Characterization of the subunit composition and structure of native adult glycine receptors

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SUMMARY

The strychnine-sensitive pentameric Glycine Receptor (GlyR) mediates fast inhibitory neurotransmission in the mammalian nervous system. Only heteromeric GlyRs mediate synaptic transmission, since they contain the β subunit that permits clustering at the synapse through its interaction with scaffolding proteins. Here we show that $\alpha 2$ and β subunits assemble with an unexpected 4:1 stoichiometry to produce GlyR with native electrophysiological properties. We determined structures in multiple functional states at 3.6 – 3.8 Å resolutions and show $\alpha 2\beta$ GlyR assembly mechanism. Furthermore, we show that one single β subunit in each GlyR gives rise to the characteristic electrophysiological properties of heteromeric GlyR, while more β subunits renders GlyR non-conductive. A single β subunit ensures a univalent GlyR-scaffold linkage, which means the scaffold alone regulates the cluster properties.

KEYWORDS: heteromeric glycine receptor, Cryo-EM structure, stoichiometry, planar bilayer recording, stepwise photobleaching

INTRODUCTION

Nervous system function depends on electrical signal transmission across neuronal cells. At chemical synapses, electrical signals are transmitted through the release of neurotransmitters from the pre-synaptic neuron and resultant activation of the cognate neurotransmitter receptors in the post-synaptic neuron (Eccles, 1982). The neurotransmitter glycine mediates fast inhibitory neurotransmission through activating the strychnine-sensitive glycine receptors (GlyRs) at inhibitory synapses (Moss and Smart, 2001; Pfeiffer and Betz, 1981; Werman et al., 1967). GlyRs are expressed in both the central and peripheral nervous systems (Lynch, 2004). Defects in GlyRs cause the neurological disorder hyperekplexia (Bode and Lynch, 2014) and are implicated in autism spectrum disorders (Yu et al., 2013). GlyRs have also been found in the visual system (Wassle et al., 2009) and identified as possible therapeutic targets for inflammatory pain (Harvey et al., 2004; Lynch and Callister, 2006).

GlyRs are members in the ionotropic pentameric Cys-loop receptor superfamily. There are two major types of GlyRs, homomeric GlyRs composed of only α subunits and heteromeric GlyRs consisting of both α and β subunits. Extensive study on the homomeric GlyRs (Du et al., 2015; Huang et al., 2015; Kumar et al., 2020; Yu et al., 2021) and related Cys-loop receptors (Phulera et al., 2018; Rahman et al., 2020; Zhu et al., 2018) provided little information on the architecture and working mechanism of native adult GlyRs for the following reasons. First, homomeric GlyRs are found almost exclusively during early development while heteromeric GlyRs is the predominantly form in adult animals (Malosio et al., 1991; Weltzien et al., 2012). Second, heteromeric GlyRs exhibit very different electrophysiological properties, which is solely determined by the β subunit that occur only in the heteromeric GlyRs (Bormann et al., 1993; Lynch, 2009; Pribilla et al., 1992). Third, heteromeric GlyRs form high-density clusters at post-

synaptic membrane and mediate synaptic transmission while homomeric GlyRs do not localize at synapses (Kirsch et al., 1993; Specht et al., 2013). This is because homomeric GlyRs do not contain the β subunit with which post-synaptic scaffolding protein gephyrin directly associates (Meyer et al., 1995).

We know very little about heteromeric GlyRs that even the subunit composition has been controversial for decades (Durisic et al., 2012; Grudzinska et al., 2005; Kuhse et al., 1993; Langosch et al., 1988; Patrizio et al., 2017; Yang et al., 2012). The number and spatial arrangement of β subunits in each GlyRs are central questions in synaptic transmission since they impact how heteromeric GlyR clusters with multivalent scaffolding protein gephyrin (Sander et al., 2013; Sola et al., 2004) and regulate synaptic plasticity (Citri and Malenka, 2008; Zacchi et al., 2014; Zeng et al., 2018). Here, we characterized the function of heteromeric $\alpha 2\beta$ GlyR both in cells and in total reconstitution systems. A combination of biochemical and structural analyses revealed the molecular mechanism underlying the assembly of $\alpha 2\beta$ GlyR consisting four $\alpha 2$ and one β subunit, as well as how such molecular composition gives rise to native GlyR electrophysiological characteristics, including glycine activation, single-channel conductance and picrotoxin (PTX) inhibition. We further show how $\alpha 2\beta$ GlyR containing more than one β subunit does not conduct Cl^- upon glycine activation. Our findings lead to the conclusion that a 4:1 $\alpha 2:\beta$ stoichiometry accounts for the native physiology of heteromeric GlyRs.

RESULTS

Invariant $\alpha 2\beta$ GlyR subunit stoichiometry

We produced heteromeric $\alpha 2\beta$ GlyR through co-expressing the $\alpha 2$ and the β subunits in HEK293 cells for structural and functional characterization. We have identified $\alpha 2\beta$ GlyR constructs, $\alpha 2\text{em}$ and βem (see methods for details, denoted here $\alpha 2$ and β unless otherwise indicated), that

exhibited both good physiological function (Figure S1, A and B) and biochemical behavior, allowing unambiguous characterization of the molecular composition. Depending on the expression levels of each subunit, homomeric $\alpha 2$ GlyR, heteromeric $\alpha 2\beta$ GlyR, or a mixture of both may be produced. We used whole-cell patch-clamp electrophysiology to characterize the GlyRs residing in the plasma membrane as a function of expression levels.

Homomeric GlyRs are inhibited by the convulsant alkaloid, pore-blocking toxin picrotoxin (PTX) in the micro-molar range while native heteromeric GlyRs are more resistant with an over 10-fold higher IC_{50} (Pribilla et al., 1992; Shan et al., 2001). When transfected with only $\alpha 2$, homomeric GlyRs exhibited high affinity to PTX with an IC_{50} of $\sim 2.4 \mu M$ (Figure 1A and 1C black, Figure S1D) (Pribilla et al., 1992; Yang et al., 2007). When a moderate amount of the β subunit was co-expressed, at 1:1 $\alpha 2:\beta$ ratio (transfected DNA amounts, Figure S1C), the IC_{50} increased to $\sim 11 \mu M$ (Figure 1C green), indicating that a mixture of $\alpha 2$ and $\alpha 2\beta$ GlyR were produced. When an excess of the β subunit was expressed at 1:3 $\alpha 2:\beta$ ratio, PTX IC_{50} increased further to $\sim 30 \mu M$ (Figure 1, B and C blue). However, a greater excess of the β subunit (at 1:10 $\alpha 2:\beta$ ratio) did not result in further change (Figure 1C red).

Unlike PTX, neurotransmitter glycine binds to the GlyR extracellular domain, distant from the ion conduction pore. Based on the difference in its affinity for homomeric and heteromeric GlyR (Grudzinska et al., 2005; Pribilla et al., 1992), we used glycine as an additional probe of the formation of $\alpha 2\beta$ GlyR (Figure S1E). When only $\alpha 2$ GlyR was expressed, a $\sim 100 \mu M$ EC_{50} (Figure 1, D and F black) was observed. With both the 1:3 and 1:10 $\alpha 2:\beta$ ratio, the EC_{50} decreased to $\sim 60 \mu M$ (Figure 1, E and F blue, red). These numbers are within the range of reported glycine affinities for $\alpha 2$ and $\alpha 2\beta$ GlyRs (Grudzinska et al., 2005; Mohammadi et al., 2003; Pribilla et al., 1992). Clearly, the change in glycine sensitivity followed a similar trend as

that of PTX – the effects of β subunit expression saturate at 1:3 $\alpha 2:\beta$ ratio. This trend has two implications: first, at a 1:3 $\alpha 2:\beta$ ratio, sufficient β subunit is expressed to make $\alpha 2\beta$ GlyR the predominant form. Second, the number of β subunits in each $\alpha 2\beta$ GlyR did not further increase when the $\alpha 2:\beta$ ratio was changed from 1:3 to 1:10, since the incorporation of additional β subunits should further change the PTX and glycine affinities (Pribilla et al., 1992; Shan et al., 2001).

