout the adult central nervous system [40, 52]. Indeed it has recently been shown that homomeric $\alpha 2$ and $\alpha 3$ GlyRs mediate tonic glycine-mediated currents throughout higher brain regions [21] [53]. Homomeric GlyRs are also found presynaptically at cortical glutamatergic synapses [51, 54-56]. These presynaptic GlyRs are likely to be both $\alpha 2$ and $\alpha 3$ GlyRs, given that 1) the $\alpha 2$ GlyR has been positively identified in these locations [51] [55] and 2) a missense mutation ($\alpha 2$ -P192L, $\alpha 2$ -P185L) common to both subunits results in a common temporal lobe epilepsy phenotype [50]. When expressed presynaptically, homomeric $\alpha 2$ GlyRs would be exposed to phasically released glycine. In this case, PKA phosphorylation would prolong receptor open times and thereby prolong the depolarization of presynaptic terminals. This is likely to increase calcium influx, thereby enhancing neurotransmitter release and leading to enhanced excitability of postsynaptic neurons.

In summary, we have demonstrated that phosphorylation of S341 exerts a conformational change that propagates to the $\alpha 2$ GlyR glycine-binding site. We also found that the S341E phospho-

mimetic mutation results in slower IPSC rise and decay times. These results suggest that PKA phosphorylation can alter the structure and functional properties of the $\alpha 2$ GlyR. This information adds to the complexity of glycinergic modulatory mechanisms, and may help to identify new mechanisms by which $\alpha 2$ GlyRs may be pathologically modified or therapeutically targeted for the treatment of neurological disorders including epilepsy.

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References

1. Dutertre S, Becker CM, Betz H. Inhibitory glycine receptors: an update. *J Biol Chem.* 2012;287(48):40216-23. doi: 10.1074/jbc.R112.408229. PubMed PMID: 23038260; PubMed Central PMCID: PMC3504737.
2. Lynch JW. Native glycine receptor subtypes and their physiological roles. *Neuropharmacology.* 2009;56(1):303-9. doi: 10.1016/j.neuropharm.2008.07.034. PubMed PMID: WOS:000262625500035.
3. Zeilhofer HU. The glycinergic control of spinal pain processing. *Cell Mol Life Sci.* 2005;62(18):2027-35. doi: 10.1007/s00018-005-5107-3. PubMed PMID: 15968463.
4. Leacock S, Syed P, James VM, Bode A, Kawakami K, Keramidas A, et al. Structure/Function Studies of the alpha4 Subunit Reveal Evolutionary Loss of a GlyR Subtype Involved in Startle and Escape Responses. *Front Mol Neurosci.* 2018;11:23. doi: 10.3389/fnmol.2018.00023. PubMed PMID: 29445326; PubMed Central PMCID: PMC5797729.
5. Ehrensperger MV, Hanus C, Vannier C, Triller A, Dahan M. Multiple association states between glycine receptors and gephyrin identified by SPT analysis. *Biophys J.* 2007;92(10):3706-18. doi: 10.1529/biophysj.106.095596. PubMed PMID: WOS:000245852600034.
6. Feng GP, Tintrup H, Kirsch J, Nichol MC, Kuhse J, Betz H, et al. Dual requirement for gephyrin in glycine receptor clustering and molybdoenzyme activity. *Science.* 1998;282(5392):1321-4. doi: DOI 10.1126/science.282.5392.1321. PubMed PMID: WOS:000076982200046.
7. Specht CG, Grunewald N, Pascual O, Rostgaard N, Schwarz G, Triller A. Regulation of glycine receptor diffusion properties and gephyrin interactions by protein kinase C. *EMBO*

504 J. 2011;30(18):3842-53. doi: 10.1038/emboj.2011.276. PubMed PMID: 21829170; PubMed
505 Central PMCID: PMCPMC3173796.

506 8. Bode A, Lynch JW. The impact of human hyperekplexia mutations on glycine
507 receptor structure and function. Mol Brain. 2014;7:2. doi: 10.1186/1756-6606-7-2. PubMed
508 PMID: 24405574; PubMed Central PMCID: PMCPMC3895786.

