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The emerging role of glycine receptor $\alpha 2$ subunit defects in neurodevelopmental disorders

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Rare neurodevelopmental disorders (NDDs) are one of the most significant unmet challenges in healthcare due to their lifelong nature, high management costs, and recurrence within families. This review will focus on newly-emerging genetic forms of NDDs resulting from variants in the glycine receptor (GlyR) $\alpha 2$ subunit gene. Studies using *Gla2* knockout mice have convincingly demonstrated that GlyR $\alpha 2$ is essential for cortical interneuron migration and progenitor homeostasis. Genetic inactivation of GlyR $\alpha 2$ impairs the capacity of apical progenitors to generate basal progenitors, resulting in an overall reduction of projection neurons in the cerebral cortex. As a result, microcephaly is observed in newborn *Gla2* knockout mice, as well as defects in neuronal morphology, increased susceptibility to seizures, and defects in novel object recognition, motor memory consolidation, righting reflexes, novelty-induced locomotion in the open field test, and motivational reward tasks. Consistent with these findings, we and others have identified missense variants and microdeletions in the human GlyR $\alpha 2$ subunit gene (*GLRA2*) in individuals with autism spectrum disorder (ASD), developmental delay (DD) and/or intellectual disability (ID), often accompanied by microcephaly, language delay and epilepsy. In this review, we highlight the critical role of the GlyR $\alpha 2$ subunit revealed by knockout mice and our current understanding of GlyR $\alpha 2$ pathomechanisms in human NDDs. Finally, we will consider the current gaps in our knowledge, which include: (i) Limited functional validation for GlyR $\alpha 2$ missense variants associated with human NDDs; (ii) The lack of *gain-of-function* GlyR $\alpha 2$ mouse models; (iii) Our limited knowledge of GlyR $\alpha 2$ interacting proteins. We also highlight potential future developments in the field, including routes to personalized medicines for individuals with GlyR $\alpha 2$ mutations.

KEYWORDS

autism spectrum disorder, cortical neuronal migration, epilepsy, glycine receptor, *Gla2* knockout mice, GlyR $\alpha 2$ subunit, intellectual disability, neurodevelopmental disorders

Introduction

Glycine receptors (GlyRs) are recognized as mediators of fast inhibitory synaptic neurotransmission in the spinal cord and brainstem (Breitinger and Becker, 2002), in addition to the retina and inner ear (Wässle et al., 2009; Buerbank et al., 2011). GlyRs are members of the pentameric cys-loop ligand-gated ion channel family, with other members including the nicotinic acetylcholine receptor (nAChR), serotonin type 3 receptor (5-HT₃R), and γ -aminobutyric acid type A receptor (GABA_AR) (Lynch, 2004, 2009). Members of this superfamily share a common pentameric, symmetrical configuration of subunits arranged around a central, ion-conducting pore that traverses the cell membrane (Du et al., 2015; Huang et al., 2015; Yu et al., 2021; Zhu and Gouaux, 2021; Gibbs et al., 2023). Each GlyR subunit comprises an N-terminal signal peptide (SP); a large, agonist-binding extracellular domain

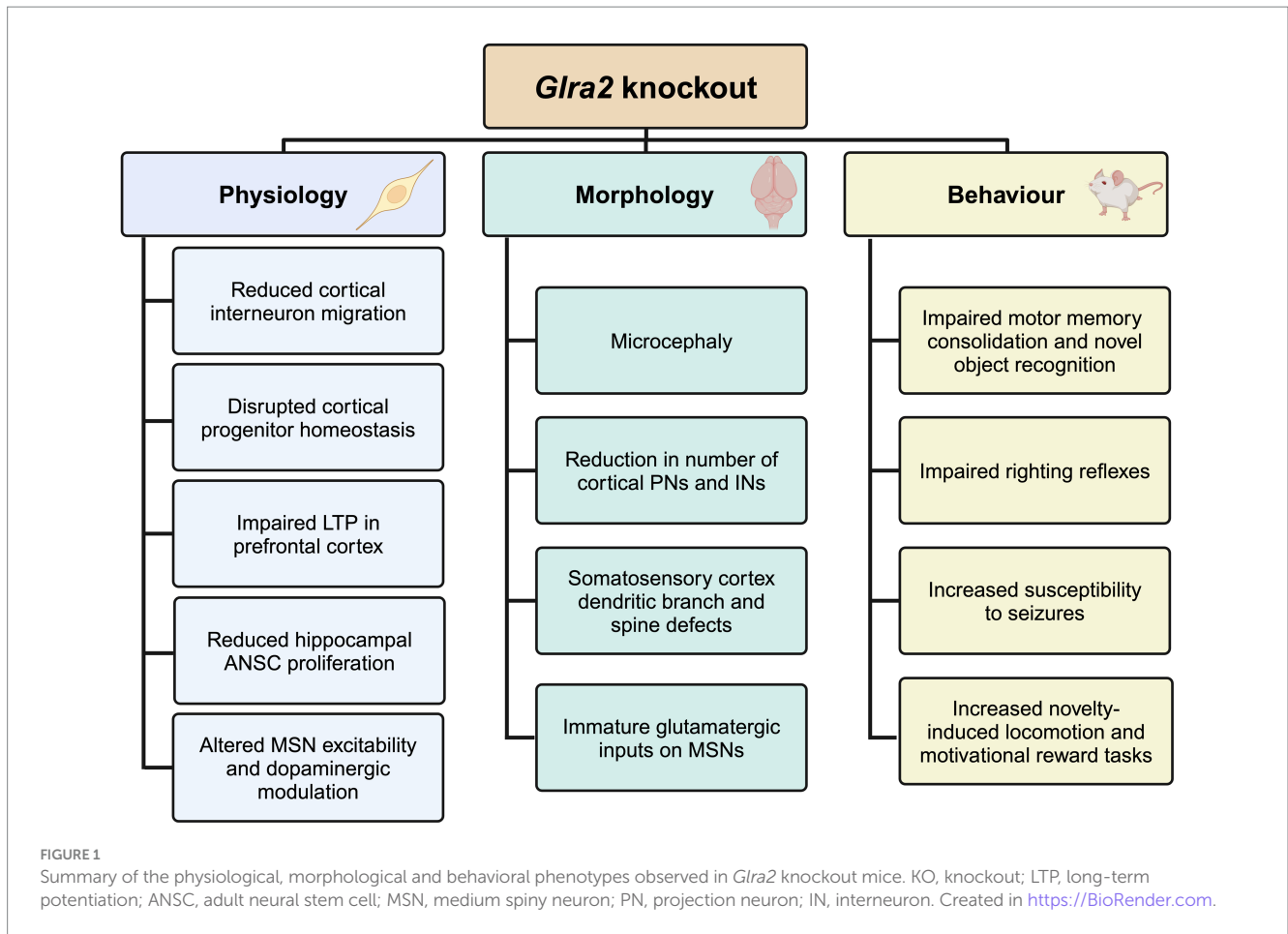
(ECD); four membrane-spanning domains (TM1-TM4) connected by loops of varying length and a short extracellular C-terminus. Five evolutionarily conserved, yet distinct GlyR subunits have been described in humans and rodents: the GlyR α 1- α 4 and β subunits. These subunits assemble into pentameric homomeric α or heteromeric $\alpha\beta$ receptors. The stoichiometric configuration of heteromeric GlyRs has been a matter of contention. However, the recent analysis of GlyRs via cryo-electron microscopy suggests that heteromeric GlyRs have an invariant 4 α :1 β configuration (Yu et al., 2021; Zhu and Gouaux, 2021). Given their evolutionarily conserved nature, the GlyR α subunits share a high degree of sequence identity ($\geq 80\%$) (Grenningloh et al., 1990; Matzenbach et al., 1994). The region featuring the least sequence similarity is the TM3-TM4 intracellular domain (ICD), which mediates interactions with accessory proteins, such as gephyrin (Prior et al., 1992; Meyer et al., 1995), collybistin (Breitinger et al., 2021) and syndapin I (Del Pino et al., 2014; Troger et al., 2022), as well as providing a site for post-translational modification, including phosphorylation and ubiquitination (Harvey K. et al., 2004; Langlhofer and Villmann, 2016).

