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GLYCINERGIC TRANSMISSION: PHYSIOLOGICAL, DEVELOPMENTAL AND PATHOLOGICAL IMPLICATIONS

Hosted by
Robert J. Harvey and Jean-Michel Rigo

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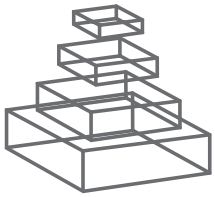

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GLYCINERGIC TRANSMISSION: PHYSIOLOGICAL, DEVELOPMENTAL AND PATHOLOGICAL IMPLICATIONS

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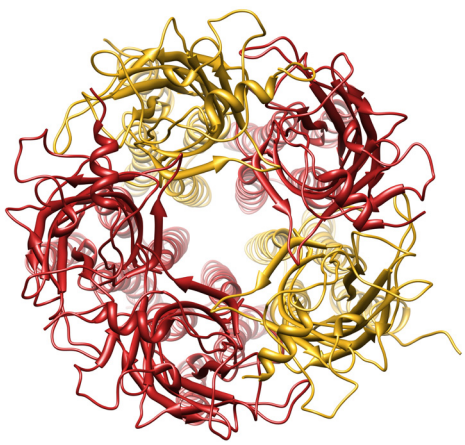

Inhibitory glycine receptors (GlyRs) containing the alpha1 and beta subunits are well known for their involvement in an inherited motor disorder (hyperekplexia) characterised by neonatal hypertonia and an exaggerated startle reflex.

However, it has recently emerged that other GlyR subtypes (e.g. those containing the alpha2, alpha3 and alpha4 subunits) may play more diverse biological roles. New animal models of glycinergic dysfunction have been reported in zebrafish (bandoneon, shocked), mice (cincinatti, Nmf11) and cows (CMD2).

In addition, key studies on neurotransmitter transporters for glycine (GlyT1, GlyT2, VIAAT) have also revealed key roles for these presynaptic and glial proteins in health and disease.

Molecular modelling and structure/function studies have also provided key insights into allosteric signal transduction mechanisms and the diverse pharmacology of glycine receptors.

This Research Topic aims to bring together experts in thefield of glycinergic transmission, and invite research articles or topical reviews to provide an up-to-date perspective on the insights into receptor, transporter and synaptic function that can be gained by the study ofglycinergic transmission.

Image credit is to Victoria James, UCL School of Pharmacy (victoria.james.11@ucl.ac.uk).

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EDITORIAL
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Glycinergic transmission: physiological, developmental and pathological implications

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The last few years have seen remarkable developments in our understanding of the physiology, pharmacology and genetics of inhibitory glycinergic synapses. In part, this has been due to the development of new resources such as specific antisera recognizing glycine receptor (GlyR) and transporter (GlyT) subtypes, but also the characterization of new mouse, zebrafish and bovine genetic models of glycinergic dysfunction. What is also evident is the high quality and impact of the research conducted in this field. This is reflected in the reviews and research articles in this Special Issue entitled "Glycinergic transmission: physiological, developmental and pathological implications".

The study of inhibitory synaptic transmission has a long and illustrious history, as documented by Callister and Graham (2010). Key *in vivo* experiments on spinal glycinergic synapses conducted in the 1950s and 1960s helped to define key concepts in chemical neurotransmission and the distinct pharmacological and electrophysiological properties of what we now know to be inhibitory GlyRs containing the α1 and β subunits. This major adult GlyR isoform predominates in the spinal cord and brainstem (Baer et al., 2009) and has a major role the control of spinal motor reflex circuits. Defects in the corresponding genes, *GLRA1* and *GLRB*, result in an inherited motor disorder in humans known as hyperekplexia, characterized by neonatal hypertonia and an exaggerated startle reflex. Modern genetics techniques (Davies et al., 2010) have revealed that hyperekplexia is best thought of as a synaptopathy, since mutations in *SLC6A5* – encoding the presynaptic glycine transporter GlyT2 – can also cause startle disease. Other GlyR subtypes, such as those containing the α2, α3 and α4 subunits, may play more diverse biological roles in retinal circuitry (Wässle et al., 2009) and central inflammatory pain sensitization (Harvey et al., 2009). GlyR α2 and α3 subunit transcripts are also unusual in that they undergo both alternative splicing and cytidine to uracil RNA editing (C to U), resulting in a proline to leucine substitution (P185L in α3, P192L in α2) that confers high agonist sensitivity and pharmacology to "edited" GlyRs (Legendre et al., 2009). GlyR transcript editing may promote the generation of sustained chloride conductances associated with tonic inhibition and is modulated by brain lesions, suggesting a possible involvement with pathogenic processes. These "orphan" GlyR subtypes may also have key roles in peripheral tissues, since GlyRs have been located on sperm and neutrophils. However, in renal, liver and endothelial cells, where glycine protects from cell death, caution should be applied in attributing these functions to classical GlyRs and GlyTs (Van den Eynden et al., 2009). Certainly, not all cell types that express GlyR subunit mRNAs or polypeptides exhibit GlyR-mediated membrane conductance changes. It is also noteworthy that NMDA receptors composed of the NR1 and NR3 subunits lack glutamate-binding sites and can be activated by glycine alone. It is therefore imperative to understand the synaptic location and pharmacology of this "excitatory" GlyR (Madry et al., 2010).

So what does the future hold for the study of glycinergic transmission? Certainly, GlyRs have a far richer pharmacology than has been appreciated until now. The advent of high throughput screening techniques using anion-sensitive EYFP has enabled automated electrophysiology approaches to be applied in the search for new GlyR-active compounds and subtype-specific modulators (Gilbert et al., 2009). In addition, further study of spontaneous or knockout models of GlyR and GlyT dysfunction has the potential to reveal new roles for these synaptic proteins. In particular, the biological roles of the GlyR α2 and α4 subtypes still remain enigmatic. The embryonic/neonatal GlyR α2 subtype has previously been linked to roles in synaptogenesis, cell fate/paracrine transmitter release in the developing cortex/spinal cord and retinal photoreceptor development. It was therefore somewhat surprising that *Glra2* knockout mice did not show a clear behavioral phenotype. This is most likely due to the "rewiring" of neuronal circuits during development allowing compensatory mechanisms to mask certain phenotypes. For example, the loss of GlyR α3 in a knockout model results in both presynaptic and postsynaptic compensation in the spinal cord. Lamina II synapses that typically express both α3β GlyRs show an elevated glycine release probability, with no changes in quantal content onto α1β GlyRs, which continue to mediate synaptic transmission. Phenotypes revealed to date in *Glra3* knockout mice have exclusively been linked to G-protein coupled receptor pathways influencing PKA-mediated phosphorylation of GlyR α3. In fact, these were only evident because α1β GlyRs are not modulated by PKA phosphorylation. Whilst new knock-in models expressing dominant-negative mutations might overcome this issue, other model organisms will undoubtedly play an important role. For example, zebrafish have a full complement of GlyR and GlyT genes and are amenable to developmental and genetic analysis using *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis, gene-traps and rapid targeted gene "knockdown" using antisense morpholinos (Ganser and Dallman 2009; Chalphin and Saha 2010; Hirata et al., 2010). Curiously, the gene encoding GlyR α4 is thought to be a pseudogene in humans due to a stop codon in *GLRA4* exon 9, causing a protein truncation between membrane-spanning domains M3 and M4. However, this finding may need revisiting in the light of recent resequencing studies that highlight that certain genes on the X chromosome are intact in some individuals but contain non-sense or frameshift changes in other apparently normal

control subjects. It would therefore seem that some genes that are apparently inert in some humans may be active in others. It is also certain that additional defects involving glycinergic transmission remain to be identified. Not all cases of hyperekplexia can be explained by mutations in the genes encoding the adult GlyR α1β isoform or GlyT2, implying that researchers are either missing mutations in important gene regulatory elements, or in other genes involved in the formation/function of glycinergic synapses (Davies et al., 2010). In addition, several hyperekplexia-like syndromes in animals remain unresolved, such as inherited myoclonus in Peruvian Paso horses and familial reflex myoclonus in labrador retrievers. Lastly, although we know much about the cellular transport and membrane dynamics of GlyRs (Dumoulin et al., 2010) – mediated in part by the multifunctional protein gephyrin -our knowledge concerning proteins associated with GlyRs and GlyTs is still painfully thin. The development of reliable antibodies that function in immunoprecipitation and the application of modern proteomics techniques to the study of glycinergic synapses is therefore a priority for the future.

We thank all contributors for their interesting and informative articles and the reviewers for their constructive and thoughtful suggestions.

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REVIEW ARTICLE
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Localization of glycine receptors in the human forebrain, brainstem, and cervical spinal cord: an immunohistochemical review

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Inhibitory neurotransmitter receptors for glycine (GlyR) are heteropentameric chloride ion channels that are comprised of four functional subunits, alpha1–3 and beta and that facilitate fast-response, inhibitory neurotransmission in the mammalian brain and spinal cord. We have investigated the distribution of GlyRs in the human forebrain, brainstem, and cervical spinal cord using immunohistochemistry at light and confocal laser scanning microscopy levels. This review will summarize the present knowledge on the GlyR distribution in the human brain using our established immunohistochemical techniques. The results of our immunohistochemical labeling studies demonstrated GlyR immunoreactivity (IR) throughout the human basal ganglia, substantia nigra, various pontine regions, rostral medulla oblongata and the cervical spinal cord present an intense and abundant punctate IR along the membranes of the neuronal soma and dendrites. This work is part of a systematic study of inhibitory neurotransmitter receptor distribution in the human CNS, and provides a basis for additional detailed physiological and pharmacological studies on the inter-relationship of GlyR, $GABA_AR$ and gephyrin in the human brain. This basic mapping exercise, we believe, will provide important baselines for the testing of future pharmacotherapies and drug regimes that modulate neuroinhibitory systems. These findings provide new information for understanding the complexity of glycinergic functions in the human brain, which will translate into the contribution of inhibitory mechanisms in paroxysmal disorders and neurodegenerative diseases such as Epilepsy, Huntington's and Parkinson's Disease and Motor Neuron Disease.

Keywords: human brain, glycine receptor, immunohistochemistry

INTRODUCTION

Glycine receptors (GlyR) belong to the superfamily of ionotropic receptors that include $GABA_A$, acetylcholine receptors and glutamate (Rajendra et al., 1997; Cascio, 2002; Colquhoun and Sivilotti, 2004). They are also part of the superfamily of ligand-gated ion channels, the Cys-loop receptors (Connolly and Wafford, 2004). Recently accumulated knowledge demonstrates that the receptor structure of the Cys-loop family has an important impact on the function of the receptor (Connolly and Wafford, 2004). GlyRs are important inhibitory receptors in the central nervous system, and are especially prominent in the brainstem and spinal cord (Altschuler et al., 1986; Alvarez et al., 1997). GlyRs are strychnine sensitive and are involved in regulating inhibitory chloride influx through chloride channels to stabilize the resting potential of neurons. GlyRs form pentamers assembled from a range of subunits (currently α1–4, and β subunits), (Langosch et al., 1990; Grudzinska et al., 2005). In the human, only four known functional GlyR subunits have been identified, α1–α3 and β (Lynch, 2004) which are most likely to exist in heteromeric αβ combinations. Defects in mammalian glycinergic neurotransmission can result in a complex motor disorder characterized by an exaggerated startle reflex and neonatal hypertonia, known as hyperekplexia (Andrew and Owen, 1997; Bakker et al., 2006). In humans, missense and nonsense mutations in the GlyR α1 gene (*GLRA1*) and the GlyT2 transporter gene (*SLC5A6*) are the major cause of this disorder (Shiang et al., 1993; Rees et al., 1994, 2001, 2006; Gomeza et al., 2003), although mutations in the GlyR β subunit (Rees et al., 2002), and the GlyR clustering protein gephyrin (GPHN; Rees et al., 2003) have also been reported. GlyRs containing the α3 subunit have increasing therapeutic importance, as they are involved in downstream signaling of inflammatory pain in the spinal cord (Harvey et al., 2004) and may offer novel avenues for the treatment of inflammatory pain. Despite these studies, the location of GlyR subunit combinations in human adult brain and spinal cord remain largely uncharacterized (Probst et al., 1986; Naas et al., 1991), and the biological and genetic basis of many cases of hyperekplexia and paroxysmal movement disorders remain unresolved (Harvey et al., 2008).

Early studies showed the presence of GlyR in rat and human cerebral cortex, although levels of IR were lower than those seen in spinal cord (Naas et al., 1991). GlyR-IR was prominent in apical dendrites of pyramidal neurons in layers III and IV (Naas et al., 1991). GlyRs are also present in other higher cognitive areas such as the hippocampus, where they contribute to both short- and long-term plasticity (Keck and White, 2009), in the developing

neocortex (Flint et al., 1998), and in the mammalian amygdala of the limbic system (Danober and Pape, 1998; McCool and Botting, 2000; Dudeck et al., 2003). In addition, GlyR signaling was detected in non-neuronal cells (den Eynden et al., 2009).

In our ongoing investigations, we have utilized immunohistochemical staining methods (Waldvogel et al., 2006) to analyse the expression and synaptic localization of GlyRs in the human brainstem and spinal cord. The antibodies utilized in these studies produce a punctate immunolabelling pattern in the rat, cat or human brain (Triller et al., 1985, 1987; Kirsch and Betz, 1993; Todd et al., 1996; Alvarez et al., 1997; Colin et al., 1998; Geiman et al., 2000, 2002). In addition to GlyRs, we have described the distribution of gephyrin in the human brain (Waldvogel et al., 2003, 2009), a multifunctional protein responsible for the clustering of GlyRs at inhibitory synapses (Fritschy et al., 2008).

Recently we reported on the distribution of GlyRs in the human brain basal ganglia (Waldvogel et al., 2007). The human brain basal ganglia are a group of large subcortical nuclei involved in the complexity of motor and mood control. They include the striatum (caudate nucleus and putamen), the external (GPe) and internal (GPi) segments of the globus pallidus (GP), the substantia nigra (SN), which has two subdivisions, the substantia nigra pars compacta (SNc) and the substantia nigra pars reticulata (SNr), and also include the subthalamic nucleus (Graybiel, 1995). The caudate and putamen project to the output nuclei of the basal ganglia, the GPe, GPi and the SNr. The substantia nigra pars compacta contains dopaminergic, pigmented neurons which project to various regions of the forebrain especially the striatum. The neurons of the substantia nigra pars reticulata are mainly GABAergic and project to the thalamus, superior colliculus and brainstem regions (Smith et al., 1998). The predominant inhibitory receptors in the human basal ganglia are ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors (Waldvogel et al., 1999, 2004), but there is convincing evidence from observations in animals (Sergeeva, 1998; Darstein et al., 2000; Sergeeva and Haas, 2001) and in the human brain (Naas et al., 1991) that inhibitory GlyRs also exist in the mammalian basal ganglia. Also, there is evidence that glycinergic mechanisms modulate dopaminergic, cholinergic and GABAergic functions in the striatum and the substantia nigra (Cheramy et al., 1978; Kerwin and Pycock, 1979; de Montis et al., 1982; Sergeeva, 1998; Darstein et al., 2000) and these mechanisms can be influenced by the action of ethanol (Darstein et al., 1997).

This work is a review article and summarizes these studies of the post-mortem human brain utilizing antibodies directed against GlyR subunits and immunohistochemical procedures to visualize the regional and cellular distribution of GlyRs in the forebrain and the various major nuclei of the basal ganglia, the striatum, globus pallidus, substantia nigra and the cervical spinal cord at the light and confocal laser scanning microscope levels. These investigations are specifically directed to investigate the distribution of GlyRs in these human brain regions in order to elucidate the role GlyRs may play in inhibitory mechanisms modulating human brain function.

IMMUNOHISTOCHEMICAL PROCEDURES

BRAIN TISSUE

For this study, the human brain tissue was obtained from the Neurological Foundation of New Zealand Human Brain Bank (Department of Anatomy with Radiology, University of Auckland, Auckland, New Zealand). The University of Auckland Human Subject Ethics Committee approved the protocols used in these studies. For further details see Waldvogel et al. (2006). Microtome-cut brain sections of adult human brain were processed for immunohistochemical staining as previously described (Waldvogel et al., 2006). In brief, the human brains were fixed by perfusion through the basilar and internal carotid arteries, first with phosphate-buffered saline (PBS) with 1% sodium nitrite, followed by 15% formalin in 0.1M phosphate buffer, pH 7.4. After the perfusion, blocks were dissected out and kept in the same fixative for 24 h. These blocks were cryoprotected in 20% sucrose in 0.1 M phosphate buffer with 0.1% Na-azide for 2–3 days, and then in 30% sucrose in 0.1 M phosphate buffer with 0.1% Na-azide for a further 2–3 days. Sectioning of the blocks was performed on a freezing microtome at a thickness of 50–70 μm. The sections were collected in PBS with 0.1% sodium azide (PBS-azide) and stored for immunohistochemical processing.