509 9. Ahmadi S, Lippross S, Neuhuber WL, Zeilhofer HU. PGE(2) selectively blocks
510 inhibitory glycinergic neurotransmission onto rat superficial dorsal horn neurons. Nat
511 Neurosci. 2002;5(1):34-40. doi: 10.1038/nn778. PubMed PMID: 11740501.

512 10. Harvey RJ, Depner UB, Wassle H, Ahmadi S, Heindl C, Reinold H, et al. GlyR
513 alpha3: an essential target for spinal PGE2-mediated inflammatory pain sensitization.
514 Science. 2004;304(5672):884-7. doi: 10.1126/science.1094925. PubMed PMID: 15131310.

515 11. Zeilhofer HU, Wildner H, Yevenes GE. Fast synaptic inhibition in spinal sensory
516 processing and pain control. Physiol Rev. 2012;92(1):193-235. Epub 2012/02/03. doi:
517 10.1152/physrev.00043.2010. PubMed PMID: 22298656.

518 12. Acuna MA, Yevenes GE, Ralvenius WT, Benke D, Di Lio A, Lara CO, et al.
519 Phosphorylation state-dependent modulation of spinal glycine receptors alleviates
520 inflammatory pain. J Clin Invest. 2016;126(7):2547-60. doi: 10.1172/JCI83817. PubMed
521 PMID: 27270175; PubMed Central PMCID: PMCPMC4922714.

522 13. Bregman H, Simard JR, Andrews KL, Ayube S, Chen H, Gunaydin H, et al. The
523 Discovery and Hit-to-Lead Optimization of Tricyclic Sulfonamides as Potent and Efficacious
524 Potentiators of Glycine Receptors. J Med Chem. 2016. doi: 10.1021/acs.jmedchem.6b01496.
525 PubMed PMID: 28001399.

526 14. Lynch JW, Callister RJ. Glycine receptors: a new therapeutic target in pain pathways.
527 Curr Opin Investig Drugs. 2006;7(1):48-53. PubMed PMID: 16425671.

- 529 15. Lynch JW, Zhang Y, Talwar S, Estrada-Mondragon A. Glycine Receptor Drug
530 Discovery. *Adv Pharmacol.* 2017;79:225-53. doi: 10.1016/bs.apha.2017.01.003. PubMed
531 PMID: 28528670.
- 532 16. Xiong W, Cheng K, Cui T, Godlewski G, Rice KC, Xu Y, et al. Cannabinoid
533 potentiation of glycine receptors contributes to cannabis-induced analgesia. *Nat Chem Biol.*
534 2011;7(5):296-303. Epub 2011/04/05. doi: nchembio.552 [pii]
535 10.1038/nchembio.552. PubMed PMID: 21460829.
- 536 17. Xiong W, Cui T, Cheng K, Yang F, Chen SR, Willenbring D, et al. Cannabinoids
537 suppress inflammatory and neuropathic pain by targeting alpha3 glycine receptors. *J Exp*
538 *Med.* 2012;209(6):1121-34. doi: 10.1084/jem.20120242. PubMed PMID: 22585736; PubMed
539 Central PMCID: PMC3371734.
- 540 18. Avila A, Nguyen L, Rigo JM. Glycine receptors and brain development. *Front Cell*
541 *Neurosci.* 2013;7:184. doi: 10.3389/fncel.2013.00184. PubMed PMID: 24155690; PubMed
542 Central PMCID: PMC3800850.
- 543 19. Avila A, Vidal PM, Dear TN, Harvey RJ, Rigo JM, Nguyen L. Glycine receptor
544 alpha2 subunit activation promotes cortical interneuron migration. *Cell Rep.* 2013;4(4):738-
545 50. doi: 10.1016/j.celrep.2013.07.016. PubMed PMID: 23954789; PubMed Central PMCID:
546 PMC3763372.
- 547 20. Avila A, Vidal PM, Tielens S, Morelli G, Laguesse S, Harvey RJ, et al. Glycine
548 receptors control the generation of projection neurons in the developing cerebral cortex. *Cell*
549 *Death Differ.* 2014;21(11):1696-708. doi: 10.1038/cdd.2014.75. PubMed PMID: 24926615;
550 PubMed Central PMCID: PMC4211368.
- 551 21. McCracken LM, Lowes DC, Salling MC, Carreau-Vollmer C, Odean NN, Blednov
552 YA, et al. Glycine receptor alpha3 and alpha2 subunits mediate tonic and exogenous agonist-
553 induced currents in forebrain. *Proc Natl Acad Sci U S A.* 2017;114(34):E7179-E86. Epub