We further used Fluorescence Size Exclusion Chromatography (FSEC) to test whether the number of β subunit in each $\alpha 2\beta$ GlyR stayed the same irrespective of expression levels of $\alpha 2$ and β subunits. Since the β subunit has a higher molecular weight than that of $\alpha 2$, changes in $\alpha 2:\beta$ stoichiometry should result in changes of elution volumes. As control experiments, we co-expressed $\alpha 2$, and the higher molecular weight fluorescent fusion $\alpha 2$ -Tsi-GFP (see methods), over a wide range of expression ratios (virus MOI, see methods) (Morales-Perez et al., 2016). A clear shift in the peak elution volume was observed (Figure 1G and Figure S1F, left). This finding supports that α subunits are able to form GlyRs with arbitrary stoichiometries (Griffon et al., 1999; Kuhse et al., 1993). In contrast, when co-expressing $\alpha 2$ and β , the peak elution volume did not change over the same range of virus ratios (Figure 1H and Figure S1F, right). These observations point to an invariant subunit stoichiometry irrespective of expression levels of $\alpha 2$ and β , consistent with our and previously reported electrophysiology data (Durisic et al., 2012; Kuhse et al., 1993). Clearly, GlyRs are unlike other members of the Cys-loop receptor family in which variability in stoichiometry has been shown (Morales-Perez et al., 2016).

Planar lipid bilayer electrophysiology

Although electrophysiological properties of recombinantly expressed homomeric GlyR have been characterized (Cascio et al., 2001), functional reconstitution of heteromeric GlyR has not

been reported. We employed the planar lipid bilayer method to characterize the electrophysiology of purified $\alpha 2\beta$ GlyR *in vitro* (Figure 2A) (Mueller et al., 1962; Wang et al., 2014). In this method, a synthetic bilayer was created using the painting method across a ~ 100 μm hole. Purified $\alpha 2$ ($\alpha 2$ -Tsi-GFP) and $\alpha 2\beta$ (expressed with 1:3 $\alpha 2$: β ratio) GlyRs were reconstituted into proteoliposomes and subsequently incorporated into the synthetic membrane through salt-assisted spontaneous fusion. GlyR electrophysiological properties were thus characterized in a chemically defined environment.

The $\alpha 2$ GlyR exhibited rare spontaneous openings in the absence of ligands (Figure 2C left). When 200 μM glycine was applied to the same membrane, robust channel activation was observed (Figure 2C middle). This observation is consistent with the low ‘basal’ activity and strong glycine activation found in cellular membranes (Schmieden et al., 1989; Sontheimer et al., 1989) (See Figure 1). A histogram of the current levels (Figure 2E black) led to the identification of a ~ 75 pS single-channel conductance. This value is consistent with one major conductance observed in cells (~ 70 pS) (Bormann et al., 1993; Cascio et al., 1993; Lynch, 2009). Subsequent application of 10 μM competitive antagonist strychnine (Becker et al., 1988) on the same side of the membrane almost completely inhibited $\alpha 2$ GlyR activity (Figure 2C right). Very similar pharmacology – low basal activity (Figure 2D left), strong glycine activation (Figure 2D middle) and strychnine inhibition (Figure 2D right) – was observed for the $\alpha 2\beta$ GlyR. However, the $\alpha 2\beta$ GlyR had a much lower single-channel conductance of ~ 48 pS (Figure 2E blue), which recapitulates the major conductance of heteromeric GlyRs both heterologously expressed and in native adult tissues (~ 50 pS) (Ali et al., 2000; Bormann et al., 1993; Lynch, 2009; Takahashi et al., 1992). The single channel conductance of both $\alpha 2$ and $\alpha 2\beta$ GlyRs remained constant in bilayers made with either polar lipids extracted from brain tissues or an artificial mimic (see

Methods), indicating minimal lipid effects in these mixtures. The uniformity of single channel conductance (Figure 2E) further confirms a homogeneous subunit stoichiometry of the purified $\alpha 2\beta$ GlyR, since the unique sequence features in the M2 helix of the β subunit has been shown to alter GlyR conductance (Bormann et al., 1993). Therefore, based on the similarity of its electrophysiological properties to native heteromeric GlyRs (Ali et al., 2000; Bormann et al., 1993; Lynch, 2009; Takahashi et al., 1992), we believe this purified $\alpha 2\beta$ GlyR is likely to be the dominant, physiological form of $\alpha 2\beta$ GlyR.

4:1 $\alpha 2\beta$ GlyR structures in the closed, desensitized and open states

The $\alpha 2\beta$ GlyR structures were resolved in complex with the neurotransmitter glycine, as well as a competitive antagonist strychnine in saposin nanodiscs (Flayhan et al., 2018). Reconstructed cryo-EM maps contained densities of $\alpha 2\beta$ GlyR (Figure 3A grey), as well as a single extra satellite near the intracellular side (Figure 3A green) resulting from the GFP fusion in the intracellular M3-M4 loop of the β subunit (see methods for details), suggesting that each GlyR contains only one β subunit. Within a mask excluding this satellite density (Figure 3A translucent blue surface), the $\alpha 2\beta$ GlyR maps reached overall resolutions of 3.6 – 3.8 Å (Figure S2), allowing for unambiguous amino acid registration for the majority of the receptor (Figure S3). One β subunit (Figure 3B blue) and four $\alpha 2$ subunits (Figure 3B grey) were identified in each pentameric $\alpha 2\beta$ GlyR in the atomic models, resulting in an unexpected but unequivocal 4:1 subunit stoichiometry.

Two structural classes were present in the GlyR-strychnine complex (Figure S2A). In both classes, the ion-conduction pores were tightly constricted near the 9' amino acid residues $\alpha 2$: L268 and β : L285 (Figure 3, D and E), too narrow (< 1.8 Å radii) for even non-hydrated Cl⁻ to pass through (Figure 3C orange). The antagonist strychnine was present in all 5 neurotransmitter

binding pockets in both structures (Figure S4, A and B left). These binding pockets are in an apo/resting conformation where the loop Cs are in the “uncapped” configuration (Yu et al., 2021) (Figure S5, G and I blue). The differences between these two structures are manifested in the transmembrane domain (TMD) near the intracellular leaflet. One class (closed 1) is pseudo-5-fold symmetrical across the whole receptor (Figure S4, B and C), while the other class (closed 2) has the β TM2 tilted toward the ion conduction pore and one of the α 2 TM2 tilted out (Figure S4D). Since both structures exhibit functional characteristics of a closed conduction pore, we believe they represent two possible sub-conformations in the closed state of heteromeric GlyR.

In the α 2 β GlyR-glycine complex, we also identified two distinct structural classes (Figure S2H). In both classes, the conduction pores near the 9' position widened and no longer cause constriction as in the GlyR-strychnine structures. The radii were larger than 1.8 Å (Figure 3C black) that allow partially hydrated Cl⁻ to pass through. In one class, the ion conduction pore narrowed near the -2' amino acid residues α 2: P257 and β : A274, resulting in a new constriction near the intracellular side (Figure 3F). Glycine densities were observed in all the 5 neurotransmitter binding pockets (Figure S4B middle) that are in the agonist-bound conformation (Figure S5, D-F). These features suggest that this structural class represents the desensitized functional state. In contrast, an asymmetrical widening near -2' position in the other structural class removed constriction (Figure 3G and Figure S4F). The resultant pore radii were larger than 1.8 Å all across (Figure 3C). All 5 neurotransmitter binding pockets were in the agonist-bound conformation even though clear densities of glycine were observed for only 3 (Figure S4B right). Since there is no constriction near the 9' or the -2' positions and the pore size allows for partially hydrated Cl⁻ to pass through, similar to homomeric GlyRs in the open state

(Kumar et al., 2020; Yu et al., 2021), we believe this class represents a structure of $\alpha 2\beta$ GlyR in the open functional state.

Structural determinants of $\alpha 2\beta$ GlyR assembly and electrophysiology

The $\alpha 2\beta$ GlyR shares similar overall structures with homomeric GlyRs (Du et al., 2015; Kumar et al., 2020; Yu et al., 2021). The constriction sites in the ion conduction pathway, 9' in the closed state and -2' in the desensitized state, are similar (Figure 3). In addition, the neurotransmitter binding sites are highly conserved across the α/β , β/α and α/α interfaces and exhibit similar structural changes upon agonist binding (Figure S5). These conservation in binding sites explain the small difference in glycine affinity between $\alpha 2\beta$ and $\alpha 2$ GlyRs (Figure 1F) (Grudzinska et al., 2005; Pribilla et al., 1992). Despite apparent similarities, several features in the β subunit give rise to the unique properties of native GlyRs.