PRIMARY ANTIBODIES

All antibodies used were dissolved in immunobuffer consisting of either 1% goat or 1% donkey serum in PBS with 0.2% Triton X-100 and 0.4% thimerosal (Sigma). Three different GlyR antibodies were tested for the detection of GlyRs in the human brain. These were:

(i) the mouse monoclonal antibody mAb4a raised against amino acids 96–105 in the 48 kDa α1-subunit of the GlyR, and which also recognizes the 58 kDa β-subunit of the GlyR (Alexis Biochemicals; Pfeiffer et al., 1984; Schroder et al., 1991; Kirsch and Betz, 1993);

(ii) a mouse monoclonal antibody mAb2b raised against amino acids 1–10 of the 48 kDa GlyR α1 subunit (Alexis Biochemicals; Pfeiffer et al., 1984); and

(iii) a rabbit polyclonal antibody AB5052 (RGlyR) raised against a peptide from the N-terminus of human α1 GlyR subunit (sequence of the immunizing peptide is ARSTKPMSPSDFLDKLMGC; manufacturer's technical information) with cross reactivity to the α2 subunit (Chemicon, Temecula, CA, USA; Geiman et al., 2002). The rabbit polyclonal AB5052 was used mainly for double labeling studies (see below), and has been used reliably to label GlyR clusters in rat spinal cord and brainstem (Geiman et al., 2002; Liu and Wong-Riley, 2002; Rubio and Juiz, 2004), and human brain (Waldvogel et al., 2007).

The anti-GlyR antibodies used were directed to various different sequences of the GlyR subunits and detected either the α1 subunit only (mAb2b), the α1 and α2 subunits (AB5052), or recognized an epitope conserved in all known α and β subunits (mAb4a; Pfeiffer et al., 1984; Schroder et al., 1991; Kirsch and Betz, 1993). The mAb4a and AB5052 antisera recognized the expected two protein bands (48 and 58 kDa) on Western blot of human basal ganglia tissue homogenates (Waldvogel et al., 2007). The staining patterns of the various GlyR antibodies were generally similar in the regions tested. The anti-GlyR antibodies produced similar patterns of staining except that the mAb4a which recognizes all subunits was consistently more intense than the other GlyR antibodies which selectively recognize α subunits.

Other primary antibodies used to identify various cell phenotypes in the basal ganglia were as follows:

(1) Polyclonal rabbit anti-parvalbumin antiserum (SWANT, Bellinzona, Switzerland; PV-28, also named PV4064, manufacturer's information) was produced in rabbits immunized with rat muscle parvalbumin. The antiserum against parvalbumin was previously characterized by western blotting of mouse brain tissue (Schwaller et al., 2004; Collin et al., 2005), in which the serum recognized a single protein band of the appropriate molecular weight. The specificity of the polyclonal anti-parvalbumin antiserum was previously confirmed by absorption experiments using purified proteins and recombinant rat parvalbumin (Xu et al., 2006).
(2) Polyclonal rabbit anti-calretinin antiserum (SWANT, Bellinzona, Switzerland; 7699/4) was produced in rabbits immunized with recombinant human calretinin. The antiserum against calretinin was previously characterized by Western blotting of monkey and rat brain extracts (Schwaller et al., 1993), in which the serum recognized a single protein band of the appropriate molecular weight.

For the detection of gephyrin, the monoclonal mouse antibody mAb3B11 was used which was raised against the first half of gephyrin's E-domain (Synaptic Systems; Germany). The gephyrin antiserum recognized a single protein band (93 kDa) on Western blot of rat brain (manufacturer's technical information), transfected gephyrin variants (Smolinsky et al., 2008), and human brain (Waldvogel et al., 2009) and stained a pattern of protein distribution in the human brain that is identical with previous reports (Baer et al., 2003; Waldvogel et al., 2003).

SINGLE IMMUNOPEROXIDASE LABELING

Using standard immunohistochemical procedures, adjacent series of sections were selected and processed free-floating in tissue culture wells. Sections were washed in PBS and 0.2% Triton X-100 (PBS-Triton) and pretreated for antigen retrieval using a protocol modified from that of Fritschy et al. (1998), before being processed for immunohistochemistry. Sections for antigen retrieval were transferred to six-well tissue culture plates and incubated overnight in 0.1 M sodium citrate buffer, pH 4.5, and transferred to 10 ml of fresh sodium citrate buffer solution. Following this the sections were microwaved in a 650 W microwave oven for 30 s and allowed to cool before washing (3 × 15 min in PBS-Triton). The sections were then washed in PBS-Triton, incubated for 20 min in 50% methanol and 1% H_2O_2, washed again (3 × 15 min) in PBS-Triton, and incubated in primary antibodies for 2–3 days on a shaker at 4°C. The mouse monoclonal antibody mAb2b was used at dilution 1:2,000 and mouse monoclonal antibody mAb4a was used at a dilution of 1:2,000–1:5,000; the rabbit polyclonal antibody AB5052 (RGlyR) was used at a dilution of 1:500–1:1,000. The primary antibodies were washed off (3 × 15 min; PBS-Triton) and the sections incubated overnight in species-specific biotinylated secondary antibodies (1:500; Sigma; Jackson Laboratories). The secondary antibodies were washed off (3 × 15 min; PBS-Triton) and the sections incubated for 4 h at room temperature in ExtrAvidin, 1:1,000 (Sigma). The sections were reacted in 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma) and 0.01% H_2O_2 in 0.1 M phosphate buffer, pH 7.4, for 15–30 min to produce a brown reaction product. A nickel-intensified procedure was also used in which 0.4% nickel ammonium sulphate was added to the DAB solution to produce a blue-black reaction product (Adams, 1981). The sections were washed in PBS, mounted on gelatin chrom-alum coated slides, rinsed in distilled water, dehydrated through a graded alcohol series to xylene, and coverslipped with DPX (BDH, Poole, UK).

Routinely a few sections were processed as control sections to determine nonspecific staining using the same immunohistochemical procedures detailed above except that the primary antibody was omitted from the procedure. In addition, sections were stained for Nissl substance with Cresyl Violet according to standard techniques.

IMMUNOFLUORESCENT DOUBLE LABELING

For immunofluorescent double-labeling confocal laser scanning microscopy, sections were incubated in a cocktail of monoclonal and polyclonal antibodies (monoclonal antibody mAb4a diluted 1:2,000; polyclonal rabbit antibody AB5052 against GlyR subunits diluted 1:500; monoclonal mAb3B11 against gephyrin diluted 1:1,000; polyclonal rabbit antibody against parvalbumin diluted 1:500; polyclonal rabbit antibody against calretinin diluted 1:500). The sections were processed using the same procedure as the single-labeled sections. They were incubated in primary antibodies for 2–3 days on a shaker at 4°C, washed, and then incubated in species-specific fluorescent secondary antibody directly linked to AlexaFluor 488 or AlexaFluor 594 (1:400; Molecular Probes) or CY3 (1:500). Control sections where the primary antibody was omitted showed no IR. The sections were washed, mounted on slides with Citifluor (Agar Scientific, Stanstead, UK) or ProLongGold (Invitrogen), and viewed using a Zeiss LSM510 or Leica SP2 confocal laser scanning microscope. Confocal laser scanning microscope digital images were collected and contrast optimized using Adobe Photoshop software. Linear methods were used to enhance image brightness and contrast for presentation, applied to all images with the same parameters unless otherwise indicated.

GlyR-IMMUNOREACTIVITY (IR) IN THE HUMAN FOREBRAIN, BRAINSTEM, AND CERVICAL SPINAL CORD

The principal aim of these studies was to immunohistochemically investigate the localization and distribution of GlyRs in the human forebrain, brainstem and cervical spinal cord at the regional and cellular levels. Sections from representative regions of the human brainstem and spinal cord were immunohistochemically labeled for GlyR subunits, and were microscopically examined to determine the distribution of specific immunoreactivities generated by these antibodies. Light and confocal laser scanning microscope analysis of the distribution of GlyR subunits revealed robust and punctate GlyR-IR in the human brainstem and spinal cord regions.

The antibodies assessed for these studies were selected to investigate the staining patterns of GlyRs based on their staining properties in human tissue. The GlyR monoclonal antibody mAb4a produced the strongest signal with the least background and was used for the majority of the GlyR staining in this study. It is predominantly the results of the antibodies mAb4a and mAb2b that are presented in this paper, although some illustrations from the polyclonal rabbit

antibody AB5052 (termed RGlyR) are included where appropriate. The anti-GlyR antibodies demonstrated a similar distribution, although mAb4a consistently produced a stronger signal probably due to recognizing all subunits of the GlyR whereas the others selectively recognized α subunits.

When the staining pattern of single labeled adjacent sections was compared, the staining pattern of the three antibodies mAb4a, mAb2b and RGlyR was essentially the same at both the regional and cellular level (**Figures 1 and 2**). In double labeling studies where both the rabbit anti-GlyR antibody and the mAb4a antibody were incubated together, the staining pattern was very similar and showed an overlapping distribution on neuronal cell membranes (Waldvogel et al., 2007).

GlyRs IN THE FIRST CERVICAL SEGMENT OF THE SPINAL CORD

The labeling of GlyRs was consistently high throughout the cervical spinal cord (Baer et al., 2003; Waldvogel et al., 2009). GlyR-IR was evident throughout the gray matter of the spinal cord (**Figures 1A,B**) and was especially intense in the dorsal (DH) and ventral horns (VH; **Figures 1A,B**). The neuropil of lamina II of the dorsal horn showed the most intense GlyR-IR in the spinal cord (**Figures 1A,B**). At high magnification a dense network of cell bodies and fine dendritic processes was evident with punctate GlyR-IR on their membranes in lamina II and III (Baer et al., 2003). In the ventral horn, moderate to high levels of intense GlyR puncta were observed on cell bodies and dendritic processes (Baer et al., 2003).

GlyRs IN THE MEDULLA OBLONGATA

GlyR-IR produced a heterogeneous distribution in the gray matter of the middle and lower medulla oblongata (Baer et al., 2003; Waldvogel et al., 2009). The hypoglossal nucleus labeled with high intensities of GlyR-IR, and the dorsal motor nucleus of the vagus nerve consistently showed moderate GlyR-IR (Baer et al., 2003). High levels of GlyR-IR were present in the spinal trigeminal nucleus; Sp5O in the lower pons, Sp5C in the lower medulla oblongata at the level of the pyramidal decussation (**Figures 1C,D**). The cuneate nucleus (Cu) and the gracile nucleus (Gr) demonstrated small patches of high GlyR-IR (**Figures 2C,D**). At the cellular level a dense network of intensely labeled punctate GlyR-IR outlining small and large sized dendritic processes was observed in both of these nuclei (Baer et al., 2003). Similarly, neurons in the magnocellular region of the reticular formation in the medulla oblongata demonstrated GlyR-IR outlining the membranes of soma and processes (**Figures 2E,F**). The inferior olivary complex and the accessory olivary nuclei revealed moderate to high levels of GlyR-IR (Baer et al., 2003). At the cellular level, the inferior olivary complex and accessory olivary nuclei displayed high levels of punctate GlyR labeling on dendritic processes and neuronal cell bodies (Baer et al., 2003).

GlyRs IN THE BASAL GANGLIA

At low magnification the caudate nucleus (CN) and putamen (P) displayed a heterogeneous moderate to low level of labeling for GlyRs (**Figures 1G,H**) whilst the globus pallidus showed a relatively very low level of labeling (Waldvogel et al., 2007), and the substantia nigra (SN) in the midbrain a relatively high level of labeling (**Figures 1E,F**). The caudate-putamen displayed a distinct heterogeneous pattern of staining with regions of very pale patches of

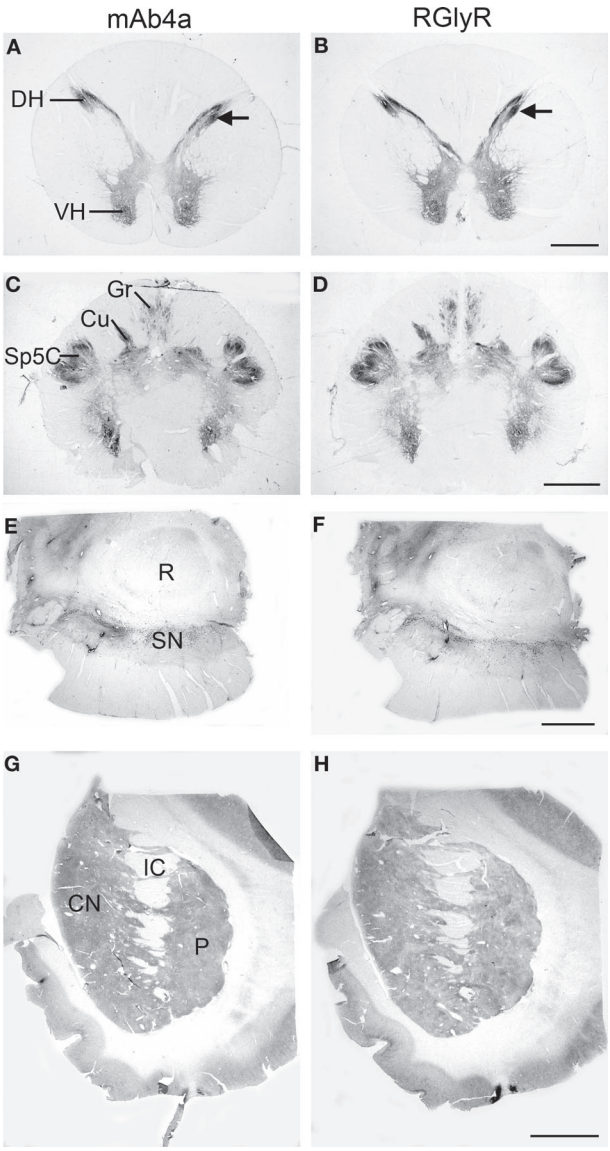

FIGURE 1 | Regional localization of glycine receptors at the cervical level of the human spinal cord (A,B), pyramidal decussation in the lower medulla oblongata (C,D), substantia nigra (E,F) and basal ganglia at the level of the caudate-putamen (G,H) visualized by DAB staining of adjacent sections using monoclonal antibody mAb4a (A,C,E,G) and rabbit polyclonal antibody AB5052 (B,D,F,H). (A,B) Light macroscopic images of sections of the first cervical segment of the spinal cord stained for GlyR with mAb4a **(A)** and AB5052 **(B)** antibody showing especially intense labeling in lamina II of the dorsal horn (DH) and moderate to high levels of GlyR-IR in the gray matter of the ventral horn (VH) and dorsal horn. **(C,D)** Macroscopic images of sections of the pyramidal decussation stained for GlyR with mAb4a **(C)** and AB5052 **(D)** antibody revealing patchy GlyR-IR in the gracile nucleus (Gr) and the cuneate nucleus (Cu) and intense GlyR-IR in the spinal trigeminal nucleus (Sp5C). **(E,F)** Macroscopic images of sections of the substantia nigra (SN) stained for GlyR with mAb4a **(G)** and AB5052 **(H)** antibody showing high levels of GlyR-IR in the regions of the substantia nigra pars compacta and the substantia nigra pars reticulata. **(G,H)** Macroscopic images of sections of the basal ganglia at the level of the caudate-putamen stained for GlyR with mAb4a **(E)** and AB5052 **(F)** antibody suggesting a heterogeneous distribution of GlyR in the caudate nucleus (CN) and putamen (P) separated by the internal capsule (IC). Scale bar = 2 mm in **(A,B)**; 2.5 mm in **(C,D)**; 0.5 cm in **(E–H)**.

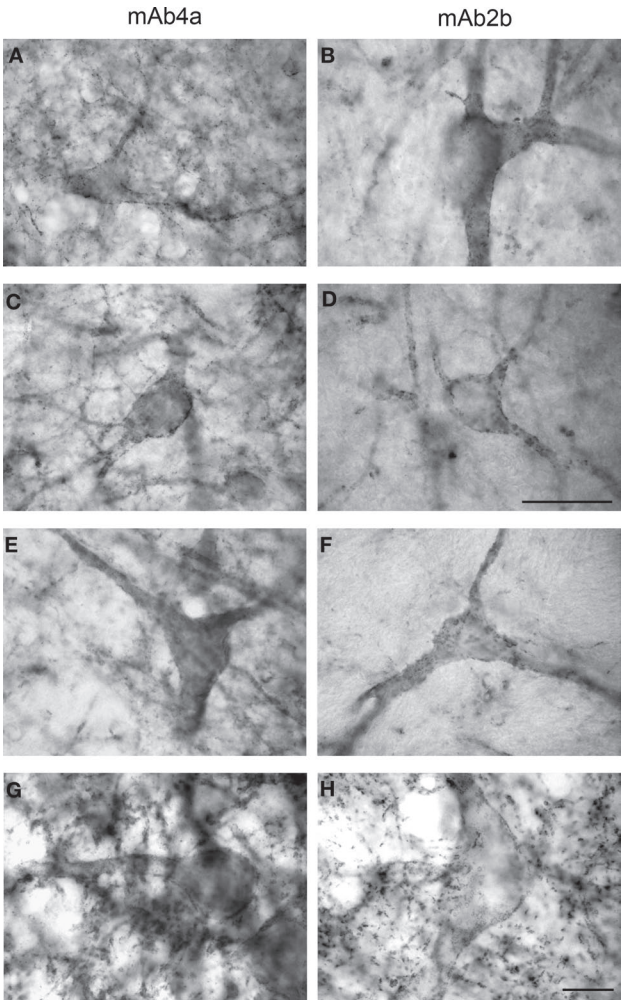

FIGURE 2 | Glycine receptor labeling on neurons in the human midbrain and medulla oblongata visualized by DAB staining of adjacent sections using monoclonal antibody mAb4a (A,C,E,G) and monoclonal antibody mAb2b (B,D,F,H). (A,B) Neurons in the periaqueductal grey area of the midbrain showing high levels of punctate GlyR-IR along their soma and processes, revealing similar IR patterns using mAb4a **(A)** or mAb2b **(B)**. **(C,D)** Neurons in the superior colliculus of the midbrain immunoreactive for mAb4a **(C)** and mAb2b **(D)** demonstrating GlyR-IR outlining the membranes of soma and processes. **(E,F)** Neurons in the magnocellular region of the reticular formation in the medulla oblongata immunoreactive for mAb4a **(E)** and mAb2b **(F)** revealing GlyR-IR along the neuronal soma and processes. **(G,H)** High levels of immunoreactive GlyR are present on neurons in the hypoglossal nucleus of the upper medulla stained for mAb4a **(G)** and mAb2b **(H)**. Scale bar = 50 μm in **(D)** applies to **(C–F)**; 10 μm in **H** [applies to **(A,B,G,H)**].

staining surrounded by a more densely staining background which resembled the striosome and matrix compartmental organization of the striatum (**Figures 1G,H**).