- 2017/08/09. doi: 10.1073/pnas.1703839114. PubMed PMID: 28784756; PubMed Central
PMCID: PMCPMC5576794.
22. Morelli G, Avila A, Ravanidis S, Aourz N, Neve RL, Smolders I, et al. Cerebral
Cortical Circuitry Formation Requires Functional Glycine Receptors. *Cereb Cortex*. 2016.
doi: 10.1093/cercor/bhw025. PubMed PMID: 26891984.
23. Seo DO, Motard LE, Bruchas MR. Contemporary strategies for dissecting the
neuronal basis of neurodevelopmental disorders. *Neurobiol Learn Mem*. 2018. doi:
10.1016/j.nlm.2018.03.015. PubMed PMID: 29550367.
24. Iossifov I, O'Roak BJ, Sanders SJ, Ronemus M, Krumm N, Levy D, et al. The
contribution of de novo coding mutations to autism spectrum disorder. *Nature*.
2014;515(7526):216-21. doi: 10.1038/nature13908. PubMed PMID: 25363768; PubMed
Central PMCID: PMCPMC4313871.
25. Pilorge M, Fassier C, Le Corrionc H, Potey A, Bai J, De Gois S, et al. Genetic and
functional analyses demonstrate a role for abnormal glycinergic signaling in autism. *Mol
Psychiatry*. 2015. doi: 10.1038/mp.2015.139. PubMed PMID: 26370147.
26. Piton A, Gauthier J, Hamdan FF, Lafreniere RG, Yang Y, Henrion E, et al. Systematic
resequencing of X-chromosome synaptic genes in autism spectrum disorder and
schizophrenia. *Mol Psychiatry*. 2011;16(8):867-80. doi: 10.1038/mp.2010.54. PubMed
PMID: 20479760; PubMed Central PMCID: PMC3289139.
27. Zhang Y, Ho TNT, Harvey RJ, Lynch JW, Keramidas A. Structure-Function Analysis
of the GlyR alpha2 Subunit Autism Mutation p.R323L Reveals a Gain-of-Function. *Front
Mol Neurosci*. 2017;10:158. doi: 10.3389/fnmol.2017.00158. PubMed PMID: 28588452;
PubMed Central PMCID: PMCPMC5440463.
28. Han L, Talwar S, Wang Q, Shan Q, Lynch JW. Phosphorylation of alpha3 glycine
receptors induces a conformational change in the glycine-binding site. *ACS Chem Neurosci*.

579 2013;4(10):1361-70. doi: 10.1021/cn400097j. PubMed PMID: 23834509; PubMed Central
580 PMCID: PMCPMC3798985.

581 29. Talwar S, Lynch JW. Investigating ion channel conformational changes using voltage
582 clamp fluorometry. *Neuropharmacology*. 2015;98:3-12. doi:
583 10.1016/j.neuropharm.2015.03.018. PubMed PMID: 25839896.

584 30. Dixon CL, Zhang Y, Lynch JW. Generation of Functional Inhibitory Synapses
585 Incorporating Defined Combinations of GABA(A) or Glycine Receptor Subunits. *Front Mol*
586 *Neurosci*. 2015;8:80. doi: 10.3389/fnmol.2015.00080. PubMed PMID: 26778954; PubMed
587 Central PMCID: PMCPMC4688394.

588 31. Zhang Y, Dixon CL, Keramidas A, Lynch JW. Functional reconstitution of
589 glycinergic synapses incorporating defined glycine receptor subunit combinations.
590 *Neuropharmacology*. 2015;89:391-7. doi: 10.1016/j.neuropharm.2014.10.026. PubMed
591 PMID: 25445488.