Glycosylation sites of the β subunit are required for proper assembly of $\alpha 2\beta$ GlyR. Based on sequence homology, two N-glycosylation sites have been predicted (Griffon et al., 1999; Schaefer et al., 2018). β :N220 is specific to the β subunit and located to the N-terminus of loop C, closer to the neurotransmitter binding pocket compared with $\alpha 2$ (Figure 4A, Figure S3). The β :N220A mutation abolished glycosylation and prevented proper assembly of the $\alpha 2\beta$ GlyR (Figure S5J). β :N36 was believed homologous to $\alpha 2$:N45 (Griffon et al., 1999; Schaefer et al., 2018) but turned out to locate near the vestibule (Figure 4A and B). Although the resolution was not sufficient for modeling, density maps show that the glycan extends into the vestibule (Figure 4B). β :N36A mutation also prevented expression of the $\alpha 2\beta$ GlyR (Figure S5J), suggesting its essential role in heteromeric GlyR assembly. Glycosylation at this site is likely a reason for the invariant 4:1 $\alpha 2$: β stoichiometry since the vestibule is not large enough to accommodate more than one β :N36 glycan.

The unique amino-acid composition of the pore-lining TM2 of β subunit (Figure S6) resulted in electrophysiological properties specific to heteromeric $\alpha 2\beta$ GlyR. First, two negatively-charged amino-acid residues, β :E297 and β :E290, poised near the extracellular side of transmembrane pore. The resultant negative electrostatic potential (Figure 4C) most likely reduced the local concentration of Cl^- , decreasing ion conductance. Indeed, the lower single-channel conductance of heteromeric GlyRs (Figure 2E) compared with homomeric GlyRs has been functionally attributed to these residues (Bormann et al., 1993). Second, the bulky side chain of 6' β :F282 narrows the pore radii in all functional states compared with homomeric GlyRs (Figure 4, C and D). This explains why heteromeric GlyRs exhibit much lower affinity to the pore blocking PTX: PTX binds near 6' position (Kumar et al., 2020) and β :F282 side chain constitutes steric hindrance. Supporting this conclusion, mutating β :F282 to amino acids with smaller side-chains rendered high-affinity blocking of heteromeric GlyRs just like with homomeric GlyRs (Pribilla et al., 1992).

Only 4:1 $\alpha 2:\beta$ GlyR conducts

The negative charges of β :E297 and β :E290 and reduced pore radii due to the bulky β :F282 predict that heteromeric GlyRs containing more than one β subunit should be impaired in Cl^- conduction. A hypothetical model of $(\alpha 2)_3\beta_2$ was constructed based on our open/desensitized structures using a $\alpha 2$ - β - $\alpha 2$ - β - $\alpha 2$ (meta) configuration where two conduction barriers formed (Figure 4E). First, negative electrostatic potentials in the pore near β :E297 and β :E290 indicate greatly reduced local Cl^- concentration as well as raised electrostatic barrier. Second, the two β :F282 sidechains resulted in tight constriction ($<1.8\text{\AA}$) even in the open/desensitized states. To test whether heteromeric GlyR with more than one β subunit is indeed deficient in Cl^- conduction, we generated a chimeric construct, βchim , by combining $\alpha 2$ -ECD and β -TMD, to

overcome the invariant 4:1 stoichiometry in wild-type $\alpha 2\beta$ GlyR. The elution peak of $\alpha 2:\beta$ chim exhibited a left-shifting trend as $\alpha 2:\beta$ chim expression ratios changed from 1:3 (Figure 4F solid blue) to 1:10 (Figure 4F solid red), and to 0:1 (Figure 4F solid green), suggesting arbitrary $\alpha 2:\beta$ chim stoichiometry and homomeric β chim GlyR formation.

Although $\alpha 2$ and β chim formed GlyR with arbitrary stoichiometry biochemically, only the 4:1 configuration conducted Cl^- . First, the amplitudes of glycine-activated currents decreased as more β chim is expressed with respect to $\alpha 2$ (Figure 4G, 1:3 and 1:10). When only the β chim is expressed, glycine elicited no current (Figure 4G, 0:1). The reduction in current is a result of GlyR being non-conductive when 2 or more β chim are incorporated, for the following reasons. First, small change in expression levels (<4 folds, Figure 4F) does not account for decimated current levels (>1000 folds). Second, PTX affinities stayed the same as native heteromeric GlyRs in all expression levels (Figure 4H). Since the incorporation of each β chim contributes equal amount of steric hindrance at β :F282 to destabilizing PTX binding, a same PTX affinity indicates a fixed number (1) of β subunit in each conductive GlyR. Higher β chim expression levels drive the population of the only conductive species, 4:1 $\alpha 2:\beta$ chim GlyR, to other stoichiometries, therefore decreasing total measurable current. The “dominant negative” effect reported earlier of a similar chimeric β construct also supports this conclusion (Kuhse et al., 1993).

$\alpha 2\beta$ GlyR distribution in the cellular membrane

Single-molecule counting allows quantification of membrane proteins in native cell membranes (Patrizio and Specht, 2016; Schmidt et al., 1996). Most research using this method suggested 2 or 3 β subunits in each $\alpha 1\beta$ and $\alpha 3\beta$ GlyRs (Durisic et al., 2012; Patrizio et al., 2017). Since we have already demonstrated an invariant 4:1 $\alpha 2:\beta$ stoichiometry for the $\alpha 2\beta$ GlyR, this method allowed us to understand how $\alpha 2\beta$ GlyR is distributed in cell membranes.

One or two homomeric $\alpha 2$ GlyRs were observed in each diffraction limited fluorescent spot (~250 nm). We used an $\alpha 2$ -GFP fusion (see methods), which is known to form pentameric GlyR (Du et al., 2015; Huang et al., 2015; Langosch et al., 1988), to determine the probability, p_m , of GFP being mature and fluorescent under our expression conditions. In 2295 diffraction-limited fluorescent spots, many had photobleaching steps ranging from 1 to 5 (Figure 5, A and C) while a significant number had between 6 and 10 steps (Figure 5, B and C), suggesting the presence of two $\alpha 2$ GlyRs in some spots. Using a dual-binomial model in data fitting (Figure 5C), we derived a p_m of ~62%, with 67% of the spots containing 1 and 33% of the spots containing 2 $\alpha 2$ -GFP GlyRs. Coincident with our findings, multiple $\alpha 1$ GlyRs being present in a single spot has also been reported (McGuire et al., 2012).

Up to three $\alpha 2\beta$ GlyRs were found residing within diffraction limited distance. When $\alpha 2\beta$ GlyR was expressed with GFP fusion in only the β subunit, bleaching steps ranged from 1 to 3 (Figure 5, D-F). Since each $\alpha 2\beta$ GlyR contains only 1 β subunit, 3 bleaching steps necessitates no less than 3 $\alpha 2\beta$ GlyR within diffraction limited distance. Indeed, using a tri-binomial model with p_m fixed at 62%, we derived the fraction of spots containing 1, 2 and 3 $\alpha 2\beta$ GlyRs to be 36%, 33% and 31%, respectively (Figure 5F light blue, see methods). As expected, our data cannot be explained with only one $(\alpha 2)_2\beta_3$ GlyR being in each spot (Figure 5F, dashed lines).

DISCUSSION

Heteromeric GlyRs are the predominant form of GlyRs in adult animals. Research on embryonic/early developmental forms of GlyRs, the homomeric GlyRs, provided very little information on how adult heteromeric GlyRs work. Even the subunit stoichiometry was disputed for decades (Burzomato et al., 2003; Durisic et al., 2012; Grudzinska et al., 2005; Kuhse et al., 1993; Patrizio et al., 2017; Yang et al., 2012). In this work, we developed a system that allowed

characterization of heteromeric GlyRs both in cells and *in vitro*. Our results showed unequivocally that each physiological $\alpha 2\beta$ GlyR contains only one β subunit and revealed the molecular mechanism underlying such arrangement. In addition, we identified structural features that gave rise to the specific electrophysiological properties, including glycine activation, single-channel conductance and picrotoxin blocking, of heteromeric GlyRs and showed why GlyRs containing more than 1 β subunit do not conduct Cl^- . We further found in cellular membranes that multiple GlyRs may exist within diffraction limited distance (~ 250 nm). Through these findings, we pin-pointed the only physiological form of $\alpha 2\beta$ GlyR that conducts Cl^- , and revealed its working mechanism.