Neurons in both the pars compacta and pars reticulata regions of the substantia nigra were immunoreactive for GlyRs. Both the mAb4a and RGlyR antibodies stained most intense on the pars reticulata neurons and less intense on the pigmented pars compacta neurons (**Figures 1E,F**; Waldvogel et al., 2007). In the substantia nigra pars compacta intense IR was localized in the region of the pigmented neurons (Waldvogel et al., 2007) and was observed on both the cell bodies and primary dendrites of pigmented neurons. The distal dendrites of SNc neurons did not appear to be immunolabeled (Waldvogel et al., 2007). The IR in the pars compacta region often highlighted groups of pigmented neurons scattered mainly throughout the dorsal tier. On closer inspection, not all pigmented neurons were coated with GlyRs and counts of the pigmented neurons indicated that approximately 80% of the pigmented neurons in the substantia nigra pars compacta showed IR for either mAb4a or RGlyR on their cell membranes (Waldvogel et al., 2007).

The large neurons of the substantia nigra pars reticulata (SNr) that were intensely labeled by GlyRs had elongated cell bodies approximately 30-μm long with long branching dendrites often with a bipolar orientation (Waldvogel et al., 2007). SNr neurons were generally more intensely labeled for GlyRs than the substantia nigra pars compacta neurons. High-resolution confocal laser scanning microscopy revealed that the GlyRs were localized in clusters; the clusters were often conspicuously arranged in discrete circular patterns on the cell surface membranes of the soma and proximal dendrites of the pars compacta and pars reticulata neurons and proximal dendrites (Waldvogel et al., 2007).

At the cellular level, neurons in the periaqueductal grey area (**Figures 2A,B**) and the superior colliculus (**Figures 2C–F**) of the human substantia nigra demonstrated GlyR-IR on the cell surface membranes of the soma and proximal dendrites. In agreement with previous reports (Baer et al., 2003), GlyR 'hot spots' were detected (**Figures 2 and 3**).

GlyRs AND GEPHYRIN

We have shown previously that gephyrin is ubiquitously distributed in the human brain (Waldvogel et al., 2003) and associated with GlyRs in the human brainstem (Baer et al., 2003). Gephyrin-IR was observed in sections at the first cervical segment of the human spinal cord (**Figures 3H,K**). Gephyrin- and GlyR-IR was evident throughout the gray matter of the spinal cord and was especially intense in the dorsal and ventral horns (**Figures 1A,B**; Baer et al., 2003; Waldvogel et al., 2009). At high magnification a dense network of cell bodies and fine dendritic processes was evident with punctate gephyrin- and GlyR-IR on their membranes in lamina I, II and III (**Figures 3G–L**; Baer et al., 2003). **Table 1** illustrates the relative intensity of GlyR-IR and gephyrin-IR in the human medulla oblongata and spinal cord (Baer et al., 2003; Waldvogel et al., 2009).

CHEMICAL CHARACTERISTICS OF GlyR IMMUNOREACTIVE NEURONS IN THE HUMAN BASAL GANGLIA

Various markers were used to determine the chemical phenotype of cells that displayed GlyR IR in the basal ganglia (Waldvogel et al., 2007). Double labeling immunohistochemical studies were performed using markers to striatal interneurons: cholineacetyltransferase, parvalbumin, calretinin and neuropeptide Y in order to further characterize which striatal interneurons had GlyRs on their surface membranes (Waldvogel et al., 2007). The substantia nigra pars compacta neurons were recognized by their neuromelanin pigmentation.

Previously, six different types of neurons were identified in the striatum on the basis of cellular and dendritic morphology, chemical neuroanatomy, and $GABA_A$ receptor subunit configuration

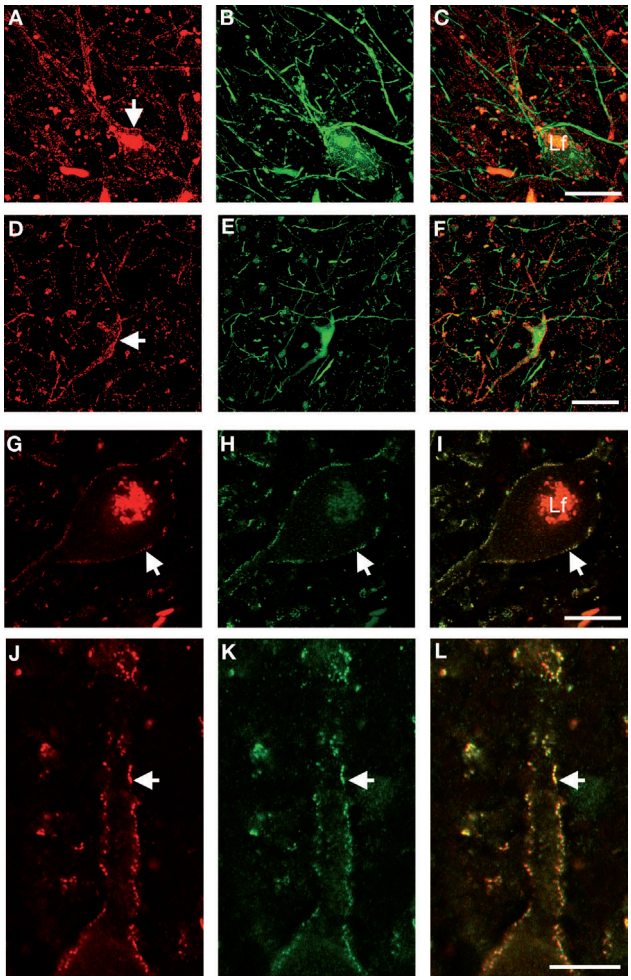

FIGURE 3 | High-resolution confocal laser scanning microscopy images of neurons in the substantia nigra pars reticulata (A–C), putamen (D–F), and first cervical segment of the spinal cord (G–L) of the human CNS double-labeled for GlyR (left column) and other cell markers (middle column) with combined images shown in the right column. (A–C) Neuron in the substantia nigra pars reticulata is labeled red for GlyR [mAb4a in **(A)**, arrow] and immunopositive for rabbit anti-parvalbumin [green in **(B)**], green unlabeled parvalbumin processes in the picture resemble axon bundles, combined images in **(C)**. **(D–F)** Neuron in the putamen that is outlined with GlyR-IR [mAb4a, red in **(D)**, arrow] is labeled with rabbit anti-calretinin immunoreactivity [green in **(E)**], combined images in **(F)**. **(G–I)** Large neuron in the spinal cord that is outlined with rabbit anti-GlyR immunoreactivity [red in **(G)**] is labeled with mouse anti-gephyrin immunoreactivity [green in **(H)**], combined images in **(I)**. Note the high degree of overlap of both markers, exemplified by the arrow highlighting the colocalized IR patterns. **(J–L)** High magnification of a process of a large motoneuron in the cervical spinal cord that shows punctate GlyR-IR [red in **(J)**] and punctate gephyrin-IR [green in **(K)**] demonstrating the high degree of colocalization of the GlyR and gephyrin IR patterns [yellow in **(L)**]. Lf in **(C)** and **(I)** indicates lipofuchsin autofluorescence in the cell body. Scale bar = 25 μm in **(C)** [applies to **(A–C)**], **(F)** [applies to **(D–F)**]; **(G)** [applies to **(G–I)**], and **(J)** [applies to **(J–L)**].

(Waldvogel et al., 1999). We determined that GlyRs are localized predominantly to interneurons characterized neurochemically by IR for three of these six striatal neuron types (cholineacetyltransferase, parvalbumin, or calretinin; Waldvogel et al., 2007). Only very few

Table 1 | Relative intensity of GlyR-IR and gephyrin-IR in the human medulla oblongata and spinal cord.

Brain region	GlyR-IR	Gephyrin-IR
Dorsal motor nucleus of the vagus nerve	+++	+++
Hypoglossal nucleus	++++	+++
Solitary nucleus	++	++
Gracile nucleus	+++	++
Spinal trigeminal nucleus	++++	+++
Accessory olivary nuclear complex	++++	+++
Inferior olive	+++	+++
Lateral reticular nucleus	++	++
Spinal cord lamina II	++++	++++
Spinal cord dorsal horn	++++	+++
Spinal cord ventral horn	++++	+++

+, weak levels of IR; ++, moderate levels of IR; +++ high levels of IR; ++++, most intense levels of IR.

of the striatal GlyR-IR positive neurons were double labeled with parvalbumin, corresponding to type (i) neurons (**Figures 3A–C**; Waldvogel et al., 2007).

Previously we demonstrated that the globus pallidus has a mixed population of GlyR positive neurons that may represent different types of interneurons and possibly includes a subpopulation of the projection neurons (Waldvogel et al., 2007). Small numbers of GlyR positive pallidal neurons were labeled with parvalbumin or calretinin (**Figures 3D–F**). Most neurons in the human globus pallidus are labeled with parvalbumin or calretinin (Waldvogel et al., 1999), therefore the low number of the GlyRs positive neurons double labeled with parvalbumin or calretinin would suggest they are not projection neurons, but may be local interneurons (Waldvogel et al., 2007).

CONTEXTUAL SUMMARY OF IMMUNOHISTOCHEMICAL FINDINGS

Using immunohistochemical methods at the light and confocal laser scanning microscopic levels, our results suggest that GlyRs are widely distributed in the human forebrain, brainstem, and cervical spinal cord. The methods used were optimized for structural tissue preservation and receptor stability of the postmortem human brain to limit background staining and lipofuscin artifacts (Waldvogel et al., 2006).

ANTIBODY CHARACTERIZATION

It is established that mAb2b- and 4a-IR represent postsynaptic structures (Triller et al., 1985; Altschuler et al., 1986) and also intracellular sites of GlyR protein synthesis, transport and aggregation (Hoch et al., 1989). Sequence comparison of human and rat GlyR cDNAs reveals a complete conservation of the epitope for the mAb4a antibody (Schroder et al., 1991). In rat brain, Mab4a produces both punctate and diffuse somatic labeling, suggesting that the Mab4a antigens are not restricted to postsynaptic membrane specializations (Kirsch and Betz, 1993). GlyR, using mAb4a, has demonstrated localization of GlyR at postsynaptic sites on human cortical neurons (Naas et al., 1991). Intracellular labeling was also reported for the anti-GlyR AB5052 (Geiman et al., 2002).

Although the receptor clusters are mainly postsynaptic a proportion of extrasynaptic and cytoplasmic staining is observed (Levi et al., 1999; Dumoulin et al., 2000). Using the antibodies Mab2b, Mab4a, and AB5052, it is not possible to exclusively detect the GlyR β subunit, which contains the gephyrin binding domain (Meyer et al., 1995) or to differentiate between α2 and/or α3 subunit-containing GlyRs. To establish whether gephyrin is only associated with β subunit containing GlyRs in human brain, differential studies using β, α2 and α3 subunit-specific antibodies are required. These additional GlyR subunit-specific antibodies are not yet available.

DISTRIBUTION OF GlyRs IN THE FOREBRAIN, BRAINSTEM, AND CERVICAL SPINAL CORD OF THE HUMAN BRAIN

The major findings in this study are that GlyRs are highly expressed on various subpopulations of neurons in the different nuclei of the forebrain, brainstem, and cervical spinal cord of the human brain (Baer et al., 2003; Waldvogel et al., 2007, 2009). In the forebrain, GlyR IR is most abundant in the substantia nigra, and is found on particular interneuronal cell types in the striatum (Waldvogel et al., 2007). Furthermore, GlyR-IR was detected scattered throughout the globus pallidus and substantia nigra, and was also localized to medullary laminae regions surrounding the two segments of the globus pallidus.

At the regional level, the most intense staining was observed in the dorsal and ventral horns of the spinal cord, the spinal trigeminal nuclear complex, and the motor nuclei associated with the autonomic nervous system such as the dorsal motor nucleus of the vagus nerve and motor cranial nerve nuclei such as the hypoglossal nucleus. These areas revealed intense GlyR labelling and therefore glycinergic inhibitory control of this nucleus is intense and possibly predominant. The patches of high IR in the cuneate and gracile nuclei may be associated with particular sensory inputs as the inputs to these nuclei are all topographically organized (Baer et al., 2003). Other brainstem nuclei such as the inferior olivary complex displayed moderate to high levels of GlyR-IR (Waldvogel et al., 2007).

The substantia nigra contained the highest levels of GlyR-IR in the human basal ganglia. High levels were observed in the region of the substantia nigra pars compacta. On closer investigation the GlyR-IR was localized on pigmented neurons and dendritic processes in the substantia nigra pars compacta region. Approximately 80% of the pigmented neurons had GlyRs located on their cell bodies and proximal dendrites. This would suggest that a large number of dopaminergic neurons possess GlyRs. This may indicate that the dopaminergic neurons of the substantia nigra pars compacta may be divided into different subgroups depending on the various receptor types which are expressed on their cell membranes (Waldvogel et al., 2007). The neurons of the substantia nigra pars reticulata were intensely reactive for GlyRs. These were large rounded or elongated neurons with long branching neurons characteristic of the large projection neurons of the substantia nigra pars reticulata. In the human striatum, GlyRs are localized predominantly to interneurons characterized neurochemically by IR for three of these six striatal neuron types (choline acetyltransferase, parvalbumin, or calretinin) (Waldvogel et al., 2007).

CELLULAR AND SUBCELLULAR GlyRs LOCALIZATION

At the cellular level, GlyR-IR was present on the plasma membranes of the soma and dendrites of neurons throughout the human substantia nigra, pons, medulla oblongata and cervical spinal cord (Baer et al., 2003; Waldvogel et al., 2007, 2009). Using light microscopy, punctate labeling for GlyRs was observed on the membranes of neuronal somata and processes.

Confocal laser scanning microscopy revealed GlyR 'hot spots' (brightly stained puncta) reflecting synaptic localization and aggregation (Grunert and Wassle, 1993; Kirsch et al., 1996). The confocal laser scanning microscopy data in the human are consistent with GlyRs as observed in rat spinal cord (Geiman et al., 2002). In addition to GlyR cluster 'hot spots,' GlyR-IR was observed along the entire length of neuronal membranes. A similar observation was reported for cat spinal cord ventral horn neurons where the density and topographical organization of GlyR clusters varied in different neuronal types and in different dendritic regions (Alvarez et al., 1997).

COMPARING THE DISTRIBUTION OF GlyRs IN HUMAN AND RODENT BRAINSTEM AND SPINAL CORD

The current data show that the general distribution of GlyR subunits detected in the human rostral and caudal pons, rostral medulla oblongata and the cervical spinal cord are generally in good agreement with immunohistochemical and immunoenzymatic electron microscopy studies in the rat brainstem and spinal cord (Triller et al., 1985, 1987; van den Pol and Gorcs, 1988; Rampon et al., 1996; Alvarez et al., 1997; Geiman et al., 2002). The subcellular distribution of GlyRs in the mammalian retina has also been examined (Wässle et al., 1998). Using *in situ* hybridization techniques, GlyR mRNAs were detected in somata and dendrites of most neurons of the ventral horn of rat spinal cord (Racca et al., 1997); and in the rat spinal trigeminal nucleus, principal trigeminal nucleus, gracile and cuneate nuclei (Sato et al., 1991). Another rat brain study showed high levels of IR for the neurotransmitter glycine in the hypoglossal nucleus, gracile nucleus, spinal trigeminal nucleus and raphe nucleus (Rampon et al., 1996). The GlyR-IR which we have detected in the human is in agreement with these studies in the rat brain (**Table 2**).

GEPHYRIN INVOLVEMENT

Numerous studies have revealed that gephyrin is present at glycinergic and GABAergic synapses (Fritschy et al., 2008). Virtually complete colocalization of GlyR-IR and postsynaptic gephyrin-IR has been established in the ventral horn of rat spinal cord (Triller et al., 1985, 1987; Todd et al., 1995, 1996; Colin et al., 1998). Another study suggested that most GlyRs in the rabbit retina colocalize with gephyrin (Zucker, 1998). Previously we demonstrated that gephyrin is widely distributed in the human brain and spinal cord, and that a large proportion of the GlyRs in the brainstem and spinal cord show punctate IR that co-localizes with gephyrin (Baer et al., 2003; Waldvogel et al., 2003, 2009). The studies reviewed here establish the association of gephyrin and GlyRs in human brainstem and spinal cord indicating an association of gephyrin and GlyRs, implicating similar functions for gephyrin in human brain as that reported for rodent brain. Thus, gephyrin is likely to play a fundamental role in the organization of major types of inhibitory synapses at postsynaptic membranes in human brain.