592 32. Shan Q, Lynch JW. Chimera construction using multiple-template-based sequential
593 PCRs. *J Neurosci Methods*. 2010;193(1):86-9. doi: 10.1016/j.jneumeth.2010.08.033. PubMed
594 PMID: 20817030.

595 33. Pless SA, Dibas MI, Lester HA, Lynch JW. Conformational variability of the glycine
596 receptor M2 domain in response to activation by different agonists. *J Biol Chem*.
597 2007;282(49):36057-67. doi: 10.1074/jbc.M706468200. PubMed PMID: 17911099.

598 34. Pless SA, Lynch JW. Ligand-specific Conformational Changes in the $\alpha 1$ Glycine
599 Receptor Ligand-binding Domain. *J Biol Chem*. 2009;284(23):15847-56.

600 35. Du J, Lu W, Wu S, Cheng Y, Gouaux E. Glycine receptor mechanism elucidated by
601 electron cryo-microscopy. *Nature*. 2015;526(7572):224-9. doi: 10.1038/nature14853. PubMed
602 PMID: 26344198; PubMed Central PMCID: PMCPMC4659708.

36. Lynch JW, Rajendra S, Pierce KD, Handford CA, Barry PH, Schofield PR. Identification of intracellular and extracellular domains mediating signal transduction in the inhibitory glycine receptor chloride channel. *EMBO J.* 1997;16(1):110-20. doi: 10.1093/emboj/16.1.110. PubMed PMID: 9009272; PubMed Central PMCID: PMC1169618.
37. Yang Z, Cromer BA, Harvey RJ, Parker MW, Lynch JW. A proposed structural basis for picrotoxinin and picrotin binding in the glycine receptor pore. *J Neurochem.* 2007;103(2):580-9. doi: 10.1111/j.1471-4159.2007.04850.x. PubMed PMID: 17714449.
38. Xiong W, Wu X, Li F, Cheng K, Rice KC, Lovinger DM, et al. A common molecular basis for exogenous and endogenous cannabinoid potentiation of glycine receptors. *J Neurosci.* 2012;32(15):5200-8. doi: 10.1523/JNEUROSCI.6347-11.2012. PubMed PMID: 22496565; PubMed Central PMCID: PMCPMC3334839.
39. Lynch JW. Molecular structure and function of the glycine receptor chloride channel. *Physiol Rev.* 2004;84(4):1051-95. doi: 10.1152/physrev.00042.2003. PubMed PMID: WOS:000224094500001.
40. Malosio ML, Marqueze-Pouey B, Kuhse J, Betz H. Widespread expression of glycine receptor subunit mRNAs in the adult and developing rat brain. *EMBO J.* 1991;10(9):2401-9. Epub 1991/09/01. PubMed PMID: 1651228; PubMed Central PMCID: PMCPMC452935.
41. Kirsch J, Betz H. Glycine-receptor activation is required for receptor clustering in spinal neurons. *Nature.* 1998;392(6677):717-20. Epub 1998/05/16. doi: 10.1038/33694. PubMed PMID: 9565032.
42. Levi S, Vannier C, Triller A. Strychnine-sensitive stabilization of postsynaptic glycine receptor clusters. *J Cell Sci.* 1998;111 (Pt 3):335-45. Epub 1998/04/29. PubMed PMID: 9427682.