Clustering of GlyR at post-synaptic densities is crucial for its function in neurotransmission. Since GlyR clustering relies on a specific interaction between the β subunit and the scaffolding protein gephyrin (Kirsch et al., 1993; Meyer et al., 1995), little can be learned from homomeric GlyRs since they do not contain the β subunit. Our results have strong implications in GlyR clustering as follows (Figure 6). Since gephyrin assembles into a multimeric form with one GlyR β subunit binding site in each monomer, the number of β subunits in each GlyR determines how GlyRs are clustered – having only 1 β subunit requires the gephyrin to cluster by itself, GlyRs are recruited to the already formed gephyrin clusters. Having more than 1 β subunit in each GlyR would allow multi-valent interactions between the gephyrin and GlyR, inducing cluster formation. This is reminiscent of the phase-separated systems where some factors serve as scaffolds and others as clients (Banani et al., 2016) – the 4:1 $\alpha 2:\beta$ GlyR can only function as clients (Figure 6A) whereas additional β subunits would allow GlyR to act as scaffolds of the inhibitory post-synaptic density (Figure 6B). Our data shows that functional GlyRs can only serve as clients, indicating that synaptic plasticity of a given glycinergic synapse may be regulated through two mechanisms: the

physical properties of post-synaptic density, for examples size and compactness, can be regulated through gephyrin, while the intensity of glycinergic transmission can be further tuned through GlyRs diffusion in and out of clusters. Clearly, more research is required to gain complete understanding.

Our results do not exclude the possibility of alternative subunit composition in native neuronal cells. First, we focused on the $\alpha 2\beta$ GlyR, in contrast to $\alpha 1\beta$ and/or $\alpha 3\beta$ GlyR reported previously, raising the possibility that 4:1 is specific to the $\alpha 2\beta$ GlyR. However, this seems unlikely because of the high sequence homology at the N-terminus and subunit interfaces (Figure S5 and S6). Second, unidentified chaperons and other factors might produce GlyRs with multiple β subunit in specific neuronal cells. In this case, since GlyRs containing multiple β subunits are non-conductive upon glycine-activation (Figure 4), these GlyRs will not function as ionotropic receptors, but instead as scaffolding proteins (Figure 6B). Multiple GlyRs residing within one diffraction-limited spot (~250 nm in diameter) indicates that the number of β subunits in each spot do not necessarily equal the number of β subunits in each GlyR. Since we found this for both homomeric and heteromeric GlyRs, and in the absence of over-expressed gephyrin, the mechanism and physiological significance of this phenomenon calls for further investigations.

ACKNOWLEDGMENTS

We thank Robbie Boyed for preparing tissue culture, molecular biology and protein biochemistry tools for this project; Dr. Khuloud Jaqaman, Dr. Xian Cheng and Dr. Bruno Rocha Azevedo for help in photobleaching data collection and analysis; Dr. Ryan Hibbs and Jeong Joo Kim for input to biochemistry experiments and comments on the manuscript; Dr. Roderick MacKinnon, Dr. Youxing Jiang and Dr. Xiao Tao for comments on the manuscript; All members of the Wang

laboratory for helpful discussion. Cryo-EM data were collected at the University of Texas Southwestern Medical Center Cryo-EM Facility, which is funded by the CPRIT Core Facility Support Award RP170644. **Funding:** This work is supported by the Welch Foundation No. I-2020-20190330.

AUTHOR CONTRIBUTIONS

H.Y. performed the experiments and analyzed the data. X.B. collected the Cryo-EM data and reconstructed density maps. W.W. conceived the project, performed experiments, and analyzed data. W.W., H.Y. and X.B. composed the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

FIGURES

Figure 1. Saturable effects of β subunit expression levels

(A and B) Representative recordings of GlyR inhibition by 10 μ M PTX in the presence of 100 μ M glycine at $\alpha 2:\beta$ expression ratios of 1:0 (A) and 1:3 (B). Normalized current was calculated by dividing the currents immediately after (open triangle) PTX application by that before (closed triangle).

(C) Dose response of PTX with IC₅₀ resulting from Hill fits (lines) listed. Data are represented as mean $\pm$ SEM (n = 3-6 cells).

(D and E) Glycine activation at $\alpha 2:\beta$ ratios of 1:0 (D) and 1:3 (E). Glycine concentrations are in μ M.

(F) Dose response of glycine with EC₅₀ resulting from Hill fits (lines) listed. Data are represented as mean $\pm$ SEM (n = 3-6 cells).

1 (G and H) FSEC elution profiles of (G) $\alpha 2:\alpha 2$ -Tsi-GFP ratio of 3:1 (red), 1:1 (green) and 1:3
2 (blue) and (H) $\alpha 2:\beta$ ratio of 3:1 (red), 1:1 (green) and 1:3 (blue). Dashed line denotes 1:3 $\alpha 2:\alpha 2$ -
3 Tsi-GFP. Peak elution volumes are listed.
4 See also Figure S1.
5
6

Figure 2. Electrophysiology of GlyRs in planar lipid bilayers.

(A and B) Illustration of (A) the recording apparatus and (B) GlyR reconstituted into artificial bilayers.

(C and D) Current traces of (C) α₂ and (D) α₂β GlyRs at -50 mV membrane potential. Solution on both sides contained (in mM): 10 potassium phosphate pH 7.4, 150 KCl and 1 EDTA.

Glycine (Gly.) and strychnine (Str.) concentrations were 200 μM and 10 μM, respectively.

(E) Histogram of all points from traces of α₂ homomer (color in black) and α₂β heteromer (color in blue). The conductance of α₂ and α₂β heteromer are listed.

Figure 3. Overall structures of $\alpha 2\beta$ GlyR in complex with glycine and strychnine.

(A) One $\alpha 2\beta$ GlyR-strychnine cryo-EM density map containing GlyR (grey) and a GFP blob (green), with a mask excluding GFP (translucent blue).

(B) Cartoon representations of $\alpha 2\beta$ GlyR atomic model viewed parallel to the membrane (black lines) plane (left) and down the extracellular side (right). Grey: $\alpha 2$, blue: β , orange: strychnine, cyan sticks: N-linked glycans.

(C) Pore radii plotted against the distance along the pore axis from the Ca position of 0' Arginine.

(D-G) Ion permeation pathways of (D) strychnine-bound closed 1, (E) closed 2, and (F) glycine-bound desensitized, (G) open states. Purple, green and red mesh represents radii of >3.3 Å, 1.8-3.3 Å and < 1.8 Å, respectively. Sidechains of pore-lining residues are highlighted as sticks.

See also Figure S2, S3, S4 and S5.

Figure 4. Features of the β subunit underlying $\alpha 2\beta$ GlyR physiology.

(A) N-linked glycosylation sites on $\alpha 2$ (grey) and β (blue) subunits facing away the vestibule.

(B) cryo-EM density originating from β :N36 (blue) and extending into the vestibule. Map is low-pass filtered and 6 Å and shown as translucent surface.

(C) Ion permeation pore of $\alpha 2\beta$ GlyR with β : E297, E290 and F282 shown as spheres. Electrical potentials are shown as color gradient from red (-2 kT/e) to blue (2 kT/e).

(D) Pore radii in the closed, open and desensitized states of $\alpha 2\beta$ (see Figure 3C) and homomeric $\alpha 1$ GlyRs (PDB ID: 3JAD, 6PM6, 6PM5, respectively). Regions exhibiting clear differences between $\alpha 2\beta$ and $\alpha 1$ are shaded.

(E) Ion permeation pore of hypothetical $(\alpha 2)_3(\beta)_2$ represented as in (C).

(F) FSEC of $\alpha 2$ co-expressed with β (dashed) and β chim (solid) at ratios of 1:3 (blue), 1:10 (red) and β alone (light green).

(G) Whole-cell current activated by 100 μ M glycine ($n = 5-6$ cells, mean $\pm$ SEM).

(H) PTX dose response curves of $\alpha 2:\beta$ and $\alpha 2:\beta$ chim expressed at 1:3 and 1:10 ratios ($n = 3-6$ cells, mean $\pm$ SEM).

See also Figure S3, S5 and S6.

Figure 5. Step-wise photobleaching of GlyRs in HEK293 cells.

(A and B) Representative fluorescence intensities (black) and idealization (grey) of $\alpha 2$ -GFP exhibiting (A) 5 and (B) 10 photobleaching steps.

(C) Histogram of the $\alpha 2$ -GFP GlyR steps from 2295 spots after correction for missed events (black). A dual-binomial model was used to fit the data (grey), resulting in a GFP maturation rate pm of 62%.