Table 2 | Comparison of the distribution of relative intensity of GlyR-IR in human and rodent brainstem and spinal cord (van den Pol and Gorcs, 1988; Rampon et al., 1996; Baer et al., 2003).

Brain region	GlyR-IR on cell bodies in human	GlyR-IR on processes in human	GlyR-IR on cell bodies in rodent	GlyR-IR on fibers in rodent
Dorsal motor nucleus of the vagus nerve	++	+++	0 to +	++
Hypoglossal nucleus	+++	++++	++	++++
Solitary nucleus	++	++	+	++
Gracile nucleus	+++	+++	++	+++
Spinal trigeminal nucleus	++++	++++	++ to ++++	+++
Cuneate nucleus	+++	+++	+	+++
Locus coeruleus	++	++++	0 to +	+++
Dorsal raphe nucleus	+++	+++	0 to +	++
Accessory olivary nuclear complex	++	++++	+++	++++
Inferior olive	+++	+++	+++	++++
Lateral reticular nucleus	+	++	++	+++
Spinal cord lamina II	++++	++++	++ to ++++	++ to ++++
Spinal cord dorsal horn	+++	++++	++ to ++++	++ to ++++
Spinal cord ventral horn	+++	++++	++ to ++++	++ to ++++

0, no detectable IR; +, weak levels of IR; ++, moderate levels of IR; +++ high levels of IR; ++++, most intense levels of IR.

Studies in rodents have revealed that gephyrin directly interacts with key regulators of microfilament dynamics, namely profilin I and IIa and microfilament adaptors of the mammalian enabled (Mena)/vasodilator stimulated phosphoprotein (VASP) family (Giesemann et al., 2003). This interesting link may play an important role in receptor density and dynamics at inhibitory synapses including activity-dependent remodeling of synaptic structures (Neuhoff et al., 2005). Although the scenario in human CNS is not known, high-resolution microscopy analysis suggested that not all GlyR α1 subunit-IR co-localizes with gephyrin, indicating that there may be other mechanisms involved in GlyR localization, presumably at presynaptic or extrasynaptic sites. The distribution of other major GlyR subtypes in human brain (α2, α3) remains to be determined.

Recent exciting data revealed that gephyrin and the cell adhesion molecule neuroligin 2 interact and that deletion of neuroligin 2 in mice disturbs glycinergic synaptic transmission (Poulopoulos et al., 2009). Neuroligins are important for the assembly of synaptic specializations, and complexes of neuroligin 2, gephyrin and collybistin are sufficient for cell-autonomous clustering of inhibitory neurotransmitter receptors (Poulopoulos et al., 2009). The role of neuroligins at inhibitory synapses in the human CNS requires further attention, but these recent findings in rodents demonstrate that neuroligins can be regarded as important organizer proteins at postsynaptic glycinergic synapses.

Furthermore, gephyrin has multiple functions and is required for the biosynthesis of molybdenum cofactor (Feng et al., 1998; Fritschy et al., 2008). In an extensive search for human gephyrin transcript heterogeneity, we identified neuronal gephyrin isoforms created from the alternative splicing of five exons, also termed cassettes (C3, C4A–D) in the central linker region of the protein (Rees et al., 2003). The biological role of these gephyrin isoforms is not clear. At least one of these variants (cassette C3) is constitutively spliced into non-neuronal gephyrin (Rees et al., 2003), but otherwise the biological role of this neuronal heterogeneity is unclear. This is particularly true since most known interactors of gephyrin, including GlyR β, profilins, Mena/VASP (Giesemann et al., 2003) and collybistin (Harvey et al., 2004) all bind to the C-terminal MoeA homology domain. One hypothesis is that gephyrin cassettes may create or destroy sites for additional protein–protein interactions, perhaps controlling the accumulation of specific signalling pathways or cytoskeletal interactions in a brain-region specific manner. Also, these isoforms might play a role in GlyR clustering and/or might be differentially expressed during development and/or in healthy versus diseased brain tissue. In addition, defined gephyrin isoforms might be selectively distributed to synaptic or extrasynaptic sites and/or might be enriched at glycinergic synapses. Future work will elucidate whether gephyrin-/GlyR-IR patterns are changed by neuropathological disorders, and whether these IR patterns could represent novel biomarkers or indicators for neurodegenerative disease processes, by comparing and contrasting the immunoreactive patterns observed between normal and neuropathological brain samples. Also, given the limitations of immunoreactive approaches, future work will evaluate the development of other technology platforms (for example, Quantum dot nanotechnologies) to enhance detection specificity for GlyR.

FUNCTIONAL CONTEXT AND LINKAGES

The source of the neurotransmitter glycine which presumably acts on the GlyRs found in the human basal ganglia is not clear, but studies in the rat show that glycinergic IR terminals are present in regions of the ventral pallidum and basal forebrain (Rampon et al., 1996). This may indicate that other regions of the basal ganglia such as the lateral medullary lamina between the putamen and external segment of the globus pallidus and the medial medullary lamina between the external segment of the globus pallidus and internal segment of the globus pallidus examined previously (Waldvogel et al., 2007) in the human basal ganglia may also be innervated by

glycinergic neurons. Since the greatest concentration of glycine and GlyRs are predominantly found in the brainstem it is possible that nuclei in the brainstem may be the source of the glycinergic input to the basal ganglia.

Our findings, consistent with findings in the rat (Darstein et al., 2000), suggest that GlyRs are found on cholinergic interneurons in the human striatum (Waldvogel et al., 2007). Functional studies in the rat striatum have demonstrated that GlyRs can be activated within the striatum and that glycine application can induce acetylcholine release in the striatum (Darstein et al., 1997, 2000). Therefore the same mechanism may also operate in the human striatum. In addition, dopamine can be released in the striatum by application of glycine (Yadid et al., 1993) and is blocked by strychnine. Also, strychnine-sensitive glycinergic responses have been recorded in the rat striatum on both large cholinergic neurons and on GABAergic medium spiny neurons. One study has also established that ethanol can inhibit glycine induced responses in the rat striatum (Darstein et al., 1997), suggesting that glycine might present a new site for the functional effect of alcohol.

The pharmacological and physiological role of glycine in the substantia nigra is still unclear but studies in rats and cats have identified various effects of glycine in the substantia nigra including depression of dopamine neuron activity and reduced efflux of dopamine in the caudate nucleus (Cheramy et al., 1978; de Montis et al., 1982). In contrast, other studies demonstrated an increase of dopamine efflux in the substantia nigra with glycine application (Kerwin and Pycock, 1979), as well as glycine mediated effects of GABA transmission in the VTA region (Ye et al., 2004). Another study has implicated the role of glia as being a source of extracellular glycine which can act on GlyRs in the substantia nigra pars reticulata (Dopico et al., 2006).

Defects in mammalian glycinergic neurotransmission at post and presynapse can result in a paroxysmal motor disorder, hyperekplexia (for review see Harvey et al., 2008), and the GlyR alpha3 subunit is an essential target for inflammatory pain sensitization (Harvey et al., 2004). However, the locations of GlyR subunit combinations in human adult brain and spinal cord remain largely uncharacterized (Naas et al., 1991), and the biological and genetic basis of many cases of hyperekplexia and paroxysmal movement disorders remain unresolved. In addition, there is little data evaluating the GlyR and glycine presence in neurodegenerative diseases. Although GlyRs are changed in some pathological conditions (e.g. in the substantia nigra in Parkinson's disease), the neuropharmacology of the glycine system is still poorly understood (Lloyd et al., 1983). Moreover, glycine concentrations in Huntington's disease patients were higher compared to controls as reported for analysis of platelets (Reilmann et al., 1997), and cerebrospinal fluid (Nicoli et al., 1993). It is not known which metabolic defect might be responsible for the changes in glycine levels in Huntington's disease, but the link to the *N*-methyl-D-aspartate subclass of ionotropic glutamate receptors (NMDARs), which possess a glycine binding site, and NMDARs excitoxicity in Huntington's disease has been suggested (Reilmann et al., 1997; Fan and Raymond, 2007).

Finally, the need to increase our understanding of neuroinhibitory receptor distribution and function including GlyRs in healthy and diseased human brain tissue is reflected in the potential to create new pharmacological drugs for different neuropsychiatric disorders based on agonist activity at inhibitory neurotransmitter receptors. In summary, we provide evidence for the localization of GlyRs in neuronal populations in various regions throughout the human forebrain, brainstem, and cervical spinal cord. GlyRs are localized especially on cholinergic and calretinin positive neurons in the striatum and globus pallidus, as well as in high concentrations within the intermedullary laminae of the globus pallidus and the substantia nigra. High levels of GlyRs are also found throughout the substantia nigra where previous studies have implicated GlyRs in the modulation of dopaminergic function. It is postulated from previous studies that glycinergic mechanisms that act on glycine ion channel receptors are involved in the modulation of cholinergic, dopaminergic and GABAergic pathways in the basal ganglia. Further studies are required to establish GlyR heterogeneity in human brain using additional subunit-specific anti-GlyR antibodies. The clustering protein gephyrin directly interacts with GlyRs, and the role of gephyrin and its isoforms remains elusive with regard to the mechanisms of synaptic localization, dynamic receptor clustering and GlyR heterogeneity in human brain. Future studies are essential for establishing the biological context and basic neuroscience principles of human glycinergic neuroinhibition in normal and neuropathological conditions.

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Early history of glycine receptor biology in mammalian spinal cord circuits

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In this review we provide an overview of key *in vivo* experiments undertaken in the cat spinal cord in the 1950s and 1960s, and point out their contributions to our present understanding of glycine receptor (GlyR) function. Importantly, some of these discoveries were made well before an inhibitory receptor, or its agonist, was identified. These contributions include the universal acceptance of a chemical mode of synaptic transmission; that GlyRs are chloride channels; are involved in reciprocal and recurrent spinal inhibition; are selectively blocked by strychnine; and can be distinguished from the $GABA_A$ receptor by their insensitivity to bicuculline. The early *in vivo* work on inhibitory mechanisms in spinal neurons also contributed to several enduring principles on synaptic function, such as the time associated with synaptic delay, the extension of Dale's hypothesis (regarding the chemical unity of nerve cells and their terminals) to neurons within the central nervous system, and the importance of inhibition for synaptic integration in motor and sensory circuits. We hope the work presented here will encourage those interested in GlyR biology and inhibitory mechanisms to seek out and read some of the "classic" articles that document the above discoveries.

Keywords: chloride channel, motoneuron, Renshaw cell, strychnine, bicuculline, inhibition, synaptic transmission

INTRODUCTION: A BRIEF HISTORY OF GlyR STUDY

This review is directed at graduate students whose research focuses primarily on the genetic and molecular aspects of glycine receptor (GlyR) function, and the experienced researcher with an interest in the pivotal experiments that established the fundamentals of GlyR biology and inhibitory synaptic transmission. We use two well-understood motor circuits, located in the spinal cord, to illustrate how our current understanding of GlyR function evolved (**Figure 1**). Where possible, we refer to the crucial involvement of GlyRs in reflexes and motor behaviors because strychnine, the selective antagonist of the GlyR, has long been used to reveal mechanisms underlying motor behaviors such as locomotion. For example, at the behavioral level, strychnine administration in spinalized animals greatly facilitates reflex walking. This observation indicates ongoing (or tonic) supra-segmental inhibition is vital for normal locomotor co-ordination (Hart, 1971). Likewise, strychnine administration disturbs the normal modulation of motoneuron discharge during fictive locomotion (Pratt and Jordan, 1987). These data are consistent with inhibition being important for modulating activity in muscle groups that underlie rhythmic motor behaviors.

GlyR biology is currently undergoing a "mini" renaissance because a new form of the receptor (containing α3 subunits) was recently identified in sensory divisions of the spinal cord, specifically those involved in pain processing (Harvey et al., 2004). This basic science finding is now driving work aimed at identifying ways to selectively modulate the α3-form of the receptor and improved pain therapies (Zeilhofer, 2005; Lynch and Callister, 2006). We therefore feel it is timely to review some of the *in vivo* history of GlyR biology, which began in the cat spinal cord, and outline how current views of this receptor and its function evolved from the initial quest to understand motor control pathways and synaptic transmission in the mammalian central nervous system (CNS).

We consider the first important period for GlyR biology, and the focus of this review, to have occurred during the 1950–1960s when fundamental aspects of inhibitory synaptic transmission were being revealed by application of extracellular and intracellular recording techniques to neurons in the cat spinal cord. Early in this period, the GlyR and its endogenous agonist were yet to be identified. The next phase of GlyR research, during the 1970s - 1990s, was very much concerned with *in vitro* experimentation. This involved work on recombinant GlyRs, where the receptor was considered a "proto-typical" ligand gated ion channel, or experiments on acute spinal cord and brainstem preparations that examined the pharmacolology and physiology of native GlyRs. Considerable advantages were provided by the apparent expression of only one type of GlyR (comprised of α1 and β subunits) in the adult nervous system for structure-function studies on recombinant channels, especially compared to its close relative, the γ-amino-n-butyric acid receptor ($GABA_AR$), where multiple subtypes existed (Mody and Pearce, 2004; Sarto-Jackson and Sieghart, 2008). Work over this period has been summarized in previous reviews (Legendre, 2001; Lynch, 2004). At this time, translation of findings to the clinic was hampered by the widespread distribution of a single form of the receptor in the mammalian CNS, as well as the toxicity of its major antagonist strychnine.

These disadvantages for clinical applications were balanced by the discovery and study of naturally-occurring mutations in the GlyR in a number of species including humans, horses, dogs and mice (Floeter and Hallett, 1993; Rajendra and Schofield, 1995).

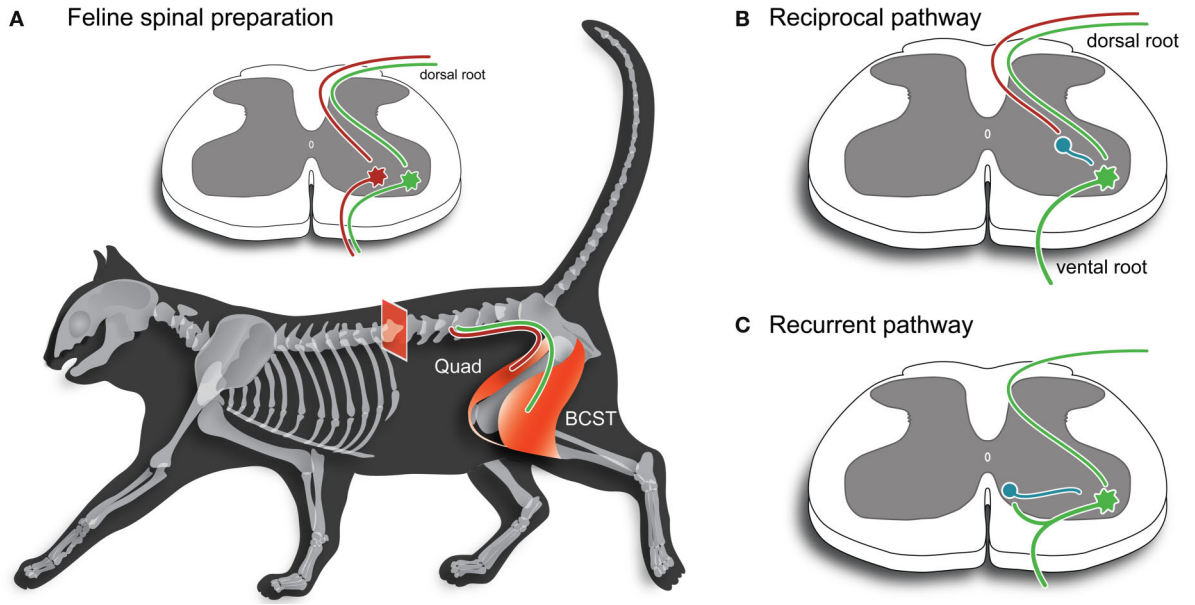

FIGURE 1 | Key spinal cord motor pathways used to study synaptic mechanisms in the mammalian CNS. (A) Schematic illustrating two hind limb muscles employed extensively in the study of spinal cord reflexes and synaptic transmission in the 1950s and 1960s. The quadriceps (Quad) and biceps-semitendonosus (BCST) muscles produce extension and flexion about the knee joint, respectively. Inset, shows a spinal cord cross-section and the monosynaptic excitatory pathway. Afferents originating in annul spiral endings of muscle spindles (1a afferents) are involved in the classic stretch reflex, which is used clinically to test the integrity of spinal circuits. These afferents (red and green axons) make monosynaptic connections with motoneurons that innervate the same (homonymous) muscle. **(B)** Inhibition is produced in motoneurons by activation of 1a afferents in antagonist muscles. In early work, this pathway was called the "direct inhibitory pathway" because stimulation of an antagonist muscle nerve (e.g., Quad) could directly inhibit responses in motoneurons innervating BCST. This inhibition is provided by an interposed 1a inhibitory interneuron (shown in blue). This pathway is now termed the "reciprocal inhibitory pathway" to better reflect its peripheral action where it ensures antagonist muscles are relaxed when agonist muscles are activated during movement. **(C)** Inhibition can also be produced in motoneurons by antidromic stimulation of agonist muscle nerves or ventral roots. This inhibition is provided via another type of inhibitory interneuron, termed a Renshaw cell (shown in blue), which is activated by motoneuron axon collaterals. This pathway is called the "recurrent inhibitory pathway".