43. Racca C, Gardiol A, Triller A. Cell-specific dendritic localization of glycine receptor alpha subunit messenger RNAs. *Neuroscience*. 1998;84(4):997-1012. Epub 1998/05/13. PubMed PMID: 9578391.
44. Flint AC, Liu X, Kriegstein AR. Nonsynaptic glycine receptor activation during early neocortical development. *Neuron*. 1998;20(1):43-53. PubMed PMID: 9459441.
45. Young TL, Cepko CL. A role for ligand-gated ion channels in rod photoreceptor development. *Neuron*. 2004;41(6):867-79. Epub 2004/03/30. PubMed PMID: 15046720.
46. Li H, Tornberg J, Kaila K, Airaksinen MS, Rivera C. Patterns of cation-chloride cotransporter expression during embryonic rodent CNS development. *Eur J Neurosci*. 2002;16(12):2358-70. Epub 2002/12/21. PubMed PMID: 12492431.
47. Young-Pearse TL, Ivic L, Kriegstein AR, Cepko CL. Characterization of mice with targeted deletion of glycine receptor alpha 2. *Mol Cell Biol*. 2006;26(15):5728-34. doi: 10.1128/MCB.00237-06. PubMed PMID: 16847326; PubMed Central PMCID: PMC1592777.
48. Marin O. Interneuron dysfunction in psychiatric disorders. *Nat Rev Neurosci*. 2012;13(2):107-20. Epub 2012/01/19. doi: 10.1038/nrn3155. PubMed PMID: 22251963.
49. Cobos I, Calcagnotto ME, Vilaythong AJ, Thwin MT, Noebels JL, Baraban SC, et al. Mice lacking *Dlx1* show subtype-specific loss of interneurons, reduced inhibition and epilepsy. *Nat Neurosci*. 2005;8(8):1059-68. Epub 2005/07/12. doi: 10.1038/nn1499. PubMed PMID: 16007083.
50. Eichler SA, Kirischuk S, Jüttner R, Schaefermeier PK, Legendre P, Lehmann TN, et al. Glycinergic tonic inhibition of hippocampal neurons with depolarizing GABAergic transmission elicits histopathological signs of temporal lobe epilepsy. *J Cell Mol Med*. 2008;12(6B):2848-66. Epub 2009/02/13. doi: 10.1111/j.1582-4934.2008.00357.x. PubMed PMID: 19210758; PubMed Central PMCID: PMC1592777.

51. Eichler SA, Forstera B, Smolinsky B, Juttner R, Lehmann TN, Fahling M, et al. Splice-specific roles of glycine receptor alpha3 in the hippocampus. *Eur J Neurosci*. 2009;30(6):1077-91. doi: 10.1111/j.1460-9568.2009.06903.x. PubMed PMID: 19723286.
52. Jonsson S, Morud J, Pickering C, Adermark L, Ericson M, Soderpalm B. Changes in glycine receptor subunit expression in forebrain regions of the Wistar rat over development. *Brain Res*. 2012;1446:12-21. Epub 2012/02/15. doi: 10.1016/j.brainres.2012.01.050. PubMed PMID: 22330726.
53. Molchanova SM, Comhair J, Karadurmus D, Piccart E, Harvey RJ, Rigo JM, et al. Tonically Active alpha2 Subunit-Containing Glycine Receptors Regulate the Excitability of Striatal Medium Spiny Neurons. *Front Mol Neurosci*. 2017;10:442. Epub 2018/01/30. doi: 10.3389/fnmol.2017.00442. PubMed PMID: 29375305; PubMed Central PMCID: PMC5767327.
54. Meier JC, Semtner M, Winkelmann A, Wolfart J. Presynaptic mechanisms of neuronal plasticity and their role in epilepsy. *Front Cell Neurosci*. 2014;8:164. Epub 2014/07/06. doi: 10.3389/fncel.2014.00164. PubMed PMID: 24987332; PubMed Central PMCID: PMC4060558.
55. Winkelmann A, Maggio N, Eller J, Caliskan G, Semtner M, Haussler U, et al. Changes in neural network homeostasis trigger neuropsychiatric symptoms. *J Clin Invest*. 2014;124(2):696-711. Epub 2014/01/17. doi: 10.1172/JCI71472. PubMed PMID: 24430185; PubMed Central PMCID: PMC3904623.
56. Kubota H, Alle H, Betz H, Geiger JR. Presynaptic glycine receptors on hippocampal mossy fibers. *Biochem Biophys Res Commun*. 2010;393(4):587-91. Epub 2010/02/16. doi: 10.1016/j.bbrc.2010.02.019. PubMed PMID: 20152805.

Significance test between groups using One Way ANOVA,
*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$