GlyR α and β subunits display distinct spatiotemporal expression profiles within the mammalian CNS. In particular, GlyR α 2 is widely expressed in brain and spinal cord during embryonic development and early postnatal life, but is replaced by GlyR α 1 and α 3 during postnatal maturation (Becker et al., 1988; Akagi et al., 1991; Malosio et al., 1991). The GlyR α 1 subunit is known for its role in providing fast synaptic inhibition within motor reflex pathways of the spinal cord (Lynch, 2009). These GlyRs are hypothesized to comprise a heteromeric α 1 β configuration, given that gephyrin is necessary for the postsynaptic localization of GlyRs and only the β subunit is capable of interacting with this scaffolding protein via the gephyrin E domain (Meyer et al., 1995; Rees et al., 2003; Harvey K. et al., 2004; Sola et al., 2004). The importance of heteromeric α 1 β GlyRs in tempering the excitability of motoneurons is evidenced by studies of the rare neurological disorder startle disease/hyperkplexia (Harvey et al., 2008; Schaefer et al., 2022), which is caused by mutations within the corresponding genes: *GLRA1* and *GRLB* (Shiang et al., 1993; Rees et al., 2002), *SLC6A5* encoding the presynaptic glycine transporter GlyT2 (Rees et al., 2006; Carta et al., 2012; Giménez et al., 2012) and *SLC7A10* encoding Asc-1, an alanine-serine-cysteine transporter (Drehmann et al., 2023). Numerous spontaneous mouse mutants with defects in the *Glr1* and *Glr2* subunit genes are also known, including *spastic*, *spasmodic*, *oscillator*, *cincinatti*, *nmf11* and *shaky*, which have proven to be outstanding models for the study of anxiety and startle phenotypes (Schaefer et al., 2022). GlyR α 3 is found in the spinal cord dorsal horn where pain-sensing nerve fibers arrive from the periphery (Harvey R. J. et al., 2004), as well as in neurons within the brainstem pre-Bötzinger complex that control breathing (Manzke et al., 2010). In both regions, GlyR α 3 is modulated by different G-protein-coupled receptors that lead to downstream changes in the phosphorylation state of GlyR α 3. The generation and analysis of knockout and knock-in mice has revealed roles for the GlyR α 3 subunit in inflammatory pain sensitization (Harvey R. J. et al., 2004; Werynska et al., 2021), rhythmic breathing (Manzke et al., 2010), auditory nerve function (Dlugaczzyk et al., 2016), as well as ethanol-related addictive behaviors (Blednov et al., 2015; San Martin et al., 2021). Lastly, GlyR α 4 impacts embryonic development, litter sizes, startle responses, and anxiety-like behaviors in mice (Nishizono et al., 2020; Darwish et al., 2023) but is a pseudogene in humans (Leacock et al., 2018).

This review will focus on the biological role of the GlyR α 2 subunit, highlighting a series of studies using newly-developed GlyR α 2 subunit knockout mice that linked this receptor subtype to multiple defects in neuronal physiology, morphology and behaviors (Figure 1). This led to the subsequent discovery of a microdeletion and missense variants in the human GlyR α 2 subunit in cases of autism spectrum disorder, intellectual disability and neurodevelopmental disorders. We highlight fundamental concepts, knowledge gaps and potential future developments in the field, including a roadmap to personalized medicines for individuals with pathogenic GlyR α 2 mutations.

Biological roles of the GlyR α 2 subunit

The GlyR α 2 subunit was initially designated as the 'neonatal isoform' (49 kDa) of the GlyR, distinguishable from the 'adult isoform' (α 1, 48 kDa) by virtue of molecular weight and an embryonic/neonatal expression pattern in rodents (Becker et al., 1988; Akagi et al., 1991). GlyR α 2 shows ubiquitous expression throughout the developing CNS (Kuhse et al., 1991; Malosio et al., 1991) reaching peak expression within the first few weeks after birth and declining in postnatal stages. GlyRs containing the α 2 or α 3 subunits exhibit significantly larger main state single-channel conductances compared to those comprised of the α 1 subunit (Bormann et al., 1993) due to a single non-conserved alanine to glycine substitution at the intracellular end of the pore-lining TM2 domain (Bormann et al., 1993). The rapid short-burst activity mediated by GlyR α 1 β channels in response to low glycine concentrations makes them well-suited to mediating inhibitory synaptic currents (Krashia et al., 2011; Zhang Y. et al., 2015). Conversely, the currents mediated by GlyR α 2 and α 2 β channels are characterized by significantly slower rise and decay times (Mangin et al., 2003; Zhang Y. et al., 2015). Mangin et al. (2003) concluded that the slow kinetic properties exhibited by GlyR α 2 channels are ill-suited to the phasic neurotransmission, but rather better equipped for tonic activation at non-synaptic sites. In line with these extrasynaptic activation kinetics, the GlyR α 2 subunit was initially assigned a key role in synaptogenesis (Kirsch and Betz, 1998; Levi et al., 1998). Levi et al. (1998) demonstrated that the chronic treatment of cultured spinal cord neurons from E14 rats with the GlyR antagonist strychnine impeded the formation of postsynaptic GlyR clusters. The inhibition of GlyR activation by strychnine affected the ability of GlyR clusters to form within the somatodendritic membrane (Levi et al., 1998). Kirsch and Betz (1998) went a step further and deduced that GlyR activation triggers synaptogenesis via membrane depolarization (i.e., Cl^- efflux), which facilitates the opening of L-type Ca^{2+} channels. The resulting Ca^{2+} influx causes the accumulation of gephyrin at the membrane, 'trapping' the newly formed GlyR clusters opposite presynaptic terminals (Kirsch and Betz, 1998). Flint et al. (1998) were the first to propose a potential role for GlyR α 2 in neocortical development, as the main conductance states of GlyRs expressed by embryonic and neonatal cortical neurons resembled α 2 channels. GlyR activation in immature cortical neurons stimulated the synaptic release of GABA from GABAergic interneurons via a rise in intracellular Ca^{2+} concentrations (Flint et al., 1998). They also hypothesized that the GlyR-mediated increase in membrane depolarization – resulting in increased intracellular Ca^{2+} – could play a role in neurogenic processes, such as cellular



migration and differentiation (Flint et al., 1998). However, proof of this concept required the generation of GlyR $\alpha 2$ knockout mice.

Lessons learnt from GlyR $\alpha 2$ mouse models

GlyR $\alpha 2$ defects in retinal signaling pathways and vision

GlyR $\alpha 2$ knockout mouse models have provided unrivaled insights into the biological role of the GlyR $\alpha 2$ subunit. The first GlyR $\alpha 2$ knockout mouse model was generated by Young-Pearse et al. (2006), who replaced exons 6 and 7 of the mouse *Gla2* locus with a phosphoglycerate kinase I promoter neomycin phosphotransferase gene cassette, resulting in a frameshift and loss of part of the ECD and TM1-TM4. This model was generated to study rod photoreceptors in the neonatal retina, which were reduced after transient knockdown of GlyR $\alpha 2$ using siRNA (Young and Cepko, 2004). The hypothesis was that GlyR $\alpha 2$ activation induced retinal progenitor cells to exit mitosis and generate rods within the photoreceptor layer (Young and Cepko, 2004). Curiously, this retinal phenotype was not observed in the GlyR $\alpha 2^{\Delta Ex6-7}$ knockout mouse model as no morphological, molecular or electroretinogram differences were reported in neonatal retinæ (Young-Pearse et al., 2006). However, later studies using knockout mice have implicated GlyR $\alpha 2$ in crossover inhibition between ON

and OFF retinal pathways (Nobles et al., 2012), as well as in the modulation of the receptive field surround of OFF retinal ganglion cells (Zhang C. et al., 2015). More recently Tian et al. (2023) examined ocular phenotypes using a novel *Gla2* mouse knockout line generated by removing exon 2 using CRISPR/Cas9 technology, resulting in a frameshift and loss of the majority of the ECD and TM1-TM4. Surprisingly, Tian et al. (2023) recorded a significant reduction in the formation of rod photoreceptors in GlyR $\alpha 2^{\Delta Ex2}$ knockout retinæ compared to wild-type littermates, in addition to deficits in rod pathway transduction as evidenced by a reduced dark-adapted electroretinography response. Other ocular defects observed in the GlyR $\alpha 2^{\Delta Ex2}$ knockouts were reduced visual acuity and corneal thickness, and mice were more myopic than their wild-type littermates (Tian et al., 2023). The reasons for the phenotypic differences in retinal phenotypes between GlyR $\alpha 2^{\Delta Ex2}$ and GlyR $\alpha 2^{\Delta Ex6-7}$ knockout lines remain unclear.