Importantly, humans and animals with GlyR mutations exhibited markedly similar motor phenotypes that collectively have been termed "startle diseases". The animal models provided insight into diseases involving tremor and spasticity, and how they might be treated (Simon, 1995). The existence of naturally-occurring murine mutants provided new research directions, because GlyRs could now be examined both genetically and behaviorally within a single species. Work on native GlyRs in brainstem and spinal motor neurons in murine mutants provided insights, at the level of intact synapses, into mechanisms underlying the exaggerated motor responses observed in humans and animals with GlyR defects (Biscoe and Duchen, 1986; von Wegerer et al., 2003; Graham et al., 2006). Additionally, GlyR levels and subunit composition could be manipulated in mice and then studied at the channel, synapse, and behavioral levels of analysis (Hartenstein et al., 1996; Becker et al., 2000). Recent work has also allowed *in vivo* electrophysiological analysis of neuron excitability and spinal cord circuits in deeply anesthetized mice with GlyR mutations (Graham et al., 2007a).

The past decade has seen an increased focus on GlyR function in regions of the nervous system that process sensory information. Interestingly, these experiments have been conducted in sensory regions of the spinal cord where GlyR mutations don't appear to have the catastrophic effects observed in the motor system. Recent work on dorsal horn neurons suggested inhibitory tone is maintained, at least in some GlyR mutants, by compensatory changes in $GABA_AR$ expression and altered potassium channel function on postsynaptic neurons (Graham et al., 2003, 2007b). These findings are important for our understanding of the co-regulation of the two major fast inhibitory channels and the mechanisms by which excitability is regulated in neuronal circuits. Importantly, such insights are only available after re-examination of *in vitro* findings in intact (*in vivo*) preparations using electrophysiological and behavioral analysis. Such inquiry is now entirely feasible so that discoveries made using genetic/molecular techniques can be re-examined at both the circuit and systems levels of investigation.

UNDERSTANDING AND DEFINING CENTRAL INHIBITORY MECHANISMS: DISCOVERIES MADE IN THE CAT SPINAL CORD

It is now well accepted that two classic reflex pathways exist within the CNS, which play an important role in fast synaptic inhibition on motoneurons in spinal cord circuits. The circuitry and principle neuron types involved in these pathways are highlighted in **Figure 1**. Such circuit diagrams are often presented as the "first slide" in seminars or talks to emphasize the importance of GlyRs and inhibitory neurotransmission in CNS function. We now take for granted how GlyRs, along with closely related $GABA_ARs$, shape the output of motor neurons through activation of these "reciprocal" and "recurrent" inhibitory pathways. Studies on spinal cord reflexes in the cat, using extracellular recording techniques, largely

defined these circuits in the early part of the 20th century (Creed et al., 1932). Later, application of intracellular recording to spinal motoneurons in the 1950–1960s further refined our understanding of this circuitry.

CENTRAL INHIBITORY SYNAPSES AND THE CHEMICAL HYPOTHESIS OF SYNAPTIC TRANSMISSION

During the 1940s and early 1950s intense debate raged about the nature of synaptic transmission within the CNS (was it electrical or chemical?) and, to a lesser extent, what neurotransmitters and receptors were involved. The Australian Nobel Laureate Sir John (Jack) Eccles was an outspoken supporter of the electrical hypothesis of synaptic transmission, which he proposed involved direct transfer of electrical potentials between neurons (Brooks and Eccles, 1947; Eccles, 1949; Burke, 2006; Stuart and Pierce, 2006). Alternatively, those who worked on peripheral synapses, such as Sir Henry Dale, supported a chemical hypothesis (Dale, 1934). Indeed, for peripheral synapses at autonomic ganglia and neuromuscular junctions a candidate neurotransmitter, acetylcholine, had been proposed and this idea was even accepted by Eccles in the late 40s (Eccles, 1949).

For central synapses, an electrical theory of synaptic transmission had been considered to explain the extracellular potentials recorded in motoneuron pools or ventral roots following stimulation of synergist muscle nerves (i.e., monosynaptic excitation). According to the electrical hypothesis, presynaptic action potentials in synaptic endings could depolarize motoneurons by the "transfer of electric charges at the contact." However, these ideas could only be truly tested when motoneurons were impaled with intracellular microelectrodes in the early 1950s (Brock et al., 1952b; Woodbury and Patton, 1952). The development of this technique meant membrane potential and conductance changes across the motoneuron membrane could be directly measured (**Figure 2**). For excitatory monosynaptic connections, many of the previously established parameters using extracellular electrodes, such as central latency and times for synaptic delay, were confirmed. Accordingly, the electrical hypothesis adequately explained the results of experiments on excitatory monosynaptic connections between 1a afferents and synergist motoneurons (**Figure 2B**).

The electrical hypothesis was more difficult to apply in experiments where stimulation of muscle nerves *inhibited* motoneuron activity. Lloyd and co-workers had introduced the term "direct inhibition" for the reduced discharge observed in motoneurons after stimulating 1a afferents innervating antagonist muscles (Lloyd, 1946). For example, the monosynaptic reflex discharge generated in biceps-semitendinosus motoneurons could be inhibited if the nerve to the quadriceps was stimulated immediately (~ 0.5–1 ms) beforehand. This form of inhibition was subsequently termed "reciprocal inhibition" to better reflect the function of this pathway in stretch reflexes involving antagonistic pairs of muscles (Jankowska et al., 1965). Eccles later proposed the so-called "Golgi hypothesis" to explain reciprocal inhibition, whereby inhibitory fibers terminated on interneurons called Golgi cells, which sent their short axons to contact the inhibited motoneurons. These Golgi cells supposedly caused hyperpolarization by acting as sinks for excitatory currents (Brooks and Eccles, 1947).

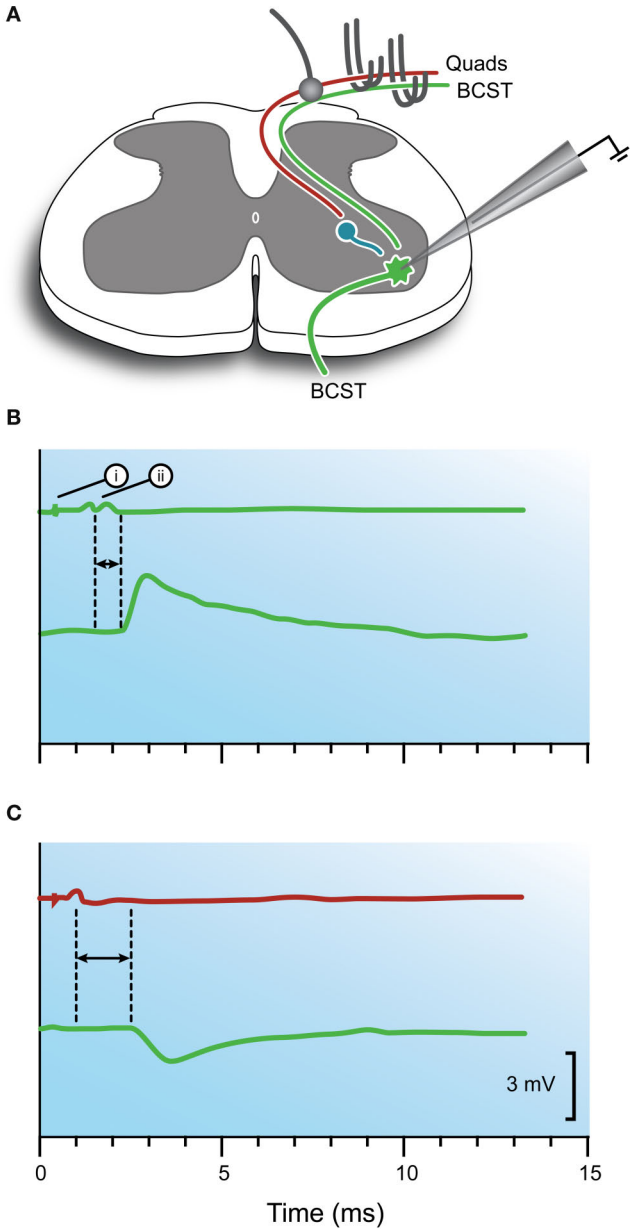

FIGURE 2 | Intracellular responses recorded in motoneurons after stimulation of monosynaptic excitatory and reciprocal inhibitory pathways. (A) Schematic showing the experimental configuration. A motoneuron innervating BCST is impaled with an intracellular recording electrode. Stimulating electrodes can activate either agonist (BCST) or antagonist (Quads) 1a afferents within muscle nerves. Arrival of the afferent volley at the dorsal root entry zone is monitored with a ball electrode. **(B)** Upper trace shows recording from ball electrode and indicates when a stimulus is delivered to the agonist muscle nerve (i) and when the volley arrives at spinal cord (ii). Bottom trace shows resulting depolarization in the motoneuron (ie, an EPSP). This response is characteristic of the monosynaptic excitatory pathway and shows the short latency between the dorsal root volley and beginning of the synaptic response (horizontal arrows). **(C)** Upper trace indicates when a stimulus is delivered to the antagonist muscle nerve and when the volley arrives at the spinal cord. Bottom trace shows hyperpolarization in the motoneuron (ie, an IPSP). Hyperpolarization and a longer latency (horizontal arrows) is characteristic of the reciprocal inhibitory pathway. Modified with permission from Figure 12 in Brock et al. (1952b).

The above views on inhibitory synaptic transmission were examined in greater detail when motoneurons were impaled with intracellular electrodes in the early 1950s. When reciprocal inhibition was studied with an intracellular microelectrode, the result was intriguing – the interior of the recorded motoneuron became transiently "more" negative to earth (**Figure 2C**). Such hyperpolarization was also noted during "secondary inhibition", the inhibition observed in extensor motoneurons after stimulating cutaneous afferents. This hyperpolarizing response was first reported in abstract form (Brock et al., 1952a). In this landmark abstract the authors stated "an electrical explanation of inhibitory synaptic action seems to be precluded" and even proposed that "inhibition is caused by a specific chemical mediator". Later in the same year, in what is now the classic manuscript on the beginning of intracellular recording in neurons, they studied hyperpolarization in more detail and concluded "…the potential change observed is directly opposite to that predicted by the Golgi-cell hypothesis, which is thereby falsified" (Brock et al., 1952b, p. 452). In the final sentence of the results section they proposed the inhibitory effect was generated in the motoneuron and not by the Golgi cell and wrote "It may therefore be concluded that inhibitory synaptic action is mediated by a specific transmitter substance that is liberated from the inhibitory synaptic knobs and causes an increase in polarization of the subjacent membrane of the motoneurone" (p. 452).

As a result of these intracellular recordings, the now familiar terms EPSP (excitatory postsynaptic potential) and IPSP (inhibitory postsynaptic potential) were proposed in a later paper to describe the transient depolarizing and hyperpolarizing responses observed in motoneurons following activation of excitatory and inhibitory synapses, respectively (Bradley et al., 1953). To some extent, the time (0.3–0.4 ms) we now assign to synaptic delay also evolved from these experiments on EPSPs and IPSPs. The longer central conduction times for reciprocal IPSPs vs. monosynaptic EPSPs (0.7 vs. 0.4 ms) could be explained by the presence of an intervening synapse, which took ~ 0.3 ms to activate, on an interposed neuron (ie, the 1a interneuron; **Figure 1B**).

Thus, based on what we know today about GlyRs in spinal inhibitory pathways, it is clear that they played a critical role in the development of one of the major tenants of modern neuroscience-both central and peripheral neurons communicate predominantly by releasing neurotransmitters from their axon terminals (ie, via chemical transmission).

CENTRAL INHIBITION IS MEDIATED BY A CHLORIDE CHANNEL

Having rapidly accepted the chemical hypothesis of synaptic transmission, Eccles and colleagues set out to determine how the "inhibitory transmitter substance" caused hyperpolarization of the postsynaptic membrane. The initial account of their findings was published in a relatively obscure journal, The Australian Journal of Science (Coombs et al., 1953), then later expanded (Eccles et al., 1954; Coombs et al., 1955b). They first noted that IPSPs generated after stimulation of the reciprocal inhibitory pathway were quite brief, with rise times and decay time constants of ~ 1 ms and ~ 4 ms, respectively. They concluded the underlying conductance change must be very rapid and be completed within ~ 2 ms. *In vivo* experiments undertaken almost four decades later using single electrode voltage clamp techniques on cat motor neurons confirmed the currents (ie, IPSCs) mediating both reciprocal and recurrent inhibition are indeed brief – they have decay times of 1.0 and 0.8 ms at 37°C, respectively (Stuart and Redman, 1990). These values also match those from voltage clamped glycine-mediated IPSCs in other spinal cord neurons after appropriate allowances for recording temperature (Takahashi and Momiyama, 1991).

Eccles and colleagues also observed that the amplitude of the reciprocal IPSP decreased over time (a few minutes) and eventually "reversed" in polarity to produce a depolarizing potential (ie, an EPSP). Importantly, this reversal only occurred when the intracellular microelectrode was filled with KCl, but never when microelectrodes contained KSO_4 or KPO_4. They proposed the reversal of the IPSP was due to diffusion of chloride ions into the recorded neuron from the highly concentrated KCl solution (3 M) within the microelectrode (**Figure 3B**). Further evidence for a chloride-mediated mechanism was provided by experiments where diffusion of chloride ions into the motoneuron was accelerated by passing negative current into the microelectrode. In such experiments, the polarity reversal was the same, but occurred more rapidly. These effects, however, were never observed on EPSPs generated when stimulating the monosynaptic excitatory pathway (**Figure 2**). After making these observations they concluded "The simplest explanation for this effect would be that the inhibitory transmitter substance causes the subsequent postsynaptic membrane to become highly permeable to chloride" (Coombs et al., 1953, p. 2).

They also suggested that a metabolic "pump" must exist for the ejection of chloride ions, much like the sodium-potassium pump proposed by Hodgkin and colleagues within axons and muscle (Hodgkin, 1951; Hodgkin and Keynes, 1953). The identity of these chloride pumps is now well established and, importantly, their activity is highly mutable. For example, during development, pump activity alters intracellular chloride concentration and changes the polarity of GlyR currents from excitatory to inhibitory (Nakayama et al., 2002). This mechanism has also been shown to be important in certain pain states whereby intracellular chloride $[Cl^-]_i$ can increase and subsequently cause GlyR current depolarization. This effectively increases the excitability of neurons involved in nociceptive processing and alters pain thresholds (Coull et al., 2003).

More heroic experiments subsequently used double-barreled microelectrodes to impale motoneurons (**Figure 3C**). One barrel was for recording and the other for passing current and manipulating membrane potential (Coombs et al., 1955a). This approach allowed the study of synaptic potentials (both EPSPs and IPSPs) over a wide range of membrane potentials, as is routinely accomplished today with patch clamp techniques. These experiments showed both EPSPs and IPSPs could be reversed by altering membrane potential. Reversal was routinely observed for IPSPs, however, for EPSPs this was usually not achieved using double-barreled microelectrodes because of capacitive coupling between the two closely spaced electrode tips. Later work using two *separate* electrodes to impale motoneurons succeeded in reversing EPSPs and confirmed a reversal potential of 0–10 mV for 1a-mediated EPSPs (Engberg and Marshall, 1979). However, the most important result from early experiments was that EPSPs and IPSPs reversed at vastly different potentials (~ 0 and −80 mV, respectively). This confirmed that