(D and E) Representative photobleaching traces (blue) and idealization (cyan) of $\alpha 2\beta$ with (D) 1 and (E) 3 steps.

(F) Histogram of the $\alpha 2\beta$ GlyR steps (blue) from 206 spots, shown with two binomial models (cyan and dashed) using pm of 62%.

Figure 6. Models of heteromeric GlyR – gephyrin clustering.

(A) GlyRs containing only one β subunit conducts Cl⁻ upon activation and interact with already-formed gephyrin clusters in a monovalent manner.

(B) GlyRs containing two or more β subunits may form multivalent interactions with gephyrin but do not conduct Cl⁻.

METHODS

Cloning of hGlyR constructs

DNAs coding human glycine receptor $\alpha 2$ (NCBI: NP_002054.1) and β (NCBI: NP_000815.1) were amplified from cDNA clones (McDermott Center, UT Southwestern Medical center). The amino acid numbering starts from the first amino acid after signal peptide. For GlyR $\alpha 2$ subunit, region encoding M3/M4 loop (residues Q317-K381) was substituted by GSSG peptide, this construct was named as $\alpha 2em$. The βem construct contained the following modifications: region encoding M3/M4 loop (residues N334-N377) was removed and GGSSAAA-monomeric enhanced green fluorescent protein (mEGFP)(Zacharias et al., 2002) -SGSGSG was inserted. A PA-tag (GVAMPGAEDDVV)(Fujii et al., 2014) and PreScission protease site (LEVLFQ/GP)(Walker et al., 1994) were inserted after signal peptide at the N-terminus. For

GlyR $\alpha 2$ -Tsi-GFP construct, region encoding M3/M4 loop (residues Q317-K381) was replaced with GGSSAAA-Tsi3(Li et al., 2013)-GSAGSAAGSG-mEGFP-SGSGSG. These mutants were cloned into a modified BacMam expression vector(Goehring et al., 2014). $\alpha 2$ wild type and $\alpha 2em$ were cloned into pLVX-IRES-ZsGreen1 vector (Clontech) for electrophysiology. The $\alpha 2em$ and βem constructs are used throughout this work and denoted $\alpha 2$ and β for simplicity unless otherwise specified.

Fluorescence-Detection Size-Exclusion Chromatography (FSEC) expression assay

The BacMam vectors containing target protein were transformed into DH10BacY competent cells (Geneva Biotech) to produce bacmids, which in turn were used to produce viruses in *sf9* insect cells. The virus titer was determined using published methods(Goehring et al., 2014; Morales-Perez et al., 2016) and the virus ratios in this work denotes the multiplicity of infection (MOI) ratios. HEK293T adherent cells were infected with viruses at different $\alpha 2:\beta$ ratio or $\alpha 2:\alpha 2$ -Tsi-GFP ratios (1:0, 20:1, 10:1, 5:1, 3:1, 1:1, 1:3, 1:5, 1:10, 1:20, 0:1, total virus amount was kept constant). Expression was induced 20 hours after infection by adding 10 mM sodium butyrate with 5 μ M strychnine added at the same time. Cells were collected 48 hours after induction by centrifugation at 20,000 g for 30 minutes at 4°C. Cell pellets were resuspended and extracted with buffer A (40 mM Tris pH 8.0, 200 mM NaCl, 0.2 mM PMSF, 0.75%(w/v) n-Dodecyl β -D-maltoside (DDM) and 0.075% (w/v) Na Cholate) for 1 hour at 4°C. After centrifuge, the supernatants were analyzed by FSEC on high-performance liquid chromatography system (HPLC, Shimadzu) equipped with fluorescence detectors set to read GFP fluorescence (excitation wavelength 480nm, emission wavelength 515nm). Due to differences in molecular

weight ($\alpha 2$ 45KDa, β 77KDa, $\alpha 2$ -Tsi-GFP 87KDa), elution volume was a function of subunit stoichiometry.

Expression and purification of hGlyR

Large scale expression of hGlyR was essentially the same as in FSEC assays except for using HEK293S GnT1⁻ cells in suspension culture instead of adherent in FSEC. HEK293S GnT1⁻ cells were infected at a density of 2.5×10^6 cells/ml using viruses encoding GlyR $\alpha 2$ and GlyR β at 1:3 ratio. Expression was induced 20 hours after infection with 10 mM sodium butyrate and 5 μ M strychnine (or no strychnine for GlyR-glycine complex) added at the same time. 48 hours later, cells were collected by centrifugation, then homogenized and extracted with buffer A for 1 hour at 4°C. PA-tag antibody (NZ-1)(Fujii et al., 2014) resin was used to capture hGlyR heteromer from the extracted supernatant and then washed with buffer B (20 mM Tris pH 8.0, 200 mM NaCl, 0.2 mM PMSF, 0.1% DDM, 0.02% Na Cholate). PreScission protease was added to release the hGlyR heteromer from PA-tag antibody resin. Eluent was concentrated and loaded onto Superose 6 increase 10/300 GL size exclusion column (GE Healthcare) in buffer C (20 mM Tris pH 8.0, 200 mM NaCl, 0.025% DDM). Peak fractions were collected and concentrated to 4 mg/ml. GlyR $\alpha 2$ -Tsi-GFP homomer was prepared the same way except for that the Tse3(Li et al., 2013) resin was used for purification. Buffer B containing 10 mM EDTA was used to elute hGlyR homomer from Tse3 resin. Concentrated proteins were used immediately.

Structure determination using single-particle cryo-EM

Reconstitution of $\alpha 2\beta$ heteromer into saposin nanodisc

Reconstitution of GlyR $\alpha 2\beta$ heteromer into saposin nanodisc was modified from the published protocol (Frauenfeld et al., 2016). After screening, a 1:30:200 molar ratio of $\alpha 2\beta$:saposin:brain polar lipids extract (BPE) (Avanti) resulted in the best reconstitution with a small void peak corresponding to lipid vesicles without scaffold. 100 μ l $\alpha 2\beta$ heteromer (4.5mg/ml) and 17 μ l BPE (13.6mg/ml, 1% DDM) were mixed and put at room temperature (RT) for 10 minutes. Then 393 μ l saposin (1.2mg/ml) was added and well mixed. The mixture was put at RT for 10 minutes, then 3.2mL dilution buffer (20mM Tris pH 8.0, 200mM NaCl, 1mM strychnine / 2mM glycine) was added. After several minutes, bio-beads SM-2 (Bio-Rad) were added to the mixture and rotated overnight at 4 °C. Next morning, the mixture was put into a new tube to remove the bio-beads, then fresh bio-beads were added again. After 8 hours, the mixture was centrifuged and loaded onto Superose 6 increase size exclusion column in SEC buffer (20mM Tris pH 8.0, 200mM NaCl, 1mM strychnine / 2mM glycine). The peak fractions containing $\alpha 2\beta$ -saposin nanodisc were concentrated to 4 mg/mL and used for EM grids preparation immediately.

Cryo-EM sample preparation, data collection and image processing

Fluorinated fos-choline 8 (Anatrace) was added into 4 mg/ml $\alpha 2\beta$ heteromer saposin nanodisc sample at a final concentration of 1 cmc just before freezing grids. 3.5 μ l sample applied to a glow-discharged Quantifoil R1.2/1.3 400-mesh gold holey carbon grid (Quantifoil, Micro Tools GmbH, Germany), blotted under 100% humidity at 4 °C and plunged into liquid ethane using a Mark IV Vitrobot (FEI). Micrographs were collected on a Titan Krios microscope (FEI) with a K3 Summit direct electron detector (Gatan) operating at 300 kV using the SerialEM software. The GIF-Quantum energy filter was set to a slit width of 20 eV. Images were recorded in the super-resolution counting mode with the pixel size of 0.83 Å. Micrographs were dose-fractioned into 32 (strychnine complex) or 50 (glycine complex) frames with a dose rate of 1.6 e⁻/Å/frame.