GlyR $\alpha 2$ defects in corticogenesis

In 2013, Harvey and Dear generated a novel GlyR $\alpha 2$ knockout line (GlyR $\alpha 2^{\Delta Ex7}$), employing the *Cre-Lox* gene targeting system to excise exon 7 of *Gla2*, resulting in the loss of TM1-TM3, and TM4 via a frameshift (Avila et al., 2013). Initially, the GlyR $\alpha 2^{\Delta Ex7}$ knockout mouse line was used to investigate the role of GlyR $\alpha 2$ in corticogenesis (Avila et al., 2013; Avila et al., 2014). Firstly, a range of

observational and functional assays including immunolabeling, Western blot analyses and electrophysiological recordings established that functional homomeric GlyR $\alpha 2$ channels were found in interneurons of the embryonic cortex (Avila et al., 2013). Acute inhibition of these channels via strychnine application to cultured embryonic brain slices decreased the migration velocity and nucleokinesis frequency of cortical interneurons (Avila et al., 2013). These observations were also reported in the embryonic brains of GlyR $\alpha 2^{\Delta Ex7}$ knockout mice, as interneurons traveling in the subventricular zone (SVZ) stream exhibited a reduction in their migration velocity and frequency of nuclear translocation (Avila et al., 2013). Building upon the observations of Flint et al. (1998) that GlyR activation in the developing cerebral cortex triggered depolarization-mediated Ca^{2+} influx, Avila et al. (2013) reported a

decreased velocity of interneuron migration in the presence of N-type and L-type voltage-gated Ca^{2+} channel blockers (omega-conotoxin and calciseptin). This suggested an integral role for Ca^{2+} influx – triggered by GlyR activation – in this process. Downstream of Ca^{2+} influx, it was noted that treatment with a myosin light chain (MLC) kinase blocker (ML-7) reduced migration velocity and nuclear translocation as was observed in the GlyR $\alpha 2^{\Delta Ex7}$ knockout or upon strychnine application to cultured embryonic brain tissue (Avila et al., 2013). Together, these results suggested that activation of homomeric GlyR $\alpha 2$ triggers depolarization-mediated Ca^{2+} influx causing the phosphorylation of MLC and activation of the myosin II complex. This results in the accumulation and contraction of actomyosin fibers at the rear of the cortical interneuron nucleus, thus promoting nucleokinesis (Figure 2; Avila et al., 2013).

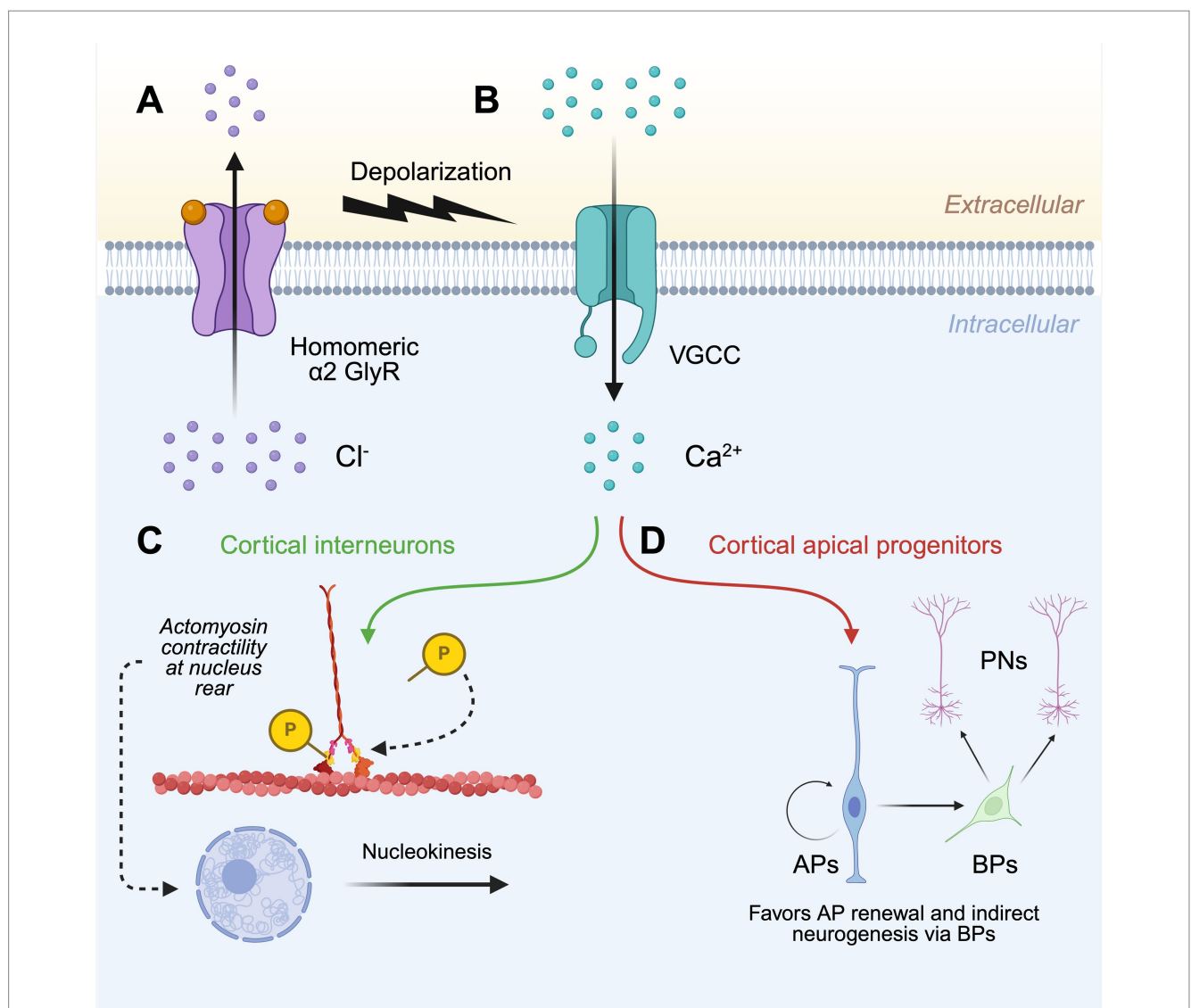


FIGURE 2

Model for the role of GlyR $\alpha 2$ in cortical development. (A) The activation of homomeric $\alpha 2$ GlyRs in the developing cerebral cortex causes membrane depolarization by facilitating Cl^- efflux. (B) Membrane depolarization triggers the opening of voltage-gated Ca^{2+} channels and subsequent Ca^{2+} influx, which within (C) cortical interneurons promotes nucleokinesis through the phosphorylation of myosin light chain, resulting in the accumulation and contraction of actomyosin at the rear of the nucleus. (D) Ca^{2+} influx also ensures the proper generation of projection neurons via the homeostatic self-renewal and differentiation of cortical apical progenitors. GlyR, glycine receptor; VGCC, voltage-gated Ca^{2+} channel; AP, apical progenitor; BP, basal progenitor; PN, projection neuron. Created in <https://BioRender.com>.

Avila et al. (2014) next sought to examine a potential role of GlyR $\alpha 2$ in the generation of cortical projection neurons. Immunolabeling and electrophysiological recordings demonstrated prolific expression of GlyR $\alpha 2$ throughout the embryonic cortex (Avila et al., 2014). In particular, GlyR $\alpha 2$ localized to apical progenitor (AP) and basal progenitor (BP) cells of the ventricular and subventricular zones, respectively (Avila et al., 2014). Gross morphological analysis of newborn GlyR $\alpha 2^{\Delta Ex7}$ knockout mice revealed microcephaly, with a significant thinning of the cerebral cortical wall (Avila et al., 2014). Given that a reduction in cortical interneurons alone could not account for the significant reduction in cortical wall thickness, one hypothesis was that this resulted from poor survival of newborn neurons and their progenitors (Avila et al., 2014). Comparison of GlyR $\alpha 2^{\Delta Ex7}$ knockout mice with controls revealed a depletion of BPs, and later a progressive depletion of APs (Avila et al., 2014). APs from GlyR $\alpha 2^{\Delta Ex7}$ knockout mice showed a propensity for direct neurogenesis, largely bypassing the generation of BPs – a cell population that acts as progenitors for the formation of projection neurons (Avila et al., 2014). Furthermore, cortical progenitor cell cycle exit was increased in GlyR $\alpha 2^{\Delta Ex7}$ knockout embryos, suggesting that loss of GlyR $\alpha 2$ causes the premature differentiation of BPs (Avila et al., 2014). Due to this predisposition for APs to undergo direct neurogenesis and BPs to prematurely differentiate, the progressive exhaustion of the cortical progenitor pool in GlyR $\alpha 2^{\Delta Ex7}$ knockout mice results in cortices with a reduced number of projection neurons in upper and deep layers (Figure 2), therefore explaining the microcephaly noted in newborn mice (Avila et al., 2014).