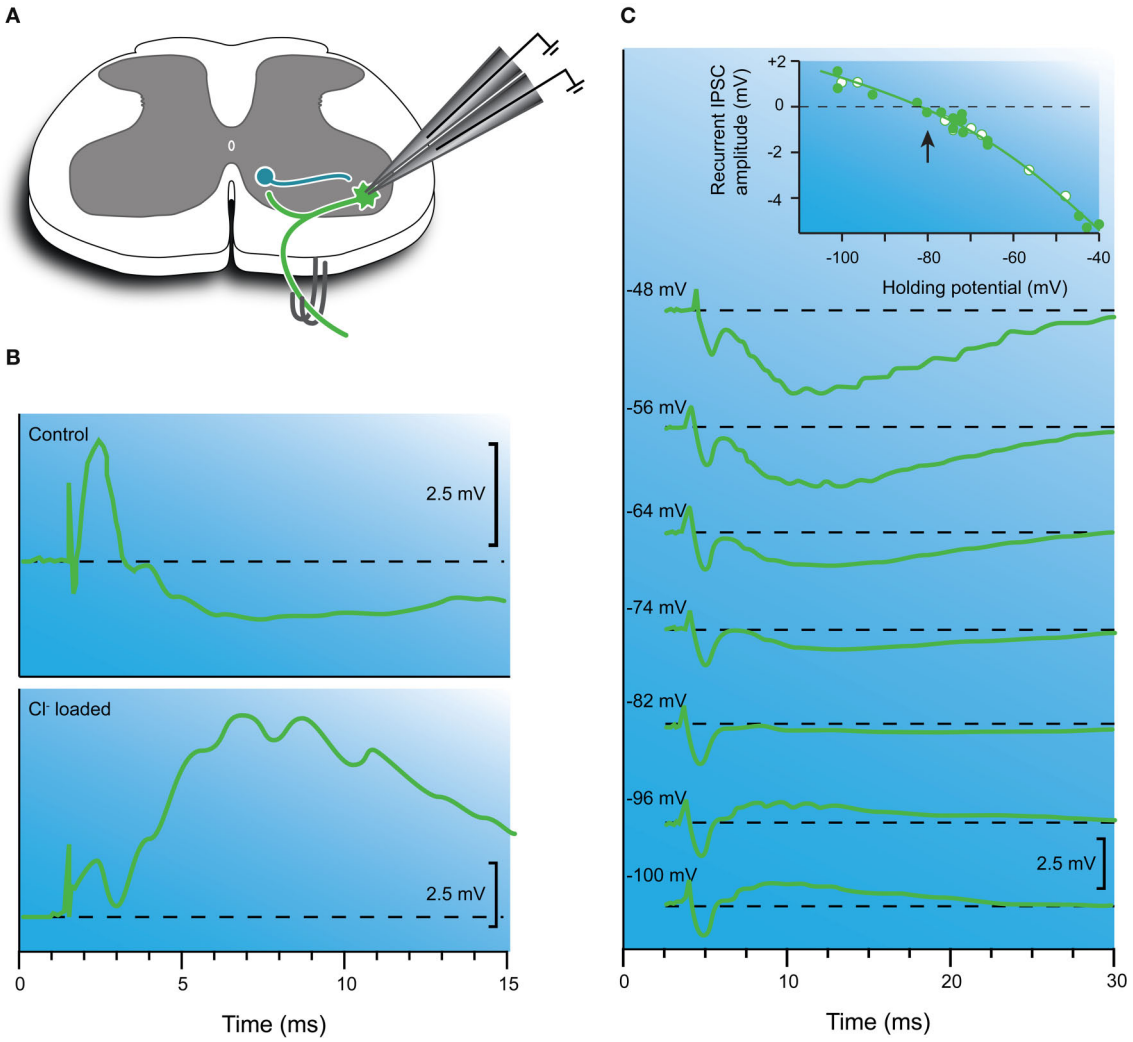

FIGURE 3 | Inhibitory synaptic transmission is mediated by chloride ions. **(A)** Schematic showing the Renshaw cell circuit and recording configuration. The recurrent inhibitory pathway is activated, by stimulating muscle nerves or ventral roots. **(B)** Recurrent IPSP recorded with a KCl-filled microelectrode before (upper trace) and after (lower trace) chloride ions have diffused out of the recording electrode into the motoneuron cytosol. Note, the IPSP has reversed (i.e., converted to an EPSP) after chloride loading. **(C)** Another experiment where recurrent IPSPs were recorded at several membrane potentials using a double-barreled intracellular microelectrode (dashed line equals baseline). One barrel recorded membrane potential and the other was used to inject current and control/hold membrane potential. In these experiments a $NaSO_4$ internal solution was used to avoid chloride loading (as shown in **B**). Note the polarity of the response varies with holding potential. The inset shows a plot of the recurrent IPSC amplitude vs. holding potential. The IPSP reversed at –80 mV, which approximates the equilibrium potential for chloride ions in CNS neurons. Modified with permission from Figures 1, 2 and 4 in Eccles et al. (1954).

EPSPs and IPSPs were due to the flow of different types of ions. Calculations based on the Nernst equation for the diffusion of ions across a semi-permeable membrane suggested that the intracellular concentration of chloride was about 7–12 mM (assuming extracellular chloride $[Cl^-]_o$ was 110 mM, the same as plasma). Subsequent *in vivo* and *in vitro* experiments on cat and rat motoneurons have confirmed that $[Cl^-]_i$ is ~ 6.5 mM when $[Cl^-]_o$ is 134 mM (Forsythe and Redman, 1988; Stuart and Redman, 1990).

In summary, these insightful experiments suggested that inhibitory synaptic transmission was mediated by a chloride conductance; that chloride ions were not equally distributed across the cell membrane; and that a membrane pump (ie, a chloride transporter) must exist to maintain the chloride gradient.

STRYCHNINE AS A SELECTIVE INHIBITOR OF CENTRAL INHIBITION

Sherrington and colleagues were the first to analyze the effects of sub-convulsive doses of strychnine on spinal cord reflexes in anesthetized cats (Owen and Sherrington, 1911). They showed strychnine reversed spinal cord reflexes, so that inhibitory reflexes became excitatory. This fit nicely with the well known convulsive effects of strychnine poisoning. In the conclusion of their paper, they proposed that strychnine "transformed the process of central inhibition into one of central excitation" (p. 240). Based on the types of experiments undertaken at the time, this explanation was entirely plausible as they were studying ventral root reflexes, driven by activity in both excitatory and inhibitory pathways (see **Figure 1**). Later experiments employing excitation of either

pure excitatory or inhibitory pathways showed convincingly that strychnine only affected inhibitory pathways (Bradley et al., 1953).

These experiments provided further support for a "chemical transmitting substance" (p. 478) that mediated central inhibition. For example, because the effect of strychnine was so rapid (within seconds following IV injection), Eccles and colleagues proposed "the most probable explanation is that strychnine and the inhibitory transmitter compete for the same steric configurations on the inhibitory postsynaptic membrane" (Bradley et al., 1953, p. 487). Similar mechanisms had been proposed to explain the action of curare on cholinergic synaptic transmission at peripheral synapses (Fatt and Katz, 1951). The final insightful prediction in their landmark 1953 paper came at the end of the discussion where they proposed "…it is evident that strychnine will provide a valuable means of investigating the nature of central inhibition." Indeed, this has proved to be the case. Strychnine subsequently played an important role in purifying and cloning the GlyR (Grenningloh et al., 1987), and confirming the involvement of GlyRs or $GABA_A$Rs in many neural pathways (Lim et al., 1999; O'Brien and Berger, 1999; Russier et al., 2002; Graham et al., 2003).

RECURRENT INHIBITION: THE RENSHAW CELL CIRCUIT

In the 1940s, Birdsey Renshaw used extracellular recording techniques to show that activity in motoneurons could inhibit the discharge of motoneurons within the same, or surrounding motoneuron pools (Renshaw, 1941, 1946). This work built on earlier work, which showed antidromic stimulation of motor axons inhibited rhythmic firing in motoneurons (Eccles and Hoff, 1932). Experimentally, this form of inhibition was most easily observed in preparations with cut dorsal roots (i.e., deafferented; **Figures 1B,3A and 4A**) where stimulation of ventral roots (ie, antidromic stimulation) produced prolonged inhibition of motoneuron discharge. Importantly, the onset (or latency) of this inhibitory response was consistent with the involvement of a synapse on an interposed interneuron, as had been proposed for the reciprocal inhibitory pathway. The time course of the inhibitory response, however, was much longer than that observed during reciprocal inhibition.

During investigation of this inhibitory pathway Renshaw made extracellular recordings from a group of neurons located ventromedial to the motoneuron pools (Renshaw, 1946). These neurons discharged in characteristic high frequency bursts (~30–50 ms duration with instantaneous firing frequencies >1000 Hz) following a *single* supra-threshold shock to ventral roots (**Figure 4B**; upper panel). Renshaw speculated that axon collaterals from motor neurons, which were known to anatomists (Cajal, 1909), excited a population of interneurons whose axons contacted motoneurons and inhibited them. At the time the mechanism surrounding this inhibition focused on the involvement of electric fields between neurons – recall the electrical theory of synaptic transmission was favored at central synapses in the 1940s. This interposed interneuron was later termed the Renshaw cell (Eccles et al., 1954) in honor of the scientist who proposed this inhibitory circuit. Ironically, Renshaw died tragically in 1948 from poliomyelitis, a disease that attacks motoneurons.

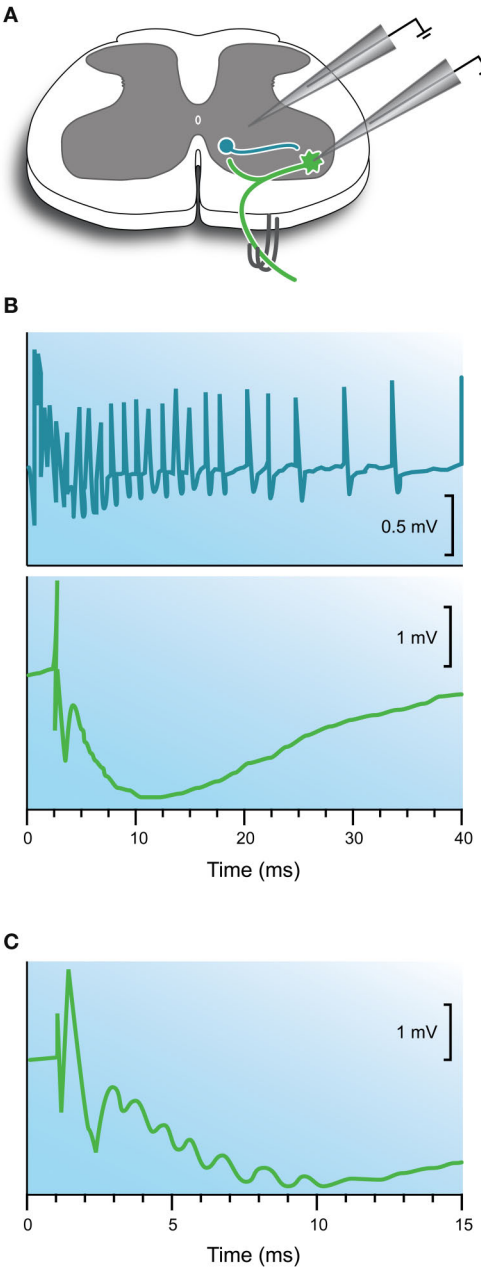

FIGURE 4 | Electrophysiological features of the recurrent inhibitory pathway. (A) Schematic showing the Renshaw cell circuit and recording methods. The recurrent pathway is activated by stimulating muscle nerves or ventral roots in deafferented preparations. In some experiments extracellular recordings are made from Renshaw cells located on the ventro-medial border of the ventral horn. In other experiments intracellular recordings are made from motoneurons. **(B)** Upper trace shows the extracellular response in a Renshaw cell following a *single* ventral root volley. Note the prolonged discharge. Lower trace shows an intracellularly recorded response in a motoneuron after a single ventral root volley. Note the long time course of the hyperpolarization compared with the response observed after stimulation of the reciprocal inhibitory pathway (**Figure 2C**). **(C)** Response shown in panel **(B)** lower on an expanded timescale. Note the prominent "ripples" in the rising phase of the hyperpolarization indicating the arrival of multiple high frequency inhibitory inputs from Renshaw cells. Modified with permission from Figures 3 and 12 in Eccles et al. (1954).

Both Renshaw and Eccles had difficulty assigning a functional role to the recurrent inhibitory pathway other than "a generalized suppressor function" (p. 558) and that the circuit could be important for limiting intense motoneuron discharge, as occurs during convulsions (Eccles et al., 1954). This suppressor function is consistent with the motor disturbances (tremor and spasticity) exhibited by mutant mice where glycinergic inhibitory synapses, including those from Renshaw cells to motoneurons, are severely disrupted (Rajendra and Schofield, 1995; Simon, 1997). To this day the Renshaw cell remains one of the most studied interneurons in the mammalian CNS, and the Renshaw pathway serves as a classic example of an "inhibitory" or negative-feedback circuit involving GlyRs (Windhorst, 1996; Gonzalez-Forero and Alvarez, 2005).

As with monosynaptic and reciprocal inhibitory pathways, the introduction of intracellular microelectrodes allowed the recurrent inhibitory circuit to be examined in more detail and, importantly, test whether observations made on reciprocal inhibition applied to recurrent inhibition (**Figures 1A,B**). Two major studies, which should be compulsory reading for new GlyR biologists, suggested similar mechanisms operated in the recurrent inhibitory pathway (Eccles et al., 1954; Coombs et al., 1955c). An IPSP could be intracellularly recorded in motoneurons following stimulation of muscle nerves or ventral roots in deafferented preparations (**Figure 4B**; lower panel). The latency of this response, measured from the arrival of an antidromic volley in the spinal cord and the onset of the IPSP, ranged from 1.1 to 1.8 ms – values that matched the central delay for reciprocal inhibition (Eccles et al., 1956a). The IPSPs often exhibited high frequency ripples on their rising phase and peaks (**Figure 4C**). This suggested convergent inputs, from neurons discharging at high frequencies, contributed to the long time course of the recurrent IPSP. These recurrent IPSPs could be reversed by passing negative current through KCl-filled electrodes (**Figure 3B**), or by manipulating membrane potential by current injection (**Figure 3C**), as was the case for reciprocal inhibition (Bradley et al., 1953; Eccles et al., 1954). Finally, intravenous injection of strychnine dramatically reduced the amplitude of the IPSP, as it did for reciprocal inhibitory connections. Thus, by the mid 50s it was clear that a *similar* neurotransmitter was responsible for the reciprocal and recurrent IPSP in spinal motor neurons.

THE SEARCH FOR THE INHIBITORY TRANSMITTER SUBSTANCE

By the mid 50s it was generally accepted that the inhibitory transmitter substance in the cat spinal cord caused permeability changes in the sub-synaptic membrane of motoneurons, and the resultant ion fluxes produced membrane hyperpolarization (Coombs et al., 1955c). Several rules pertaining to inhibitory synaptic transmission in the spinal cord were, for the most part, accepted at that time: (1) inhibitory synaptic transmission was chemically mediated; (2) both reciprocal and recurrent spinal inhibitory pathways involved a chloride conductance that reversed just below or near resting membrane potential; (3) inhibitory synaptic transmission could be markedly reduced by vascular administration of the convulsant strychnine; and (4) anatomical and electrophysiological evidence suggested clusters of neurons close to inhibited motor neurons were likely to release an inhibitory transmitter substance.

The initial search for chemical transmitter substances in the spinal cord used extracellular recordings and activation of the excitatory and inhibitory pathways shown in **Figure 1**. These early experiments, which measured responses in neurons after *vascular* administration of drugs, produced inconsistent results across laboratories and various explanations were proposed (Curtis and Eccles, 1958b). For example, it was not clear if drugs delivered into the vasculature acted within the CNS or on peripheral receptors (e.g., muscle spindles and tendon organs in muscles). There was also concern that various diffusion barriers might prevent substances getting into the CNS (Curtis and Eccles, 1958a). A significant breakthrough came with the development of multi-barreled electrodes, which allowed drugs to be delivered in the close vicinity of recorded neurons, and by extension to synapses on their somas and proximal dendrites (**Figure 5A**). These electrodes consisted of a central extracellular recording electrode, surrounded by several barrels that could apply drugs by the controlled application of current (now termed iontophoresis; (Curtis and Eccles, 1958b). The new electrodes employed ideas from earlier experiments on acetylcholine responses at neuromuscular junctions where a "breaking current" was applied to control the outflow of acetylcholine from an electrode (Del Castillo and Katz, 1955). Depending on the charge of the drug under investigation, positive or negative iontophoretic currents could be used to drive them out of electrodes. Application times could be precisely controlled, however, the exact concentrations achieved in the vicinity of recorded neurons could only be inferred, and for technical reasons, was sometimes poorly controlled.