Motioncorr2 program(Zheng et al., 2017) was used to perform 2-fold binning, motion correction and dose weighting of the movie frames. CTF correction were carried out using the GCTF program(Zhang, 2016). The following image processing steps were carried out in RELION 3.0(Scheres, 2012). Auto-picked particles were extracted and binned by 4 times and used in 2D classification. Particles in good 2D classes were selected and subjected to 3D classification using an initial model downloaded from EMDB database (EMD-6346)(Du et al., 2015). 3D classification into 6 classes yielded 2 classes with good density for the entire channel. The cryo-EM density of one GFP were identified in both classes, but present at different position with respect to the channel. As GFP is only fused to the β subunit of the receptor, this 3D classification result suggests that there is only one β subunit but four α subunits in the heteropentameric channel, and the relatively position of this single β subunit with respect to the other four α subunits are different between these two good 3D classes. To put the β subunit of both classes in the same position that would allow us to combine these two good classes, we rotated each particle from one class around the Z axis by $360^\circ/5$ through manually modifying the column “_rlnAngleRot” in the RELION selection star file. As a result, a total of 111,378 particles for GlyR-strychnine and 268,380 particles for GlyR-glycine complexes from two good 3D classes were combined, re-extracted to the original pixel size. 3D refinement of these particles with 8° local angular search and the C1 symmetry imposed led to a 3D reconstruction to overall resolutions of 3.8 – 3.9 Å, although the transmembrane region was resolved at relatively lower resolution. Therefore, the particles of the initial 3D reconstruction were subjected to partial signal subtraction to keep only the transmembrane region(Bai et al., 2015). 3D classification of these partial-subtracted particles without alignment led to two classes that showed much better density for the transmembrane domain (Figure S2A). After reverting to un-subtracted version,

CTF refinement, particle polishing, and refinement using C1 symmetry, GlyR-strychnine complex yielded two classes. One class contained 38,541 particles and reached an overall resolution of 3.8 Å (closed 1) and the other contained 44,965 particles and reached 3.6 Å (closed 2). Closed 2 class exhibited a more 5-fold symmetrical TM arrangement (Figure S5). GlyR-glycine complex also yielded two classes, one contained 55,816 particles with 3.8 Å (open) resolution and the other contained 56,419 particles and reached 3.6 Å (desensitized) resolution. Resolutions were estimated by applying a soft mask around the protein densities with the Fourier Shell Correlation (FSC) 0.143 criterion. Local resolutions were calculated using Resmap(Kucukelbir et al., 2014).

Model building and refinement

Model building of GlyR $\alpha 2\beta$ heteromer was initiated by docking the structure of $\alpha 1$ homomer strychnine-bound state(Du et al., 2015) (PDB ID: 3JAD) into the density map of GlyR $\alpha 2\beta$ heteromer using Chimera(Pettersen et al., 2004). Amino acids were mutated according to $\alpha 2$ and β sequences and rebuilt in Coot(Emsley et al., 2010). The structure model was manually adjusted in Coot, then refinement was performed with real-space refinement module with secondary structure restraints in PHENIX package(Adams et al., 2010; Liebschner et al., 2019). MolProbity was used to validate the geometries of the refined models(Williams et al., 2018). Fourier shell correlation (FSC) curves were calculated between refined atomic model and the work/free half maps as well as the full map to assess the correlation between the model and density map. Statistics of cryo-EM data processing and model refinement are listed in Table S1. Pore radii were calculated using the HOLE program(Smart et al., 1996). Figures were prepared in Chimera or PyMOL(Schrödinger, 2015). Electrostatics were calculated using Adaptive Poisson-Boltzmann plugin at 37°C and with 150 mM of monovalent anion/cations (Baker et al., 2001).

The final model of strychnine closed 2 contained the $\alpha 2$ and β subunit amino acids except the following: $\alpha 2$ subunit (total 364aa, 339aa built, 25aa not built) K1-T14, GSSG linker, K382 and E420- K425; β subunit (total 444aa, 338aa built, 106aa not built) K1-Q20, L332-N333, GGSSAAA-EGFP-SGSGSG insertion and V378-A448. Q21-R28 of β subunit were modeled as poly-alanine due to limit in resolution. The model for strychnine closed 1 is the same as closed 2 except for Q21-R28 of β subunit were not modeled. The final model of glycine bound desensitized state contained the $\alpha 2$ and β subunit amino acids except the following: $\alpha 2$ subunit (total 364aa, 338aa built, 26aa not built) K1-T14, GSSG linker, K382 and H419- K425; β subunit (total 444aa, 337aa built, 107aa not built) K1-R28, GGSSAAA-EGFP-SGSGSG insertion and V378-V443. The model for glycine bound open state is the same as desensitized state except that only 3 glycine are modeled.

Electrophysiology

Whole cell patch clamp

Plasmids bearing GlyR constructs were transiently transfected into HEK293T cells by Lipofectamine 3000 (Invitrogen) following manufacturer's protocol. For each 35 mm dish, 1.5 μ g of DNA was transfected at different α : β ratios (1:0, 1:1, 1:3, 1:10). After transfection, cells were cultured at 37°C for 6~8 hours, then transferred to 30°C cultured for ~12 hours. Whole-cell recordings were performed at room-temperature. The bath solution contained (in mM): 10 HEPES pH 7.4, 10 KCl, 125 NaCl, 2 MgCl₂, 1 CaCl₂ and 10 glucose. The pipette solution contained (in mM): 10 HEPES pH 7.4, 150 KCl, 5 NaCl, 2 MgCl₂, 1 CaCl₂ and 5 EGTA. Borosilicate glass pipettes with resistance between 2~7 M Ω were used. A Digidata 1550B digitizer (Molecular Devices) was connected to an Axopatch 200B amplifier (Molecular

Devices) for data acquisition. Analog signals were filtered at 1 kHz and subsequently sampled at 20 kHz and stored on a computer running pClamp 10.5 software. Membrane was held at -80 mV during perfusion of ligands to record GlyR current. For the heteromer recordings, as the GlyR β construct had an EGFP insertion, we used GFP fluorescence to identify the cells expressing the GlyR β subunit. Hill equation was used to fit the dose-response data and derive the EC_{50} (k) and Hill coefficient (n). For glycine activation, we used $I = I_0 + (I_{max} - I_0) \frac{x^n}{k^n + x^n}$, where I is current, I_0 is the basal current (spontaneous opening current and leak, very close to 0), I_{max} is the maximum current and x is glycine concentration. Hill equation was also used to fit picrotoxin inhibition data: $I = I_{max} - (I_{max} - I_0) \frac{x^n}{k^n + x^n}$, with same definitions of symbols except that k represents IC_{50} . Data was fitted with software OriginPro (OriginLab, Northampton, MA). Measurements were from 3–6 cells, average and S.E.M. values were calculated for each data point.

Proteoliposome reconstitution

Purified $\alpha 2\beta$ heteromer and $\alpha 2$ homomer were reconstituted into proteoliposomes using the lipid mixture composed of 3:1 (wt:wt) 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) : 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-1'-rac-glycerol (POPG). The reconstitution procedure is similar to that previously reported (Wang et al., 2014). Briefly, 20 mg/ml of the above lipid mixture was dispersed by sonication until translucent and then solubilized with 1% DDM. GlyR was diluted with reconstitution buffer (10 mM potassium phosphate pH 7.4, 150 mM KCl, 1 mM EDTA and 3 mM DTT) supplemented with 0.2% DDM to 2 mg/ml. Equal volumes of 20 mg/ml solubilized lipid mixture was combined with the diluted GlyR solutions, resulting in a final protein:lipid (wt:wt) ratios of 1:10 at a lipid concentration of ~ 10 mg/ml. Detergent was removed by dialysis against reconstitution buffer for 4 times, 12

hours each at 4°C. Bio-Beads SM2 (Bio-Rad) were added outside the dialysis bag (~1 g/500mL buffer) in the last three cycles. 20 µL aliquots of the proteoliposomes were frozen stored at –80 °C until needed.

Planar lipid bilayer recording

The planer bilayer recording experiments are similar to previously described(Wang et al., 2016; Wang et al., 2014). Briefly, 20 mg/ml of lipid mixture composed of 2:1:1(wt:wt:wt) of DOPE:POPC:POPS, or brain polar extract (Avanti), was dissolved in decane and used to form planar lipid bilayer over a ~100 µm hole on a piece of transparency film using the painting method. The same solution (10 mM potassium phosphate pH 7.4, 150 mM KCl, 1 mM EDTA) was used in both chambers. Proteoliposomes were mixed with 1M KCl at equal volume and sonicated to facilitate vesicle fusion into planer bilayer immediately before use. After vesicle fusion into planer bilayer, buffer containing glycine or strychnine was applied on the planar bilayer manually. A Digidata 1550B digitizer (Molecular Devices) interfaced to pClamp10.5 software was connected to an Axopatch 200B amplifier (Molecular Devices) for data acquisition. Analog signals were filtered at 1 kHz and subsequently sampled at 20 kHz. Recordings were performed at room temperature. Membrane voltage was held at –50 mV. The concentrations of glycine and strychnine applied to the membrane were 200 µM and 10 µM, respectively.