GlyR $\alpha 2$ defects lead to hyperexcitation and susceptibility to seizures

Morelli et al. (2017) extended this study by investigating the effect of GlyR $\alpha 2$ ablation on the morphological and functional characteristics of deep-layer neurons of the cerebral cortex. Upon examination of microcephalic GlyR $\alpha 2^{\Delta Ex7}$ knockout mice cortices, a significant reduction in interneurons, as well as upper and sub-cerebral layer projection neurons were recorded when compared to wild-type littermates (Morelli et al., 2017). In addition to a reduction in numbers, morphological defects were seen in the projection neurons and interneurons that populate layer V of the somatosensory cortex. Projection neurons and interneurons of GlyR $\alpha 2^{\Delta Ex7}$ knockout mice cortices were characterized by greater dendritic lengths, as well as increased branching points and dendritic spine numbers. Furthermore, analysis of dendritic shafts and spines found an increased abundance of postsynaptic density 95-positive boutons, suggesting a greater density of excitatory inputs upon projection neurons and interneurons in layer V of GlyR $\alpha 2^{\Delta Ex7}$ knockout somatosensory cortices (Morelli et al., 2017). Subsequent electrophysiological recordings of neonatal layer V projection neurons and interneurons within GlyR $\alpha 2^{\Delta Ex7}$ cortices established a clear shift toward excitation compared to wild-type controls (Morelli et al., 2017). This was evident by an increased frequency of excitatory postsynaptic currents (EPSCs) and a concurrent decreased frequency of inhibitory postsynaptic currents (IPSCs; Morelli et al., 2017). This purported imbalance between excitatory and inhibitory synaptic connections within layer V of the somatosensory cortices was further examined by comparing the behavior of adult GlyR $\alpha 2^{\Delta Ex7}$ knockout

and wild-type littermates upon infusion with the chemoconvulsant pentylenetetrazol (Morelli et al., 2017). GlyR $\alpha 2^{\Delta Ex7}$ knockout mice exhibited a significantly lower seizure threshold for the onset of mild and severe behaviors compared to wild-type littermates (Morelli et al., 2017). Taken together, these results suggest that GlyR $\alpha 2^{\Delta Ex7}$ knockout mice have a greater susceptibility to epileptic seizures due to the formation of aberrant somatosensory cortical circuits that are prone to overexcitation.

GlyR $\alpha 2$ defects in behavior, learning, and memory

Pilorge et al. (2016) also subjected GlyR $\alpha 2^{\Delta Ex7}$ knockout mice to a battery of behavioral tests for motor incoordination, anxiety, repetitive behaviors, and impairments in social interactions. However, GlyR $\alpha 2^{\Delta Ex7}$ knockout mice were indistinguishable from wild-type littermates with regard to these behaviors (Pilorge et al., 2016). Although GlyR $\alpha 2^{\Delta Ex7}$ knockout mice did not display defects in spatial memory as assessed by the novel location recognition task and Morris water maze, they did demonstrate impaired learning and memory in the novel object recognition task (Pilorge et al., 2016). Moreover, prefrontal cortex slices from GlyR $\alpha 2^{\Delta Ex7}$ knockout mice and littermates were subjected to high-frequency stimulation to induce plasticity, with the former displaying impaired long-term potentiation (Pilorge et al., 2016). In subsequent studies, Molchanova et al. (2017) and Comhair et al. (2018) sought to investigate the role of GlyR $\alpha 2$ in striatal medium spiny neuron (MSN) development and function. Firstly, in agreement with previous studies (Jonsson et al., 2012; McCracken et al., 2017), Molchanova et al. (2017) confirmed the expression of GlyR $\alpha 2$ by MSNs of the adult dorsal striatum via immunolabeling. The GlyR $\alpha 2$ subunit was the most abundantly expressed of the four GlyR α subunits, with both MSN populations of the dorsal striatum expressing similar levels (Molchanova et al., 2017). Inhibition of GABA_A receptors via the application of gabazine to striatal slices completely abolished IPSCs, suggesting that GlyRs are absent from synaptic sites and might instead be tonically-activated extrasynaptic receptors (Molchanova et al., 2017). Indeed, the reversal potential of glycinergic currents in MSNs was determined to be more positive than the resting membrane potential and the cells treated with strychnine were more hyperpolarized compared to those under control conditions (Molchanova et al., 2017). Therefore, these tonically-active GlyRs exert a depolarizing action upon MSNs at rest and affect the offset of evoked action potential firing (Molchanova et al., 2017). As such, MSNs treated with strychnine or those from GlyR $\alpha 2^{\Delta Ex7}$ knockouts were less excitable and fired fewer action potentials due to their more hyperpolarized resting membrane potential (Molchanova et al., 2017). To assess the potential behavioral consequences of less excitable striatal MSNs, GlyR $\alpha 2^{\Delta Ex7}$ knockouts and wild-type littermates were subjected to a battery of behavioral protocols (Molchanova et al., 2017). In line with previous studies (Young-Pearse et al., 2006; Pilorge et al., 2016; Lin et al., 2017), GlyR $\alpha 2^{\Delta Ex7}$ knockout mice showed no discernible difference in basic locomotion, habituation, and level of anxiety when compared to wild-type littermates (Molchanova et al., 2017). However, GlyR $\alpha 2^{\Delta Ex7}$ knockout mice displayed impaired motor memory consolidation during the rotarod and single-pellet reaching tests (Molchanova et al., 2017).

Comhair et al. (2018) extended these studies by examining the function of GlyR $\alpha 2$ in neonatal MSNs. Curiously, in contrast to previous studies on adult striatal MSNs (Molchanova et al., 2017), in neonatal MSNs no change in holding current was observed upon strychnine application nor were synaptic currents recorded when glutamate and GABA receptors were pharmacologically blocked (Comhair et al., 2018). This suggested that neither tonic nor phasic glycinergic signaling is present in neonatal dorsal MSNs (Comhair et al., 2018). However, despite this apparent lack of tonic or phasic signaling, distinct differences were observed in the characteristics of spontaneous and evoked action potentials between GlyR $\alpha 2^{\Delta Ex7}$ knockout and wild-type neonatal striata (Comhair et al., 2018). The frequency of action potential firing and the threshold at which action potentials began to accommodate were significantly reduced in GlyR $\alpha 2^{\Delta Ex7}$ knockout striata compared to controls (Comhair et al., 2018). Additionally, individual action potential amplitudes were lower, and their duration was longer in GlyR $\alpha 2^{\Delta Ex7}$ knockout striata (Comhair et al., 2018). These results were replicated upon strychnine application to wild-type striata, validating the involvement of GlyR $\alpha 2$ in the spontaneous activity of neonatal MSNs (Comhair et al., 2018). Functional analysis of the glutaminergic innervation of neonatal MSNs found that the frequency of miniature EPSCs was reduced in GlyR $\alpha 2^{\Delta Ex7}$ knockout striata and this impairment persisted into adulthood (Comhair et al., 2018). Unlike the aberrant dendritic morphology which was observed in projection neurons and interneurons of the cerebral cortex in GlyR $\alpha 2^{\Delta Ex7}$ knockout mice (Morelli et al., 2017), striatal MSN dendritic tree morphology and glutamatergic synapse abundance remained unaltered between the GlyR $\alpha 2^{\Delta Ex7}$ knockout and controls (Comhair et al., 2018). However, compared to wild-type striata, a reduction in the AMPA/NMDA ratio was observed in GlyR $\alpha 2^{\Delta Ex7}$ knockout mice, indicative of a greater number of silent synapses and therefore a deficit in synapse maturation (Comhair et al., 2018). To investigate whether this immature glutamatergic input onto striatal MSNs would result in impaired motor performance, neonatal GlyR $\alpha 2^{\Delta Ex7}$ knockout mice and littermate controls were examined in the righting reflex test (Comhair et al., 2018). Indeed, GlyR $\alpha 2^{\Delta Ex7}$ knockout neonates were significantly slower to right to all four paws starting from P6 compared to their wild-type counterparts (Comhair et al., 2018).