The search for the inhibitory transmitter in the spinal cord was initially driven by the view that any substance that acts as a neurotransmitter must be concentrated in the tissue where it exerts its effect. For inhibition, the amino acid, GABA, was an obvious transmitter candidate. GABA was concentrated in brain tissue (Bazemore et al., 1956), and importantly it depressed spiking in cortical neurons (Purpura et al., 1957). The depressant action of GABA in the mammalian CNS also paralleled that of the "natural transmitter substance" at a well-understood inhibitory synapse in the crayfish (Kuffler and Edwards, 1958). Based on these data, Curtis and colleagues examined the effects of GABA, and the structurally similar β-alanine, on the activity of neurons in the cat spinal cord (Curtis and Phillis, 1958; Curtis et al., 1959). Using multi-barreled electrodes they showed iontophoretic application of GABA and β-alanine suppressed spiking, reduced EPSP and IPSP amplitude, and reduced electrical excitability in spinal neurons (ie, via shunting inhibition). On the occasions where they were able to make intracellular recordings in motoneurons, the application of either compound produced *no* significant changes in resting membrane potential. This contrasted markedly with the depolarization observed when excitatory transmitter candidates (aspartate and glutamate) were applied to motoneurons (Curtis et al., 1960). In interpreting their data, they stated "All evidence indicates that these substances have a non-specific depressant action upon the whole surface membrane of neurones, *both* the chemically activated sub-synaptic regions and the remaining electrically excited postsynaptic membrane" (p. 202). In later experiments they would modify this view (Curtis et al., 1968b), however, the failure of applied amino acids

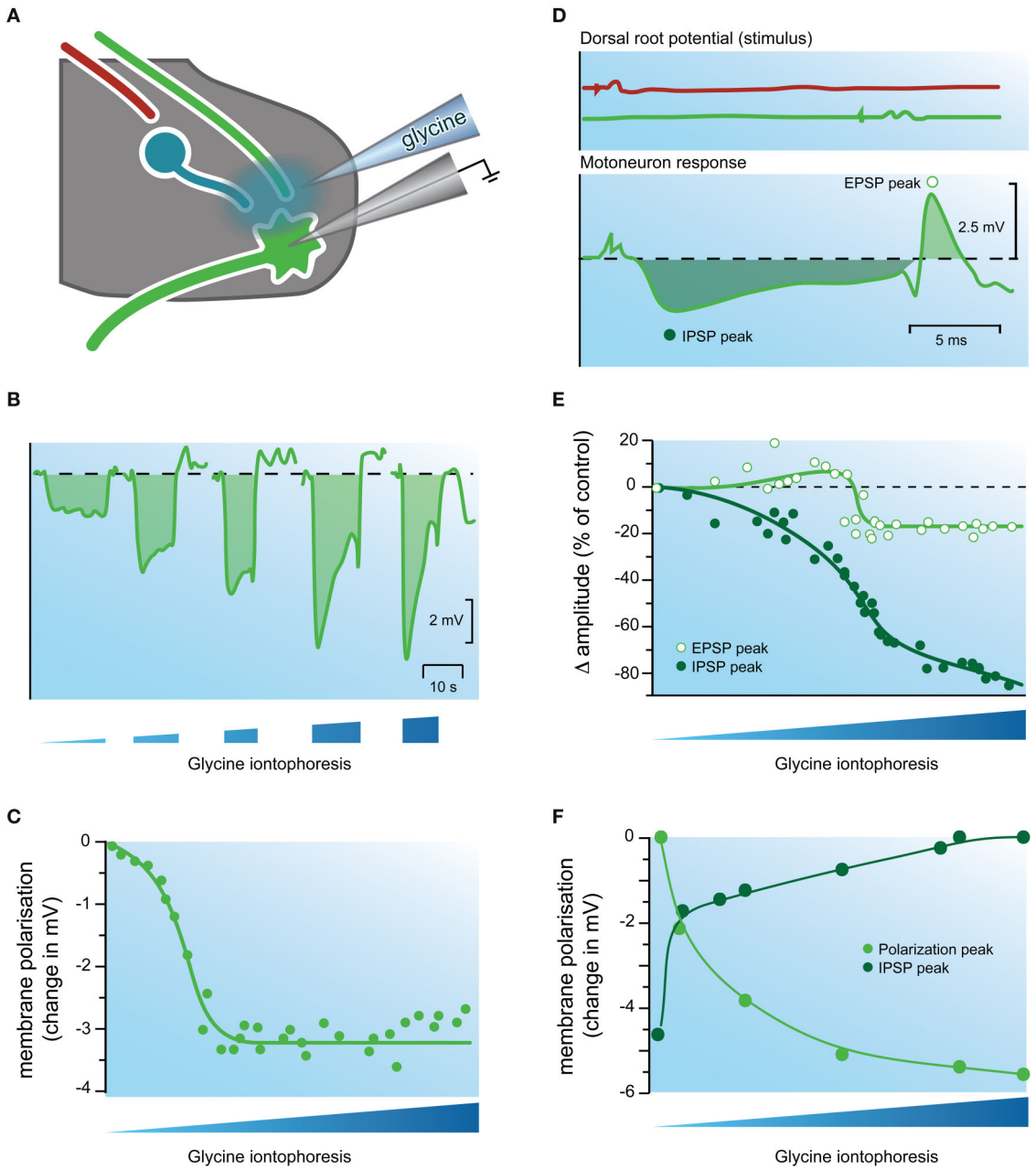

FIGURE 5 | Evidence that glycine is an inhibitory transmitter in the cat spinal cord. (A) Experimental setup showing electrodes used for extracellular iontophoresis of glycine, and intracellular recording of IPSPs in motoneurons. **(B)** Schematic showing hyperpolarizing responses recorded in a motoneuron during increasing iontophoretically-driven glycine applications. **(C)** Plot of peak membrane hyperpolarization (from data in **B**) in a motoneuron after iontophoretic application of glycine. Note the response increases to a plateau, despite continued increases in glycine iontophoresis. **(D)** Upper two traces show dorsal root potentials recorded during activation of the reciprocal inhibitory pathway (top trace), and the monosynaptic excitatory pathway (bottom trace). Lower trace shows the postsynaptic response (an IPSP followed by an EPSP) recorded in a motoneuron after stimulating the above inhibitory and excitatory pathways. **(E)** Plot of IPSP and EPSP peak amplitude (filled and open circles, respectively) during increasing iontophoresis of glycine. Note the amplitude of the IPSP is reduced far more than that of the EPSP. **(F)** Comparison of the effects of applied glycine on IPSP amplitude and membrane hyperpolarization. The IPSP amplitude (dark green circles) approaches zero as the level of membrane hyperpolarization approaches a plateau (light green circles). This experiment provided strong evidence that a similar process underlies the response to applied glycine and the IPSP. Modified with permission from Figures 4, 11 and 12 in Werman et al. (1968).

(GABA and β-alanine) to hyperpolarize motoneurons (like the synaptically released transmitter substance) meant in the early 1960s the identity of the inhibitory neurotransmitter in the cat spinal cord was still unknown.

GLYCINE IS AN INHIBITORY NEUROTRANSMITTER IN THE SPINAL CORD

The possibility that glycine might also be an inhibitory transmitter evolved more slowly. It was an amino acid, like GABA and β-alanine, and was therefore included in early structure-activity studies on

the effects of various compounds on neuron activity in the cat spinal cord (Curtis and Watkins, 1960). In surveying the effects of a large number of related amino acids of known chemical structure (50 "excitants" and 65 "depressants" listed in their Tables 1 and 2), Curtis and Watkins concluded that excitatory activity was associated with compounds possessing two acidic and one basic group, whereas depressant activity was associated with the presence of one acidic and one basic group. As specific chemical structures appeared to be linked with excitatory and inhibitory action, they also speculated on the structural features of the receptors for each type of transmitter, and the mechanisms by which excitatory and inhibitory transmitters might alter membrane permeability. In regard to the structural features of the receptor site they stated "The amino acid receptor can thus be considered to consist of an arrangement of fully or partially charged atoms or groups located within a specific region of the molecular framework of the membrane, to which region, molecules in the extracellular fluid have only limited access" (p. 133). They also proposed that binding of neurotransmitter to charged residues on the receptor could alter ionic permeability. To explain the different action of excitatory and inhibitory compounds on membrane potential they proposed only excitatory substances "result in the net entry of sodium ions into the cell, this accounting for the observed depolarization" (p. 136). These insights on the nature of ion channel behavior should seem remarkable to today's ion channel biologists who routinely use terms like charged residues, poor selectivity, and ion hydration.

During the 1960s, additional evidence accumulated for the role of glycine as an inhibitory transmitter. First, the extensive Curtis and Watkins (1960) survey had shown glycine was a depressant. Second, GABA was clearly not the only compound involved in inhibition, as vascular administration of strychnine had no effect on applied GABA, even though it was a potent inhibitor of IPSPs (Eccles et al., 1954; Curtis et al., 1959). Third, investigation of free amino acid levels in the spinal cord showed glycine levels actually exceeded those for the other candidate central transmitters, GABA and L-glutamate (Graham et al., 1967). Fourth, when glycine levels were analyzed in various regions of the cat spinal cord they were highest in the ventral horn where inhibitory interneurons were concentrated (Aprison and Werman, 1965). Accordingly, Aprison and Werman concluded "This pattern is considered to be compatible with a possible role for glycine as the major physiological postsynaptic spinal visceral inhibitory transmitter" (p. 2082). Additional support for their proposal came from studies involving manipulations that "killed off" spinal interneurons. Glycine levels in the ventral horn decreased and importantly glycine levels correlated with the extent of interneuron loss (Davidoff et al., 1967a,b). Thus, by the late 60s considerable evidence suggested glycine was a candidate inhibitory transmitter in the cat spinal cord.

As the above evidence mounted, a series of sophisticated experiments by Werman et al. (1967, 1968) examined the mechanisms by which glycine might exert its effects). They developed a side-by-side electrode configuration that allowed simultaneous *intracellular* recording from motoneurons and *extracellular* iontophoresis of glycine (**Figure 5**). This electrode arrangement allowed comparison of IPSPs evoked by stimulating inhibitory pathways (reciprocal or recurrent) and responses to iontophoresed glycine (**Figure 5B**). The most relevant findings for GlyRs were: (1) application of glycine always produced a *hyperpolarization* (~ 3.7 mV; $n = 27$) in motoneurons, and the magnitude of the hyperpolarization was inversely correlated with membrane potential; (2) as the iontophoretic current was increased (and by inference $[glycine]_o$) the magnitude of the hyperpolarization increased and plateaued (**Figures 5B,C**); (3) the hyperpolarization produced by glycine was associated with an increase in membrane conductance; and (4) as glycine application did not affect the threshold for action potential initiation, they concluded "glycine does not act on the voltage-sensitive electrically excitable membrane" (p. 86).

Werman et al. (1967, 1968) then went on to examine the effects of glycine on evoked postsynaptic potentials (ie, EPSPs and IPSPs; **Figure 5D**). The effect of applied glycine on IPSP amplitude was dramatic and in some instances application of increasing concentrations of glycine could virtually abolish the IPSP (**Figure 5E**). In contrast iontophoresis of glycine at excitatory synapses only slightly reduced the amplitude of evoked EPSPs (**Figure 5E**). They suggested the differing effect of glycine on IPSPs and EPSPs was due to the location of inhibitory and excitatory synapses on motoneuron dendritic trees – IPSPs were generated close to the soma and applied glycine was more likely to affect these synapses and reduce driving force (such as via shunting inhibition). EPSPs in contrast were generated some distance from the soma and were largely unaffected. These observations fit nicely with ideas being developed at that time on the integrative action of IPSPs and EPSPs, and the ongoing competition between inhibitory and excitatory synapses for the control of membrane potential in motoneurons and other neurons with large dendritic trees (Curtis and Eccles, 1959; Rall, 1959, 1960).

The mechanisms whereby application of increasing concentrations of glycine could virtually abolish some IPSPs were examined in more detail by comparing the amplitude of evoked IPSPs (in the reciprocal pathway) and the hyperpolarization produced by increasing $[glycine]_o$. Both IPSP amplitude and hyperpolarization showed an initial rapid change, which plateaued as glycine concentration increased (**Figure 5F**). A similar relationship was observed when glycine concentrations were progressively decreased. Together, these experiments suggested that the process underlying the evoked IPSP and the glycine response occurred in parallel – i.e., the inhibitory transmitter and glycine had identical action at the postsynaptic membrane.

To further investigate the relationship between IPSPs and glycine-induce membrane hyperpolarization, Werman and colleagues made recordings with KCl-filled electrodes and showed that both the IPSPs and responses to applied glycine could be converted to depolarizations (ie, they could be reversed). Importantly, the depolarizing IPSPs disappeared at the same membrane potential where the response to applied glycine was greatest. In their discussion they stated "glycine and the inhibitory transmitter activate the same molecular process in the postsynaptic membrane" (p. 92). Thus, if one considers the three textbook criteria for a substance to be considered a neurotransmitter (e.g., Chapter 15; Kandel et al., 2000), work in the late 1960's was close to confirming two of the major criteria for a substance to be recognized as a neurotransmitter: (1) presence – glycine is present where it should be; and (2) release and identity of action – its action on the postsynaptic membrane closely mimicked that of the naturally-occurring transmitter. The third criteria, a mechanism for removal, took longer to satisfy.

In the late 1960's there was considerable interest in processes that terminated neurotransmitter action, especially mechanisms involving *uptake*, as had been described for GABA in the cerebral cortex (Iversen and Neal, 1968). Neal (1971), subsequently showed a system for glycine uptake existed in the spinal cord and could provide a mechanism for inactivating glycine at inhibitory synapses). Moreover, the mechanism exhibited some important properties of an active transport system – specifically, the uptake process showed considerable structural specificity as uptake of [^{14}C]-glycine was unaffected by high concentrations of related amino acids such as aspartate, glutamate, valine, and GABA. Later researchers took advantage of this high-affinity uptake system and used EM-autoradiography to demonstrate that [^{3}H]-glycine density was highest in putative inhibitory synaptic terminals containing elliptical and pleomorphic vesicles (Price et al., 1976). In the 1990's the identity and important role of the glycine transporters, which rapidly remove glycine from the synaptic cleft into glia and neurons (GLYT1 and GLYT2, respectively) was firmly established (Guastella et al., 1992; Liu et al., 1993).

The possibility that glycine was an inhibitory transmitter was also explored by testing the antagonistic properties of strychnine on glycine-mediated responses. By the late 1960s, its action was considered to be competitive and reversible because strychnine blocked the action of iontophoresed glycine (Curtis et al., 1968a,b, 1969). Intriguingly, while strychnine was clearly useful for distinguishing responses mediated by glycine, it did not affect the inhibitory action of GABA. Subsequent studies explored the role of other convulsants that were structurally related to the strychnine molecule (Curtis et al., 1970). One of these was the plant alkaloid, bicuculline. When this compound was iontophoresed onto continuously discharging ventral horn neurons it was found to reversibly inhibit the action of applied GABA, but not that of glycine (**Figure 6**). Other experiments had also confirmed that strychnine did not affect the action of iontophoresed GABA (Curtis et al., 1967). Many of these findings were confirmed later in other spinal pathways. For example, Game and Lodge used extracellular recording techniques to examine the effects of iontophoresed strychnine and bicuculline on inhibitory responses in dorsal horn cells evoked by volleys in myelinated cutaneous afferents. Strychnine abolished the "early" inhibitions, and bicuculline abolished the longer latency responses. The authors concluded glycine and GABA might play differing roles in mediating inhibition in sensory pathways (Game and Lodge, 1975).

Thus by 1970, glycine and GABA were considered potential neurotransmitters at inhibitory synapses in the reciprocal and recurrent pathways in the cat spinal cord, and the action of each could be distinguished by their selective blockers strychnine and bicuculline, respectively. Later work showed glycine is widely used by other classes of spinal neurons in the ventral and intermediate grey (Fyffe, 1991), and in the dorsal horn (Game and Lodge, 1975) of the cat spinal cord. This work set the scene for future investigations when each receptor was isolated, purified, cloned and studied in cell expression systems. As stated in a recent perspective on glutamate receptor biology, it is difficult to conceive how we could study inhibitory synaptic mechanisms without the routine use of blockers of the GlyR and $GABA_A$R (Krnjevic, 2005). For example, work in the 1980s used strychnine binding to isolate and purify GlyRs from mammalian brain and reveal that the receptor was composed of

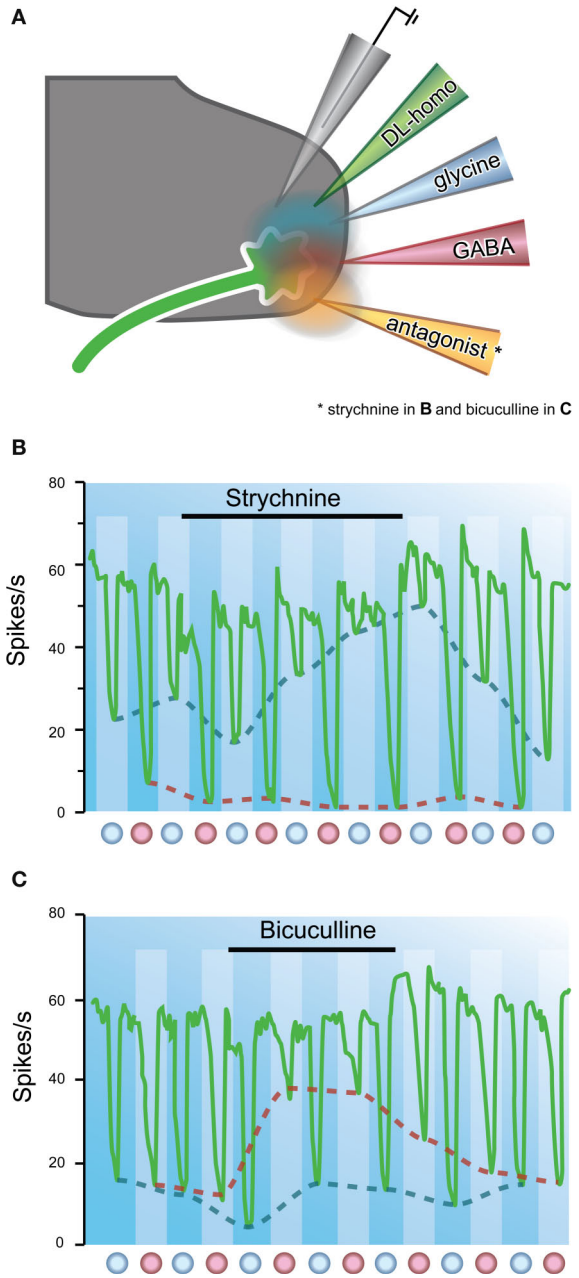

FIGURE 6 | The major inhibitory neurotransmitters can be distinguished pharmacologically. (A) Neurons are activated by continuous iontophoretic application of DL-homocysteic acid. Putative neurotransmitters and their antagonists, strychnine or bicuculline, are delivered via adjacent barrels in a multi-barrel electrode array. **(B)** Plot showing the effect of strychnine on inhibition of a Renshaw cell by applied glycine and GABA. Sustained iontophoresis of DL-homocysteic acid maintains a constant discharge rate in the target neuron. Glycine and GABA are alternatively applied at times indicated by red and blue dots, respectively. Strychnine application is indicated by horizontal bar. Note strychnine has a marked effect on glycine, but not GABA-mediated inhibition (red vs. blue dashed lines). **(C)** Similar experiment to **(B)**, but strychnine application replaced by bicuculline. Note, bicuculline has a marked effect on GABA, but not glycine-mediated inhibition (blue vs. red dashed lines). These experiments showed strychnine and bicuculline could be used to distinguish glycine and GABA-mediated synaptic transmission. Modified with permission from Curtis et al. (1970).

two membrane proteins of 48 kDa and 56 kDa – corresponding to α and β subunits, respectively (Pfeiffer et al., 1982; Langosch et al., 1988). A larger 93-kDa protein, called gephyrin, also co-purified with the GlyR. We now know that gephyrin resides on the post-synaptic side of glycinergic synapses and is critical for clustering GlyRs under release sites (Triller et al., 1985). Now of course we know that glycine is used as a neurotransmitter by numerous classes of neurons, including dorsal horn neurons.