Step-wise Photobleaching

α2-GFP or α2:β at 1:3 ratio was transfected into HEK293T cells on collagen coated glass bottom dishes using lipofectamine 3000 (Invitrogen) for expression of α2 GlyR or α2β GlyR, respectively. The transfected cells were kept in CO₂ incubator for 6 hours at 37°C and then removed to 30°C for overnight expression. Cells were unroofed(Usukura et al., 2016) and fixed

prior to imaging as follows: After 3 times wash at room temperature (RT) with Phosphate-Buffered Saline PBS (10 mM Na₂HPO₄, 1.8 mM NaH₂PO₄, 137 mM NaCl, 3 mM KCl), cells were sonicated at 20% power for 1 s using a Branson SFX550 sonicator equipped with a flat microtip in ice-cold PBS supplemented with 2% para-formaldehyde (PFA), 2 mM MgCl₂ and 1 mM CaCl₂. Unroofed cells were washed quickly with ice-cold PBS and immediately switched into PBS containing 4% PFA at RT and fixed for 15min. After 3 times washing in PBS for 10 min each, the cells were imaged within 2 hours. Total internal reflection illumination was achieved using an in-house built prism-type system using the Gem 488 laser (Laser Quantum) and components from Thorlabs at 10% power. A 1.5 OD neutral density filter was used during searching of cells to minimize photobleaching. A Leica DM6 FS microscope equipped with a 63x 1.2 NA CS2 water immersion objective and a Hamamatsu flash 4.0 V3 camera was used for imaging. Movies were collected using a PC running Metamorph (Molecular Devices) with 100 ms exposure for each frame and total exposure of 50 s. Regions of interest were subsequently cropped using ImageJ(Schneider et al., 2012) and analyzed using the PIF program(McGuire et al., 2012) to generate statistics of photobleaching steps. A dual-binomial model formulated allowing one and two pentameric GlyRs in each diffraction-limited fluorescence spot was used to fit the photobleaching step distribution of homomeric $\alpha 2$ G GlyR as follows:

$$s(n) \mid_{1 \leq n \leq 10} = \frac{(1-f) \frac{5!}{(5-n)!n!} p_m^n (1-p_m)^{5-n} \mid_{n \leq 5} + f \frac{10!}{(10-n)!n!} p_m^n (1-p_m)^{10-n}}{1-s_0}$$

$$s_0 = (1-f) \frac{5!}{(5-0)!0!} p_m^0 (1-p_m)^{5-0} + f \frac{10!}{(10-0)!0!} p_m^0 (1-p_m)^{10-0}$$

Where $s(n)$ is the fraction of spots with n photobleaching steps corrected for the fraction of non-fluorescent events s_0 . p_m and f were the fitted parameters, which denote the maturation rate of GFP and the fraction of spots with two $\alpha 2$ GlyRs, respectively. Similarly, a tri-binomial model

assuming simultaneous presence of 1, 2 and 3 GlyRs in each spot was used for the heteromeric $\alpha 2\beta$ GlyR photobleaching steps as follows

$$s(n) \mid_{1 \leq n \leq 3}$$

$$= \frac{(1 - f_1 - f_2)p_m^n(1 - p_m)^{1-n} \mid_{n \leq 1} + f_1 \frac{2!}{(2-n)!n!} p_m^n(1 - p_m)^{2-n} \mid_{n \leq 2} + f_2 \frac{3!}{(3-n)!n!} p_m^n(1 - p_m)^{3-n}}{1 - s_0}$$

$$s_0 = (1 - f_1 - f_2)(1 - p_m) + f_1 \frac{2!}{(2-0)!0!} p_m^0(1 - p_m)^{2-0} + f_2 \frac{3!}{(3-0)!0!} p_m^0(1 - p_m)^{3-0}$$

Where f_1 and f_2 were the fitted parameters which denote the corrected fractions of spots with 2 and 3 $\alpha 2\beta$ GlyRs, respectively. p_m was fixed at 0.62 (the value from $\alpha 2$ GlyR). Traces and statistics were plotted using OriginPro (OriginLab).

Western Blotting

For GlyR $\alpha 2$ subunit, a V5-tag (KEGKPIPNPLLGLDSTAA) was inserted into the $\alpha 2em$ construct after signal peptide at the N-terminus. Plasmids bearing GlyR constructs were transiently transfected into HEK293T cells by Lipofectamine 3000 (Invitrogen) following manufacturer's protocol at different $\alpha 2:\beta$ DNA ratios (1:0, 3:1, 1:1, 1:3, 1:10, 0:1). After transfection, cells were cultured at 37°C for 20 hours, then transferred to 30°C for additional ~48 hours. After detergent extraction, supernatants were mixed with an equal volume of loading buffer containing 4% SDS and 10% DTT. Then samples were analyzed using SDS-PAGE with Bio-Rad 4-15% TGX gels and transferred onto nitrocellulose membrane. Western Blot was performed with anti-V5-tag (Cell Signaling Technology, D3H8Q) for $\alpha 2$ or Anti-GFP (Santa Cruz Biotechnology, sc-9996) for β .

1 DATA AND SOFTWARE AVAILABILITY

2 Data Resources

3 Atomic coordinates of $\alpha 2\beta$ glycine receptor have been deposited in the Protein Data Bank with
 4 accession numbers 7KUY for GlyR $\alpha 2\beta$ closed state 2, 7L31 for GlyR closed state 1, 5BKG for
 5 glycine bound open state and 5BKF for glycine bound desensitized state. The corresponding
 6 cryo-EM density maps have been deposited with the Electron Microscopy Data Bank using
 7 accession numbers EMD-23041 for $\alpha 2\beta$ closed state 2 and EMD- 23148 for $\alpha 2\beta$ closed state 1,
 8 EMD-9404 for glycine bound open state and EMD-9403 for glycine bound desensitized state.

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11

SUPPLEMENTAL INFORMATION

Characterization of the subunit composition and structure of native adult glycine receptors

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Figure S1. Whole cell voltage-clamp electrophysiology and expression assays of $\alpha 2$ and β GlyRs constructs, related to Figure 1.

(A) Representative voltage-clamp recordings of glycine dose response in HEK293 cells transfected with $\alpha 2$ wt (magenta), $\alpha 2$ em (black), $\alpha 2$ em: β wt 1:10 (pink) and $\alpha 2$ em: β em 1:10 (red) DNAs.

(B) Dose response of glycine from (A). Data points with S.E.M. (n = 3-6 cells) were plotted with Hill fits and colored the same as in (A). EC50 values are listed.

(C) Western blot of $\alpha 2$ and β subunits at different DNA ratios. M: molecular weight marker, molecular weights are in kDa. See methods for details.

(D) PTX inhibition in the presence of 100 μ M glycine. $\alpha 2$: β expression ratios of 1:0, 1:1, 1:3 and 1:10 were used. The PTX and glycine concentrations are in μ M.

(E) Glycine activation at $\alpha 2$: β ratios of 1:0, 1:3 and 1:10. The glycine concentrations are in μ M.

(F) FSEC of $\alpha 2$ and $\alpha 2\beta$ GlyRs at different $\alpha 2$: β virus ratios. Left panel, FSEC of $\alpha 2$ GlyR expressed at listed $\alpha 2$: $\alpha 2$ -Tsi-GFP virus ratios. Right panel, FSEC of $\alpha 2\beta$ GlyR expressed at different $\alpha 2$: β virus ratios. Elution volumes are listed beside each virus ratio. Molecular weight of $\alpha 2$, $\alpha 2$ -Tsi-GFP and β are 45KDa, 87KDa and 77KDa, respectively.

Figure S2. Classes of $\alpha 2\beta$ GlyR density maps, related to Figure 3.