GlyR $\alpha 2$ defects increase dopaminergic signaling and enhance reward-motivated behaviors

The dorsal striatum also mediates reward-motivated behaviors (Balleine et al., 2007). Dopaminergic input from the midbrain to striatal MSNs of the direct pathway (which express dopamine D1 receptors) causes GABAergic inhibition of the internal globus pallidus and substantia nigra pars reticulata nuclei (Yager et al., 2015). The resulting excitation of the thalamus provides a 'go' signal to commence behaviors (Yager et al., 2015). Devoght et al. (2023) sought to examine the effect of GlyR $\alpha 2^{\Delta Ex7}$ knockout on dopamine-mediated striatal activity and function in adult mice. GlyR $\alpha 2$ expressed by D1-MSNs moderates dopamine-mediated activity, acting to shunt striatal cell depolarization as the membrane potential exceeds the Cl^- equilibrium potential (-54 mV) (Devoght et al., 2023). Devoght et al. (2023) therefore hypothesized that depletion of GlyR $\alpha 2$ would allow a larger

increase in striatal projection neuron (SPN) activity in response to dopamine. Indeed, optogenetically induced dopamine release on SPNs in GlyR $\alpha 2^{\Delta Ex7}$ knockout brain slices revealed a greater increase in the dopamine-mediated activity of D1-MSNs, compared to wild-type controls (Devoght et al., 2023). Consistent with this increased dopamine release, GlyR $\alpha 2^{\Delta Ex7}$ mice demonstrated an increase in novelty-induced locomotion in the open field test, with increased time spent in the center of the arena (Devoght et al., 2023). An increased locomotor response to D-amphetamine (but not cocaine) was also observed in GlyR $\alpha 2^{\Delta Ex7}$ knockout mice. GlyR $\alpha 2^{\Delta Ex7}$ knockout and wild-type mice were also subjected to an appetitive conditioning task, where difference only became apparent during highly-demanding motivational reward schedules, indicative of enhanced motivated behavior in GlyR $\alpha 2^{\Delta Ex7}$ knockout mice (Devoght et al., 2023).

GlyR $\alpha 2$ variants in human neurodevelopmental disorders

In addition to the studies documented above for GlyR $\alpha 2$ subunit knockout mice, investigation of a potential role for GlyR $\alpha 2$ dysfunction in human neurological disorders was first prompted after several large-scale sequencing projects documented rare *GLRA2* missense variants in isolated cases of autism spectrum disorder (ASD; Pinto et al., 2010; Piton et al., 2011; Iossifov et al., 2014; Pilorge et al., 2016; Supplementary Table S1). Additional clinical symptoms were reported in some individuals, including delay/loss of acquired language and seizures (Piton et al., 2011; Pilorge et al., 2016). The first structure–function study on GlyR $\alpha 2$ variants reported a microdeletion (*GLRA2* ^{$\Delta Ex8-9$}) and two *de novo* missense mutations, GlyR $\alpha 2^{N109S}$ and $\alpha 2^{R126Q}$, found in the hemizygous (XY) state in males (Pilorge et al., 2016). All three of these variants resulted in a total or partial *loss-of-function* (Pilorge et al., 2016). Microdeletion *GLRA2* ^{$\Delta Ex8-9$} was reported in a male with ASD, motor incoordination, language delay and bilateral myopia, which he had inherited from his healthy mother (Pinto et al., 2010; Pilorge et al., 2016). This microdeletion results in the truncation of GlyR $\alpha 2$ in the ICD, with loss of TM4. Although the resulting transcript appears to escape nonsense-mediated RNA decay, the truncated GlyR $\alpha 2$ subunit is not expressed at the cell surface (Pilorge et al., 2016). Given that *GLRA2* escapes X-inactivation in most tissues including the brain (Cotton et al., 2015), the mother of the proband likely remains unaffected by this microdeletion as the normal allele can compensate for the microdeletion. By contrast, GlyR $\alpha 2^{N109S}$ and $\alpha 2^{R126Q}$ missense variants cause reduced whole-cell and cell-surface expression and loss of glycine sensitivity (Pilorge et al., 2016). Consistent with this finding, molecular modeling predicted that the $\alpha 2^{R126Q}$ substitution abolishes critical hydrogen bonds within the glycine binding site (Pilorge et al., 2016). A third variant, GlyR $\alpha 2^{R323L}$, was identified in the heterozygous state in a female ASD proband, inherited from her healthy mother (Piton et al., 2011). Other clinical features included a loss of acquired words, seizures, mild motor development delay, macrocephaly and hypothyroidism (Supplementary Table S1). This case was of interest, given that microcephaly was observed in newborn *Gla2* knockout mice (Avila et al., 2014). Could a *gain-of-function* mutation explain the macrocephaly? Curiously, detailed functional analysis revealed that GlyR $\alpha 2^{R323L}$ does indeed result in a *gain-of-function* (Zhang et al., 2017) due to slower synaptic decay times, longer durations of active

periods and an increase in single-channel conductance for both homomeric $\alpha 2^{R323L}$ and heteromeric $\alpha 2^{R323L}\beta$ receptors (Zhang et al., 2017).

More recently, Chen et al. (2022) published a comprehensive functional analysis of four missense mutations GlyR $\alpha 2^{V-22L}$, GlyR $\alpha 2^{N38K}$, GlyR $\alpha 2^{K213E}$ and GlyR $\alpha 2^{T269M}$ (Supplementary Table S1). GlyR $\alpha 2^{V-22L}$ is located in the N-terminal signal peptide, and was originally in a female ASD proband with a verbal and non-verbal IQ of 63 and 103 (Supplementary Table S1). This was initially dismissed, since a variant in the signal peptide is not normally expected to disrupt GlyR $\alpha 2$ function, as it is cleaved during receptor subunit maturation and assembly. However, Chen et al. (2022) found that bioinformatic algorithms predicted that GlyR $\alpha 2^{V-22L}$ alters the signal peptide cleavage site, resulting in the retention of an additional five amino acids at the GlyR $\alpha 2$ subunit N-terminus. Biochemical analysis found that while whole-cell expression of GlyR $\alpha 2^{V-22L}$ was unaltered, cell-surface expression was significantly reduced, consistent with a partial *loss-of-function*. GlyR $\alpha 2^{N38K}$ was a *de novo* variant found in a male assigned as a 'designated unaffected sibling' of an ASD case (Supplementary Table S1; Krumm et al., 2015). However, molecular modeling revealed that the GlyR $\alpha 2^{N38K}$ variant introduces a clash with the glycan attached to residue N45, possibly impeding N-linked glycosylation of GlyR $\alpha 2$ (Chen et al., 2022). Subsequent biochemical assays demonstrated that GlyR $\alpha 2^{N38K}$ exhibits significantly reduced whole-cell and cell-surface expression (Chen et al., 2022). In line with a diminished cell-surface expression, GlyR $\alpha 2^{N38K}$ exhibited a reduced I_{max} when expressed in HEK293 cells, as well as smaller IPSC amplitudes in artificial synapses (Chen et al., 2022). This partial *loss-of-function* is consistent with previous studies of artificial N-glycosylation site mutants in GlyR $\alpha 1$, which impair receptor homo-oligomerization and transit through the ER-Golgi complex toward the cell membrane (Griffon et al., 1999; Schaefer et al., 2015, 2018). A male NDD case featuring refractory epilepsy, microcephaly, and severe developmental delay was identified by Chen et al. (2022) via diagnostic exome sequencing. GlyR $\alpha 2^{K213E}$ affects a highly conserved residue within the ECD, which is situated among important agonist-binding residues (Y209, T211 and F214; Yu et al., 2021; Chen et al., 2022). Molecular modeling demonstrated that GlyR $\alpha 2^{K213E}$ resulted in a clash with H208 in the closed state, while forming additional contacts with Y209 in the open state, suggesting that the ligand-bound open state might be favored (Chen et al., 2022). Although biochemical assays revealed a slight reduction in cell-surface expression, IPSCs generated by GlyR $\alpha 2^{K213E}$ in artificial synapses had significantly larger amplitudes, faster rise times and slower decay times, suggesting a *gain-of-function* (Chen et al., 2022).

The most common missense variant is GlyR $\alpha 2^{T269M}$, which affects a highly conserved residue within the pore-forming TM2 domain (Chen et al., 2022; Marcogliese et al., 2022). Initially identified in a female proband by a study that reported *de novo* variants in individuals with developmental disorders (Deciphering Developmental Disorders Study, 2017), this missense mutation has since been described in an additional six female NDD probands, all of whom have a *de novo* mode of inheritance (Marcogliese et al., 2022). These six cases have a diverse array of clinical features, including DD/ID, hypotonia/incoordination, ASD, inattention/hyperactivity, sleep disturbance, microcephaly, epilepsy, as well as various ocular defects (Supplementary Table S1). Using molecular dynamics simulations, Chen et al. (2022) revealed that GlyR $\alpha 2^{T269M}$ channels are predicted

to allow an increased occupancy of water and Cl^- within the channel pore (Chen et al., 2022). Consistent with these simulations, GlyR $\alpha 2^{T269M}$ homomers exhibit a significant leak current as revealed by blockage with picrotoxin, and a reduced glycine EC_{50} value (Chen et al., 2022). Balancing these findings with a reduced cell-surface expression and whole-cell I_{max} , Chen et al. (2022) categorized GlyR $\alpha 2^{T269M}$ overall as an *alteration-of-function* variant (Supplementary Table S1). Interestingly, missense mutations that result in spontaneous leak currents have also been previously reported in GlyR $\alpha 1$ in startle disease/hyperekplexia (e.g., GlyR $\alpha 1^{Q226E}$, GlyR $\alpha 1^{V280M}$, and GlyR $\alpha 1^{R414H}$). These mutations act in a similar manner, prolonging the decay of inhibitory postsynaptic currents (IPSCs) in artificial synapses and inducing spontaneous GlyR activation (Chung et al., 2010; Bode et al., 2013; Zhang et al., 2016).

Lastly, Marcogliese et al. (2022) also reported GlyR $\alpha 2$ missense variants in 13 unrelated NDD probands with heterogeneous clinical features, including ASD, developmental and cognitive delay of varying severity, motor incoordination, epilepsy, sleep disturbances, microcephaly, ocular manifestations, and dysmorphic craniofacial features (Supplementary Table S1). As previously mentioned, six of these probands are females who carry the recurrent *de novo* GlyR $\alpha 2^{T269M}$ variant (Marcogliese et al., 2022). Using a novel *Drosophila*-based functional system, Marcogliese et al. (2022) classified GlyR $\alpha 2^{T269M}$ as a *gain-of-function* allele based on overexpression of human GlyR $\alpha 2^{T269M}$ in presynaptic photoreceptors and postsynaptic neurons, reporting a significant increase in amplitudes of "OFF" transients for the GlyR $\alpha 2^{T269M}$ transgenic line. Using this system, they also classified GlyR $\alpha 2^{R225C}$ as a *loss-of-function* allele (Marcogliese et al., 2022). However, this system has substantial limitations for the study of GlyR $\alpha 2$ subunit mutants, since glycinergic neurons in *Drosophila* seem to be limited to small ventral lateral neurons (sLNvs) involved in circadian behavior (Frenkel et al., 2017). Thus, it is unclear how glycine is released onto exogenous GlyRs expressed in photoreceptors. In addition, this system does not measure defects in cell-surface trafficking or spontaneous opening of GlyRs. Other missense variants identified by Marcogliese et al. (2022) including p.F20S, p.I232M, p.A261T, p.P369T, p.P373L, p.R418Q. Another recently reported missense variant in the ICD (p.R323C; Mir et al., 2023) affects the same amino acid as the known *gain-of-function* mutant GlyR $\alpha 2^{R323L}$ (Figure 3; Supplementary Table S1).

Are defective GlyR $\alpha 2$ -accessory protein interactions a hidden cause of NDDs?

Sequence alignments reveal that p.P369T and p.P373L are located in the GlyR $\alpha 2$ TM3-TM4 ICD in a conserved polyproline helix type II (PPII), a helical secondary structure known for mediating interactions with *src* homology 3 (SH3) domain-containing proteins (Langlhofer and Villmann, 2016; Figure 3). This is of significant interest, since proline-rich stretches of the GlyR $\alpha 1$ and β subunit TM3-TM4 ICDs are involved in interactions with several accessory proteins (Del Pino et al., 2014; Langlhofer et al., 2020; Breitingner et al., 2021). A proline-rich motif in the GlyR β subunit mediates interactions with the SH3 domain of syndapin I, a neuronally expressed member of the Fes/CIP4 homology Bin-Amphiphysin-Rvs (F-BAR) family of lipid binding and remodeling proteins (Del Pino et al., 2014). Syndapin I has been linked to a physiological role in

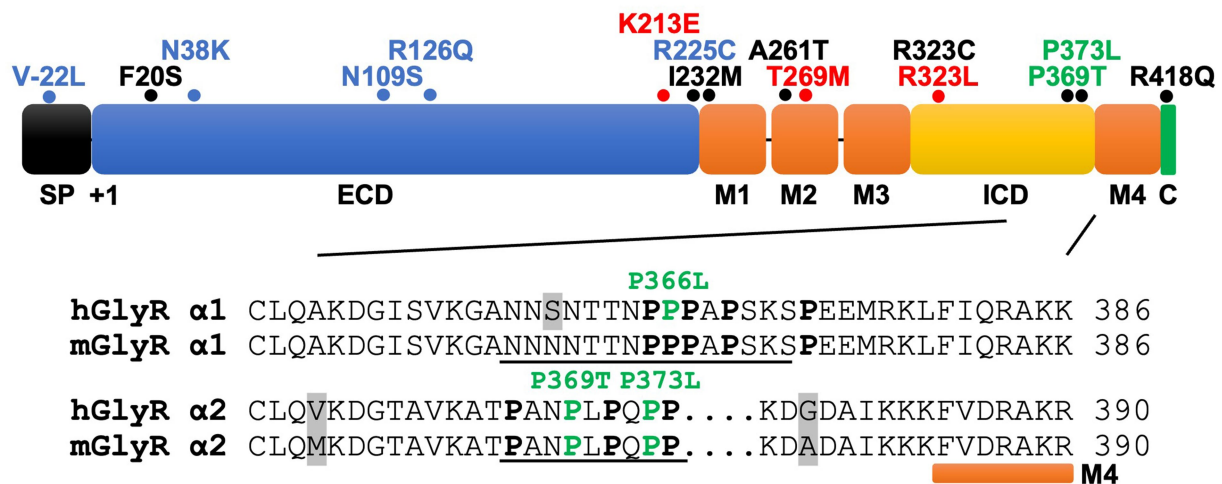


FIGURE 3

GlyR $\alpha 2$ subunit NDD mutations fall into key functional domains. Domain structure of the GlyR $\alpha 2$ subunit, including an N-terminal signal peptide (SP), extracellular domain (ECD), four membrane-spanning domains (M1–M4), the intracellular domain (ICD) and the extracellular C-terminus (C). For GlyR $\alpha 2$ missense variants (mature protein numbering), blue = loss-of-function; red = gain-of-function; green = potential altered PPIs; black = unknown. *Inset*: Alignment of mouse and human GlyR $\alpha 1$ – $\alpha 2$ subunit ICD sequences prior to M4. The GlyR $\alpha 2$ variants p.P369T/p.P373L form part of a tandem PXXP motif (underlined) and are predicted to disrupt interactions with as yet uncharacterized GlyR $\alpha 2$ interacting proteins containing a SH3 domain. While syndapin I is known to bind to a proline-rich sequence in GlyR $\alpha 1$ (underlined) and this interaction is disrupted by the GlyR $\alpha 1^{P366L}$ startle disease missense variant, there is currently no biochemical evidence that syndapin I or the RhoGEF collybistin binds to the GlyR $\alpha 2$ subunit. Proline residues are highlighted in bold type and grey shading indicates differences between mouse/human sequences.

activity-dependent bulk endocytosis (Quan and Robinson, 2013). Using a syndapin I knockout mouse model, it was found that syndapin I regulates GlyR β cluster size, density and mobility, as well as internalization via GlyR β decoupling from synaptic gephyrin scaffolds (Troger et al., 2022).