(A-G) $\alpha 2\beta$ GlyR closed states. (A) 6 classes resulting from 3D classification after signal-subtraction view along the ion conduction pore from the intracellular side. The percentages of particles in each class are indicated. The two good classes were subsequently refined. (B) The gold standard Fourier Shell Correlation (FSC) curves from the final 3D refinement of closed state 2, with (red) and without (black) mask. (C) FSC between the atomic model and half map 1 (blue), half map 2 (orange) and the full map (purple) of closed state 2. (D) Density map of closed state 2 colored according to its local resolution estimated using RESMAP. (E) The gold standard

1 FSC from the final map of closed state 1. (F) FSC between the atomic model and maps of closed
2 state 2. Color codes are the same as in (B) and (C). (G) Density map of closed state 1 colored
3 according to its local resolution estimated using RESMAP.
4 (H-N) $\alpha 2\beta$ GlyR open state and desensitized state. (H) 6 classes resulting from 3D classification.
5 The two good classes were subsequently refined. (I-J) FSC curves of open states. (K) Density
6 map of open state colored according to its local resolution (L-M) FSC curves of desensitized
7 states. (N) Density map of open state colored according to its local resolution.
8

1

2 **Figure S3. Typical density maps, related to Figure 3 and Figure 4.**

(A-B) Densities maps of representative regions of $\alpha 2\beta$ GlyR closed state 1. (A) Density maps of β -sheet, Cys-loop and M2-M3 loop in β subunit ECD; N-glycosylation on the β subunit ECD; β -sheet, Cys-loop and M2-M3 loop in $\alpha 2$ subunit ECD; Two N-glycosylation sites on $\alpha 2$ subunit ECD. (B) M1-M4 Helices of β subunit and $\alpha 2$ subunit TMD.

(C-D) Densities maps of representative regions of $\alpha 2\beta$ GlyR closed state 2. Regions are the same as (A-B).

(E-F) Densities maps of representative regions of $\alpha 2\beta$ GlyR desensitized state. Regions are the same as (A-B).

(G-H) Densities maps of representative regions of $\alpha 2\beta$ GlyR open state. Regions are the same as (A-B).

Figure S4. Symmetry of the $\alpha_2\beta$ GlyR, related to Figure 3.

(A) $\alpha_2\beta$ GlyR closed state 2 viewed parallel to the membrane with four planes indicated.

(B) Top views from the extracellular side clipped at plane 1 of closed, desensitized, and open states.

(C) Top view of planes 2-4 for closed state 1.

(D) Top view of planes 2-4 for closed state 2.

(E) Top view of planes 2-4 for desensitized state.

(F) Top view of planes 2-4 for open state. α_2 subunit is colored in gray, β in blue, strychnine in orange as spheres, glycine in yellow as spheres and NAG in cyan as sticks. The apexes of the red pentagons in panels (C-F) indicate the M2 helices positions.

Figure S5. Strychnine and glycine binding sites, related to Figure 3 and Figure 4.

(A-C) Strychnine binding sites between (A) $\beta/\alpha 2$, (B) $\alpha 2/\beta$ and (C) $\alpha 2/\alpha 2$ interfaces from closed state 2. Densities of $\alpha 2$ R126, β F142 and Strychnine are contoured at 3σ . Strychnine (orange) and the residues interacting with strychnine ($\alpha 2$: grey, β : blue) are shown as sticks. LigPlot schematic of strychnine bind sites between (A) $\beta/\alpha 2$, (B) $\alpha 2/\beta$ and (C) $\alpha 2/\alpha 2$ interfaces, showing electrostatic (dashes) and hydrophobic (eyelashes) interactions. Non-conservative residues $\alpha 2$ R126 and β F142 are underlined.

(D-F) Glycine binding sites between (D) $\alpha 2/\beta$, (E) $\beta/\alpha 2$ and (F) $\alpha 2/\alpha 2$ interfaces from desensitized state. Densities of glycine are contoured at 4σ . Glycine (yellow) and the residues interacting with glycine ($\alpha 2$: grey, β : blue) are shown as sticks. LigPlot schematic of glycine bind sites between (D) $\alpha 2/\beta$, (E) $\beta/\alpha 2$ and (F) $\alpha 2/\alpha 2$ interfaces, showing electrostatic (dashes) and hydrophobic (eyelashes) interactions.

- 1 (G) Strychnine binding sites between $\beta/\alpha 2$, $\alpha 2/\beta$ and $\alpha 2/\alpha 2$ interfaces from closed state 1.
- 2 Strychnine (carbon as orange, nitrogen as blue and oxygen as red) are shown as sticks, $\alpha 2$ and β
- 3 ($\alpha 2$: grey, β : blue) are shown as cartoon.
- 4 (H) Glycine binding sites between $\beta/\alpha 2$, $\alpha 2/\beta$ and $\alpha 2/\alpha 2$ interfaces from desensitized state.
- 5 Glycine (carbon as yellow, nitrogen as blue and oxygen as red) are shown as sticks, $\alpha 2$ and β
- 6 ($\alpha 2$: grey, β : cyan) are shown as cartoon.
- 7 (I) Overlap of glycine/strychnine binding site between $\beta/\alpha 2$ interface from desensitized/closed 1
- 8 state. The colors are same as (G) and (H).
- 9 (J) FSEC of $\alpha 2\beta$ GlyR with glycosylation site mutation. Wild type $\alpha 2\beta$ are colored as blue, $\alpha 2\beta$
- 10 with β :N32A mutation are colored as pink, $\alpha 2\beta$ with β :N220A mutation are colored as cyan.
- 11

Figure S6. Sequence alignment of GlyR subunits, related to Figure 4.

1 Signal peptides have been removed before sequence alignment. Residue conservation is
2 indicated by blue rectangle and red highlights. Secondary structure elements are indicated by
3 helices for α -helices and arrows for β -strands above the alignment. N-linked glycosylation site of
4 GlyR $\alpha 2$ and β are indicated by cyan hexagon and cyan rectangle, respectively. Non-conservative
5 residue between GlyR $\alpha 2$ and β involved in strychnine binding is indicated by blue circle. The
6 Phe282 in M2 helix of GlyR β is indicated by blue star. The alignment was generated by Clustal
7 W. Protein sequences alignment: human GLRB (Uniprot P48167), human GLRA2 (Uniprot
8 P23416), human GLRA1 (Uniprot P23415), human GLRA3 (Uniprot O75311), GLRA1em(PDB
9 accession number 3JAE), GLRA3cryst (PDB accession number 5CFB) and GluClcryst (PDB
10 accession number 4TNV).

11

Table S1.

Cryo-EM data collection, refinement and validation statistics

	$\alpha 2\beta$ GlyR, strychnine, close 2 (EMD-23041) (PDB 7KUY)	$\alpha 2\beta$ GlyR, strychnine, close 1 (EMD-23148) (PDB 7L31)	$\alpha 2\beta$ GlyR, Glycine, open (EMD-9404) (PDB 5BKG)	$\alpha 2\beta$ GlyR, Glycine, desensitized (EMD-9403) (PDB 5BKF)
Data collection and processing				
Magnification			105,000	
Voltage (kV)			300	
Electron exposure ($e^-/\text{\AA}^2$)	50			80
Defocus range (μm)		-1.0 to -3.5		
Pixel size ($\text{\AA}$)		0.83		
Symmetry imposed		<i>C1</i>		
Initial particle (no.)	472,972		555,287	
Final particle no.)	44,965	38,541	55,816	56,419
Map resolution ($\text{\AA}$)	3.6	3.8	3.8	3.6
FSC threshold	0.143	0.143	0.143	0.143
Refinement				
Initial model used (PDB code)	3JAD	3JAD	7KUY	7KUY
Model resolution ($\text{\AA}$)	3.8	4.0	4.0	3.9
FSC threshold	0.5	0.5	0.5	0.5
Map sharpening <i>B</i> factor ($\text{\AA}^2$)	-177	-131	-155	-122
Model composition				
Non-hydrogen atoms	13633	13596	13509	13470
Protein residues	1694	1686	1686	1689
Ligands	14	14	12	14
<i>B</i> factors ($\text{\AA}^2$)				
Protein	63.9	49.5	43.3	52.2
Ligand	65.3	50.7	42.1	55.4
R.M.S. deviations				
Bond lengths ($\text{\AA}$)	0.006	0.005	0.004	0.004
Bond angles ($^\circ$)	0.849	0.835	0.555	0.524
Validation				
MolProbity score	1.85	1.80	1.83	1.79
Clashscore	7.97	7.39	7.61	6.80
Poor rotamers (%)	0.14	0.28	0.14	0.35
Ramachandran plot				
Favored (%)	93.73	94.06	93.76	93.77
Allowed (%)	6.27	5.94	6.24	6.23
Disallowed (%)	0	0	0	0