Syndapin I also associates with the GlyR $\alpha 1$ subunit ICD, albeit with substantially lower affinity (Langlhofer et al., 2020). Interestingly, a missense variant associated with startle disease in the PPII helix, GlyR $\alpha 1^{P366L}$ subunit (Figure 3) has been proposed to disrupt interactions with syndapin I. GlyR $\alpha 1^{P366L}$ did not affect whole-cell or cell-surface receptor expression (Langlhofer et al., 2020). However, GlyR $\alpha 1^{P366L}$ and $\alpha 1^{P366L}\beta$ receptors had a reduced I_{max} , rapid desensitization kinetics, and exhibited spontaneous channel openings in the absence of glycine as well as a reduction in single-channel conductance (Langlhofer et al., 2020). *In vitro* analysis using peptide microarrays and tandem MS-based analysis methods suggested that binding between the PPII helix of the GlyR $\alpha 1$ subunit and the SH3 domain of syndapin I was disrupted upon introduction of the GlyR $\alpha 1^{P366L}$ mutation. However, this was not reflected within an *ex vivo* context, as incubation of whole-brain lysates from adult mice with resin-bound GlyR $\alpha 1$ and GlyR $\alpha 1^{P366L}$ peptides revealed a clear enrichment of syndapin I in the interactomes of both wild-type and mutant subunits (Langlhofer et al., 2020). Finally, Langlhofer et al. (2020) examined the neuronal distribution of syndapin I in primary hippocampal cultures virally infected with either GlyR $\alpha 1$ or GlyR $\alpha 1^{P366L}$ constructs. When compared to neurons expressing wild-type GlyR $\alpha 1$, those transfected with GlyR $\alpha 1^{P366L}$ preferentially accumulated syndapin I in the cell soma rather than neurites (Langlhofer et al., 2020).

Subsequent to these findings, Breiting et al. (2021) investigated whether GlyR $\alpha 1^{P366L}$ also interferes with binding to the RhoGEF collybistin, another SH3 domain-containing protein crucial for

gephyrin clustering at inhibitory synapses (Kins et al., 2000; Harvey K. et al., 2004). Unexpectedly, while GST-pulldown assays did reveal an interaction between the GlyR $\alpha 1$ TM3–TM4 ICD and collybistin, the site of this interaction was localized to the pleckstrin (PH) domain of collybistin, rather than the SH3 domain (Breiting et al., 2021). A comparison of the co-localization of wild-type GlyR $\alpha 1$ and GlyR $\alpha 1^{P366L}$ with collybistin in cultured hippocampal neurons demonstrated that p.P366L appeared to weaken the colocalization of GlyR $\alpha 1$ with collybistin in the cell soma, but not dendrites (Breiting et al., 2021). In accordance with these findings, the novel NDD-associated variants in the proline-rich region of the GlyR $\alpha 2$ TM3–TM4 ICD (p.P369T and p.P373L) (Figure 3) may similarly disrupt interactions with uncharacterized SH3-domain containing proteins that interact with GlyR $\alpha 2$.

Conclusions and future perspectives

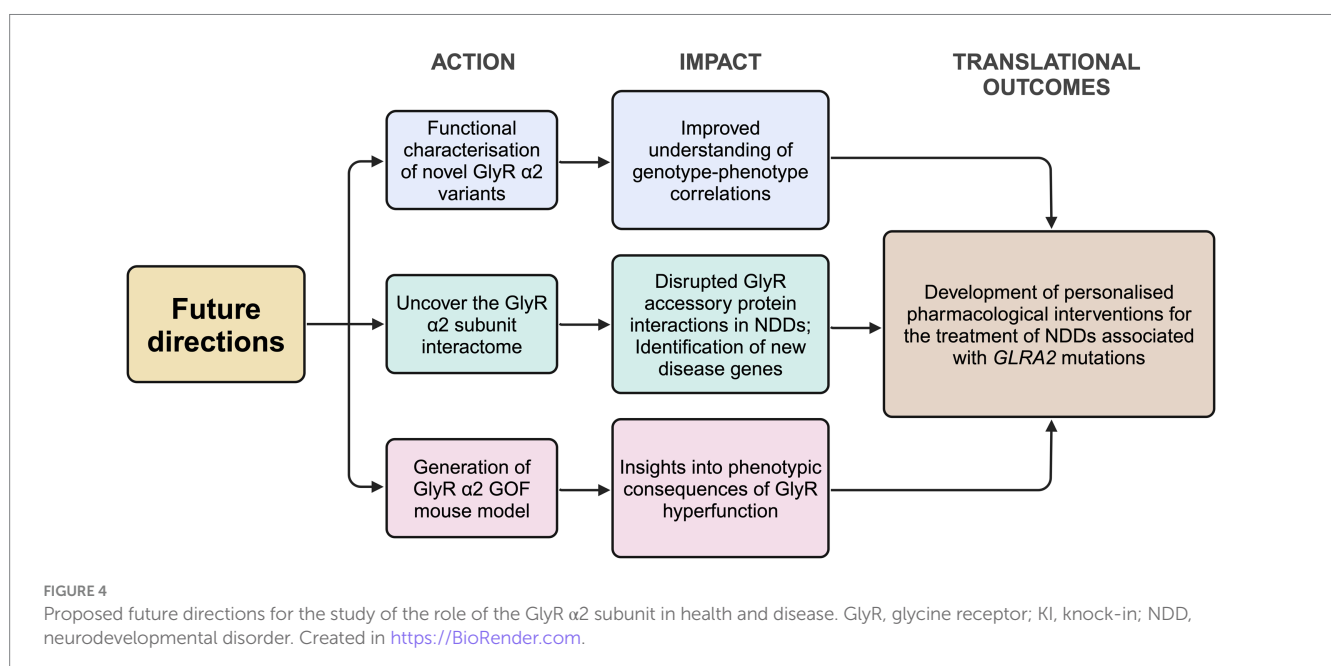
The generation and analysis of *Gla2* knockout mouse models has afforded valuable insights into the role of this GlyR subtype in health and disease (Figure 1), revealing roles in cortical progenitor homeostasis and interneuron migration (Avila et al., 2013; Avila et al., 2014), object recognition memory and motor memory consolidation (Pilorge et al., 2016; Molchanova et al., 2017), increased susceptibility to seizures (Morelli et al., 2017), defects in righting reflexes (Comhair et al., 2018), increased novelty-induced locomotion in the open field test and increased performance in motivational reward tasks (Devoght et al., 2023). GlyR $\alpha 2^{\Delta Ex7}$ knockout mice have also implicated GlyR $\alpha 2$ in ethanol-related behaviors, including binge-like drinking (San Martin et al., 2020). Since the initial discovery of a GlyR $\alpha 2$ microdeletion and missense variants in isolated cases of autism spectrum disorder with accompanying language defects and/or

seizures (Pinto et al., 2010; Piton et al., 2011; Iossifov et al., 2014; Pilorge et al., 2016), GlyR $\alpha 2$ missense variants have been identified in a spectrum of cases encompassing autism spectrum disorder, intellectual disability and neurodevelopmental disorders (Deciphering Developmental Disorders Study, 2017; Chen et al., 2022; Marcogliese et al., 2022; Mir et al., 2023). Importantly, functional studies have discovered that *GLRA2* variants can result in a loss, gain or alteration of GlyR function (Supplementary Table S1; Pilorge et al., 2016; Zhang et al., 2017; Chen et al., 2022; Marcogliese et al., 2022), perhaps explaining the broad range of clinical phenotypes observed. Further GlyR $\alpha 2$ genetic variants are expected to be identified as *GLRA2* becomes integrated into screening panels for a range of neurological disorders. In this context, there are several avenues of investigation that will enhance our understanding of the role of GlyR $\alpha 2$ in health and disease, and provide potential routes to personalized pharmacotherapies (Figure 4). However, despite these successes – it is worth noting the shortcomings of current functional studies, GlyR $\alpha 2$ mouse models and key knowledge gaps.

Firstly, while functional studies have revealed that GlyR $\alpha 2$ missense variants can result in loss, gain, or altered function, there is no clear correlation between the nature of the GlyR dysfunction and resulting clinical manifestations, severity, or disease prognosis. There are several potential confounding factors that may affect this analysis. For example, sex-specific differences have been reported in the expression of GlyR $\alpha 2$ subunit transcripts in the cortex, striatum, hypothalamus and brainstem (Ceder et al., 2024) which may influence disease severity in males versus females. In addition, NDDs often display a notable male bias in prevalence (Mendes et al., 2025). This ‘female protective effect’ suggests that females may require a higher genetic burden to manifest symptoms similar to those in males. This is relevant in for GlyR $\alpha 2$, since the corresponding gene is X-linked, meaning that defects in a single allele in males (XY) are highly likely to be pathogenic. However, there are also documented cases where *de novo* or inherited variants affecting single GlyR $\alpha 2$ alleles in females are associated with disease (e.g., p.V-22 L, p.F20S, p.I232M, p.T269M,

p.R323C, Supplementary Table S1). In this context, it is important to note that the GlyR $\alpha 2$ gene escapes X-inactivation in humans in the vast majority of tissues including the brain (Cotton et al., 2015). This means that both wild-type and defective alleles will be co-expressed, and since heteromeric $\alpha 2\beta$ GlyRs can contain four $\alpha 2$ subunits, most receptors will contain one or more defective $\alpha 2$ subunits. Lastly, seven out of the sixteen known GlyR $\alpha 2$ variants remain functionally uncharacterized (Figure 3; Supplementary Table S1). We anticipate that further exploration of sex-specific differences in GlyR $\alpha 2$ knockout mouse models and further experimental validation of GlyR $\alpha 2$ missense variants in native GlyR $\alpha 2\beta$ combinations, or in the artificial synapse system (Zhang Y. et al., 2015; Zhang et al., 2017; Chen et al., 2022), will substantially improve our understanding of genotype–phenotype correlations for GlyR $\alpha 2$.

Secondly, the discovery GlyR $\alpha 2$ missense variants affecting residues in intracellular polyproline motifs (Marcogliese et al., 2022) has also revealed a major knowledge gap – we currently do not know the identity of any SH3-domain containing proteins that interact with the GlyR $\alpha 2$ subunit. To date, only one previous study has sought to identify GlyR $\alpha 2$ interactors, using pulldown essays on rat brain extracts using a GST fusion protein containing the GlyR $\alpha 2$ subunit TM3-TM4 loop as bait (Bluem et al., 2007). This experimental design resulted in an enigmatic assortment of proteins, including components of the translational machinery [eukaryotic elongation factor 1 α (eEF1A), p70 ribosomal protein S6 kinase (rS6 kinase) and ribosomal protein S6 (rpS6); Bluem et al., 2007]. However, follow-up assays did not reveal whether these proposed interactors modulated GlyR $\alpha 2$ expression and/or function (Bluem et al., 2007), casting doubt as to whether these proteins represent genuine constituents of the GlyR $\alpha 2$ interactome *in vivo*. The GlyR $\alpha 2$ subunit TM3-TM4 loop possesses a proline-rich SH3 domain-binding motif similar to those found in GlyR $\alpha 1$ and β subunits, which have been found to bind the F-BAR and SH3 domain-containing protein syndapin I (Figure 3). For GlyR $\alpha 1$ and β subunits, interactions with syndapin I could be disrupted by mutations within intracellular polyproline motifs, such as GlyR $\alpha 1$ ^{P366L}



and GlyR $\beta^{P438A/P441A}$ (Del Pino et al., 2014; Langlhofer et al., 2020). However, there is currently no evidence that syndapin I or another key SH3-domain protein found at inhibitory synapses – the RhoGEF collybistin (Kins et al., 2000; Harvey K. et al., 2004) – binds to GlyR $\alpha 2$ *in vitro* or *in vivo*. While one study has suggested that GlyR $\alpha 1$ binds directly to collybistin, this interaction appeared to be mediated by the collybistin pleckstrin homology (PH) domain, rather than the SH3 domain (Breitinger et al., 2021). It is therefore imperative that the GlyR $\alpha 2$ interactome is re-investigated using modern proteomic methods as a matter of priority. New high-resolution methods, incorporating GlyR $\alpha 2$ knockout mouse tissue as a negative control, may reveal new components of a signaling complex associated with cortical development and function. One exciting prospect is that GlyR $\alpha 2$ subunit interactors may themselves be involved in the pathogenesis of NDDs, e.g., reciprocal SH3 domain mutations might disrupt interactions with the GlyR $\alpha 2$ subunit ICD.

Thirdly, it is clear that the generation and analysis of *Gla2* knockout mouse models has facilitated insights into the morphological, physiological and behavioral consequences of ablating GlyR $\alpha 2$ subunit expression and function (Figure 1). However, all existing mouse models (GlyR $\alpha 2^{\Delta Ex6-7}$: Young-Pearse et al., 2006; GlyR $\alpha 2^{\Delta Ex7}$: Avila et al., 2013; GlyR $\alpha 2^{\Delta Ex2}$: Tian et al., 2023) represent *loss-of-function* alleles. To fully understand the consequences of GlyR $\alpha 2$ *gain-of-function* or *alteration-of-function* variants (e.g., GlyR $\alpha 2^{T269M}$ or GlyR $\alpha 2^{R323L}$; Zhang et al., 2017; Chen et al., 2022) that have the potential to *potentiate* glycinergic signaling, novel first-in-class *gain-of-function* mouse models are required. The GlyR $\alpha 2^{R323L}$ variant represents an excellent candidate for a new mouse model of GlyR $\alpha 2$ *hyperfunction*, since this missense variant does not affect cell-surface trafficking, and incorporates into both homomeric GlyR $\alpha 2$ and heteromeric GlyR $\alpha 2\beta$ complexes. GlyR $\alpha 2^{R323L}$ also has slower IPSC decay times and longer durations of active periods in artificial synapses due to increased single-channel conductance in the presence of glycine (Zhang et al., 2017). However, the *alteration-of-function* variant GlyR $\alpha 2^{T269M}$ is also a compelling candidate – since it is associated with multiple cases of NDDs and spontaneously-opening GlyRs (Chen et al., 2022; Marcogliese et al., 2022). We anticipate that new GlyR $\alpha 2$ *gain-of-function* or *alteration-of-function* mouse lines will represent important new tools for understanding processes underlying brain development, physiology, learning/memory, seizure susceptibility and alcohol-related behaviors.

Finally, the ultimate translational outcome of this research would be personalized pharmacotherapies for individuals with GlyR $\alpha 2$ missense variants. Studies aimed at understanding exactly how disease-associated variants affect GlyR $\alpha 2$ structure and function will lead to insights into how to therapeutically-target defective GlyRs. For instance, GlyR $\alpha 2$ *loss-of-function* variants that affect cell-surface expression could potentially be treated with proteostasis regulators such as suberanilohydroxamic acid, dinoprost and dihydroergocristine, which have been found to restore surface trafficking of GABA_A epilepsy mutants (Durisic et al., 2018; Di et al., 2021). Alternatively, drugs from the GlyR allosteric ligands library (GRALL; Cerdan et al., 2020) could be tested for potentiation of wild-type GlyR $\alpha 2$ and partial *loss-of-function* mutants. Certainly, *gain-of-function* variants that potentiate glycinergic signaling may be responsive to pharmacotherapy with GlyR subunit-specific antagonists. Current candidate molecules include cyclothiazide and curcumol, both of which exert a concentration-dependent reduction in glycine-activated currents mediated by homomeric GlyR $\alpha 2$ and heteromeric GlyR $\alpha 2\beta$ channels (Zhang et al.,

2008; Wang et al., 2012). However, these and any other potential pharmacotherapies would need detailed testing in pre-clinical models, such as novel GlyR $\alpha 2$ *gain-of-function* mouse models. Certainly, the last ten years have seen an explosion of knowledge in terms of understanding the role of the GlyR $\alpha 2$ subunit in health and disease, and we look forward to seeing what insights the next ten years will bring.

Author contributions

SF: Conceptualization, Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft, Writing – review & editing. RH: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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The author(s) declared that RJH was an editorial board member of *Frontiers*, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnmol.2025.1550863/full#supplementary-material>

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