OTHER ENDURING PRINCIPLES DISCOVERED WHEN STUDYING CENTRAL INHIBITION *IN VIVO*

LOCATION OF GLYRS ON THE SOMATODENDRITC TREES

As the mechanisms underlying central inhibition in the cat spinal cord were being defined, several other important contributions to our understanding of CNS function were also uncovered. For example, in the 60s and 70s there was considerable interest in how synapse location on the somas and dendritic trees of complicated central neurons, like motoneurons, influenced their integrative or input/output properties (Rall, 1977). At that time, motoneurons were the most accessible central neuron, and excitatory and inhibitory inputs to motoneurons could be studied (**Figure 2**). The early work of Eccles and colleagues (Brock et al., 1952b) on the reciprocal inhibitory pathway proposed inhibitory synapses were located close to the spike initiating region – at least in motoneurons. This assertion was based on the extreme sensitivity of the IPSPs to intracellular chloride concentration (**Figure 3B**).

Later, an elegant study by Fyffe and colleagues examined this proposal in more detail. Four major neuron types, α motoneurons and γ motoneurons, 1a interneurons, and Renshaw cells, which together play a crucial role in spinal locomotor circuits, were electrophysiologically identified and intracellularly labeled with neurobiotin in cat spinal cord preparations (Alvarez et al., 1997). The distribution of presumptive GlyR clusters on somal, proximal and distal dendrites of each neuron type was quantified using immunohistochemistry for gephyrin, the protein that anchors GlyRs under synapses. Their data showed that GlyR cluster size and complexity generally increased with distance from the soma in α and γ motoneurons and 1a interneurons. In contrast, Renshaw cells displayed large and morphologically complex clusters, concentrated on their somas and proximal dendrites. The presence of large GlyR clusters on the small Renshaw cells suggested a powerful spinal mechanism exists to "turn off" these cells and their contribution to recurrent inhibition. The abundance of GlyRs at distal synapses suggested GlyRs can not only provide powerful shunting inhibition (Curtis et al., 1959), but also fine-tune the effects of excitation on distal synapses. These data suggested that positioning of synapses on somatodendritic trees depends on the role of the neuron in spinal cord circuits. Thus, the original postulate of Eccles regarding synapse location needs to be modified to account for the role of a neuron in circuit function.

DALE'S HYPOTHESIS AT CENTRAL SYNAPSES

In a classic paper presented to the Royal Society in 1934, Sir Henry Dale summarized the major ideas on synaptic transmission that had evolved from experiments on peripheral synapses. Based on experiments on sensory nerves (involving the axon-reflex), he suggested knowing the identity of the neurotransmitter at an axon's peripheral terminal might provide insight into the nature of its central neurotransmitter. Specifically he stated "When we are dealing with two different endings of the same sensory neurone, the one peripheral and concerned with vasodilatation and the other at a central synapse, can we suppose that the discovery and identification of a chemical transmitter of axon-reflex vasodilatation would furnish a hint as to the nature of the transmission process at a central synapse?" (p. 329). Eccles later referred to Dale's hypothesis when studying recurrent inhibition and coined the term Dale's principle (Eccles et al., 1954). He stated "the same chemical transmitter is released from all the synaptic terminals of a neurone" (p. 559). The advent of intracellular recording techniques and the increasing interest in inhibitory synaptic transmission in spinal reflex pathways allowed Dale's ideas to be tested at synapses within the CNS. Eccles et al. (1956b) took up this quest and in a series of experiments from 1952–1956 asked "Do the branches of any one nerve cell exert an excitatory synaptic action on some neurones and an inhibitory action on others?").

The recurrent inhibitory circuit provided an ideal model to test Dale's hypothesis as it was well established that acetylcholine was the neurotransmitter at the neuromuscular junction and should therefore be released from motoneuron recurrent collaterals (**Figure 1C**). Eccles and colleagues showed the extracellular responses recorded in Renshaw cells after ventral root stimulation was often enhanced when cats were given acetylcholine (intra-arterially), depressed with a cholinergic blocker (dihydro-β-erythroidine; given IV), and prolonged when animals were given an anticholinesterase (usually eserine; given IV). Together, these data suggested the synapse between recurrent motor axon collaterals and Renshaw cells, indeed uses the *same* transmitter as the peripheral neuromuscular junction. This experiment provided the first support for Dale's hypothesis at a synapse *within* the CNS.

With time, Dale's hypothesis has perhaps become overly simplified to mean one neuron, one transmitter. Dale never stated a neuron secretes a single neurotransmitter, but rather that knowledge of the neurotransmitter released at one terminal might provide clues to that released at its other terminals. As so elegantly summarized by Strata and Harvey the problem lies with use of the term transmitter in the singular (Strata and Harvey, 1999). If we accept this view, Dale's ideas do not conflict with the now well-accepted idea of neurotransmitter co-release. For example, two recent studies have shown both glycine and GABA can be concentrated in the same vesicle in synaptic terminals, released together, and act on a mixed population of glycine and GABA receptors underneath a release site (Jonas et al., 1998; O'Brien and Berger, 1999). In addition to co-release, recent work has suggested glutamate and acetylcholine can be released at the synapse between motoneuron collaterals and the inhibitory Renshaw cell (Nishimaru et al., 2005). Significantly, this study also showed that while glutamate is released centrally it is not released at peripheral terminals. Thus, there can be differences in transmitter content and release at two synapses from the same neuron. These recent observations may violate some interpretations of Dales hypothesis, but they do not violate his original suggestion that that knowledge of the neurotransmitter released at one terminal of a neuron might provide clues to that released at its other terminals. In summary, the study of inhibitory mechanisms in the spinal cord has contributed significantly to this important debate about neurotransmitter co-release and release of different neurotransmitters at two terminals of the same neuron.

CONCLUSIONS

This review has attempted to reflect on some of the important discoveries made in the cat lumbosacral spinal cord that underpin our current understanding of GlyR function and fast inhibitory synaptic transmission. These key observations and predictions were made during a golden age of *in vivo* spinal cord synaptic physiology in the 1950s–1960s and endure till this day. For example, we now accept that GlyRs mediate their effects via chemically mediated transmission, are chloride channels, are involved in spinal reciprocal and recurrent inhibition, can be selectively blocked by strychnine, can be distinguished from the $GABA_AR$ by their insensitivity to bicuculline, play a crucial role in synaptic integration, and are often preferentially located near a neuron's trigger zone. We hope our narrative on this important era will encourage those interested in GlyR biology and inhibitory mechanisms to seek out some of the "classic" articles that document these discoveries.

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The specification of glycinergic neurons and the role of glycinergic transmission in development

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Glycine's role as an inhibitory neurotransmitter in the adult vertebrate nervous system has been well characterized in a number of different model organisms. However, a full understanding of glycinergic transmission requires a knowledge of how glycinergic synapses emerge and the role of glycinergic signaling during development. Recent literature has provided a detailed picture of the developmental expression of many of the molecular components that comprise the glycinergic phenotype, namely the glycine transporters and the glycine receptor subunits; the transcriptional networks leading to the expression of this important neurotransmitter phenotype are also being elucidated. An equally important focus of research has revealed the critical role of glycinergic signaling in sculpting many different aspects of neural development. This review examines the current literature detailing the expression patterns of the components of the glycinergic phenotype in various vertebrate model organisms over the course of development and the molecular mechanisms governing the expression of the glycinergic phenotype. The review then surveys the recent work on the role of glycinergic signaling in the developing nervous system and concludes with an overview of areas for further research.

Keywords: glycine, glycinergic, review, neurotransmitter, development, embryo, phenotype

INTRODUCTION

In addition to its role in protein metabolism as the structurally simplest amino acid, glycine also serves as an important and widely distributed inhibitory neurotransmitter in the nervous system of animals from several phyla (Werman et al., 1968; Sawada et al., 1980, 1984; Mladinic et al., 1999 reviewed in Walker and Holden-Dye, 1991; Walker et al., 1996). While its role in adult neurotransmission is relatively well characterized, less is known about glycinergic signaling during development. Previous work has focused on examining the developmental expression of the glycine receptor and transporters as well as glycine immunoreactivity as a means of discerning the process whereby cells adopt a glycinergic phenotype. However several recent studies have begun to elucidate the mechanisms governing the development of the glycinergic phenotype as well as the role of glycine in neural development. For example, synaptic release of both glycine and GABA has been shown to play a determinative role in the proper development of many motor and sensory pathways, including those necessary for audition, vision, respiration, and nociception (Kirsch, 2006). Disruption of glycinergic transmission during neural development has also been shown to cause hyperekplexia, a disorder characterized by exaggerated startle responses, hypertonia and episodic neonatal apnea (Eulenburg et al., 2006; Rees et al., 2006). Differential expression of GlyR subunits over the course of development (namely $\alpha 2$) have been observed, although the function of such embryonic GlyRs have not yet been determined (Young-Pearse et al., 2006). Some evidence suggests that glycine has an early "pre" neuronal function, where taurine may act as an endogenous ligand in non-synaptic embryonic GlyR signaling to help coordinate the early neocortex, hippocampus and ventral tegmentum (Flint et al., 1998). More recently Scain et al. (2010) have described non-synaptic glycine signaling in the embryonic spinal cord.

A complete understanding of glycinergic transmission must entail knowledge of how cells acquire a glycinergic phenotype and the role of glycinergic signaling in development. This review summarizes the current understanding of glycinergic signaling in development. First, we examine the literature detailing the expression patterns of the molecular components of the glycinergic phenotype and glycinergic signaling in various vertebrate model organisms over the course of CNS development. Next we consider the molecular mechanisms governing the expression of glycinergic phenotype markers and the glycinergic phenotype. Finally, we review the current literature on the functional role of glycine signaling in the developing nervous system and then conclude with an overview of areas for further research.

DEVELOPMENTAL EXPRESSION PATTERNS OF GLYCINERGIC PHENOTYPE MARKERS

In vertebrates the biosynthesis of glycine for use in neurotransmission and general cellular metabolism is mediated by an enzyme differentially referred to as glycine synthase and glycine cleavage enzyme. This enzyme catalyzes a readily reversible reaction between carbon dioxide, ammonium ion, N^5, N^{10}-methylene tetrahydrofolate, NADH and a proton to form glycine, tetrahydrofolate and NAD^+. Immunohistochemistry and *in situ* hybridization experiments in rats have shown that the glycine cleavage system enzyme complex is expressed in embryonic neural stem/progenitor cells, neuroepithelial cells, and astrocytes (Ichinohe et al., 2004).

In addition to glycine itself, glycinergic transmission requires several additional molecular components. Two membrane-bound proteins, GlyT1 and GlyT2, members of the Na^+/Cl^- dependent neurotransmitter transporter family, serve to reuptake glycine from the synaptic cleft back into cells, with GlyT1 expressed predominantly by glial cells and GlyT2 serving as the primary neuronal glycine transporter. In addition, the vesicular inhibitory amino acid transporter (VIAAT), also commonly referred to as VGAT, transports both GABA and glycine into synaptic vesicles for later release into the synapse. Both GlyT2 and VIAAT must both be expressed for a cell to successfully perform as a glycinergic neuron.

The signaling function of glycine is mediated mainly by the glycine receptor (GlyR), a strychnine-sensitive member of the nicotinic acetylcholine receptor (nAChR) super family that is a ligand-gated chloride anion channel (reviewed in Betz and Laube, 2006). GlyR is a pentameric protein with a stochiometry reported first as 2α/3β (Becker et al., 1988; Kuhse et al., 1993; Burzomato et al., 2003) and more recently as 3α/2β using a combined site directed mutagenesis and homology modeling approach (Grudzinska et al., 2005). Four α subunits (α1, α2, α3, and α4) and one β subunit have been characterized to date. The α2 and α4 subunits are expressed earlier in development compared to α1 and α3, which are generally only seen in the adult brain (Kuhse et al., 1991). Expression of α2 is observed before synaptogenesis, suggesting this subunit may play a role in pre-synaptic glycine signaling. Splice variants have also been observed for the α1, α2 and α3 subunits. The α1 splice variant ($\alpha1^{ins}$) includes eight additional amino acids and is expressed in the brainstem and spinal cord of mature rats (Kuhse et al., 1991; Malosio et al., 1991a). The α2 splice variants (α2A and αB) differ by expression of two homologous exons and are both limited to expression in the early developing CNS (Grenningloh et al., 1990). Another α2 variant (α2*) is not generated through alternative splicing and has yet to be completely characterized (Kuhse et al., 1990). The α3 splice variant α3L has a very similar primary structure to α3 while the splice variant α3K lacks a 15 amino acid sequence. Both α3 splice variants are observed in the human fetal brain (Nikolic et al., 1998; reviewed in Legendre, 2001). While the exact role of the α and β subunits in agonist binding is unclear (Grudzinska et al., 2005), binding between the large cytoplasmic loop of the β subunit and the anchoring protein gephyrin has been well characterized (Kneussel et al., 1999). Gephyrin binding has been implicated in synaptogenesis, but uncertainty still remains over the necessity of gephyrin for synapse formation (Feng et al., 1998; Meier et al., 2000; Levi et al., 2004). In order to understand the process by which cells adopt a glycinergic phenotype, investigators have conducted numerous studies examining the ontogeny and developmental expression patterns of each of these molecular components in a variety of different species.

GLYCINE

The presence of glycine immunoreactivity (IR) in neuronal and glial cells has served as the primary marker for the development of the glycinergic phenotype and has been the subject of developmental expression studies in a number of different organisms, particularly in mammals. In mice, Allain et al. (2006) observed little IR before embryonic day 12.5 (E12.5) in the mouse spinal cord, at which time glycine IR was visualized in the somata of ventral horn cells and in fibers at the edges of the marginal zone. One day later glycine IR begins to appear in the dorsal horn, intensify in cells in the ventral horn, and decrease in the marginal zone. By E16.5 IR reaches near adult levels in both the ventral and dorsal horns. These investigators also observed the colocalization of GABA and glycine IR in a third of all glycine positive cells, with the glycinergic system maturing a day after the GABA system (Allain et al., 2006; Sibilla and Ballerini, 2008).

Glycine IR in the mammalian retina has also been extensively studied. Immunostaining for glycine of the rat retina revealed the presence of the neurotransmitter in cells of the ventricular zone and the retinal pigmented epithelium (RPE) from birth. These cells extended processes to the neuroblastic layer of the outer retinal edge, indicating that they may be developing bipolar or amacrine cells. IR positive cells were seen migrating through the ventricular zone by day postnatal day 4 (P4), when subpopulations of glycine positive amacrine cells were first observed in the inner plexiform layer (IPL). Over the next 2 days the amacrine cells of the IPL became less diffuse at the IPL borders and expression decreases in the RPE. Near adult expression is finally achieved by P11 (Fletcher and Kalloniatis, 1997).

Glycine IR has also been examined in chick embryos with positive staining first observed on E8 in the dorsal and ventral grey matter of chick embryos. The number of positive cells rises sharply 2 days later and reaches near adult expression by day 12. The majority of the IR positive neurons were observed in the dorsal horn lamina IV, V and VI. Glycine positive cells were also observed in the lamina VII and diffusely distributed throughout the spinal grey matter. In chick embryos, similar to mammals, GABA appears 4 days earlier than glycine, and is observed in the ventral horn and does not share the same distribution as glycine IR cells in the spinal grey matter (Berki et al., 1995).

Amphibian embryos have served as a classic model system for examining the development of the glycinergic phenotype (Roberts et al., 1988). In *Xenopus laevis* the first glycine positive IR cells appear in the rostal spinal cord and caudal hindbrain intermediate between ventral and dorsal at stage 22, a few hours after the neural tube closes. Glycine expression extends caudally while the more rostral regions in-fill as the embryo matures. At stage 25 only unipolar glycine IR cells were observed, but axons and growth cones can be easily visualized by stage 27. Neuron numbers can be accurately quantified up to stage 27, after which time the concentration of glycine positive cells can exceed 30 per 100 μm. By stage 37 (swimming tadpole stages), glycine IR in the spinal cord is mostly dorsal, with clear axons descending ventrally (Roberts et al., 1988).

Because of its unique phylogenetic position as a jawless fish, lamprey has been the focus of comparative expression studies for neurotransmitter markers including glycine. Glycine IR was first observed in the caudal rhombencephalon at E5, followed by the diencephalon of early prolarvae and then the mesencephalon by P15. Within these areas, glycine immunostaining occurred in CSF-c cells of the preoptic nucleus and postoptic-buberal cell group, post-optic commissural region, ventral and dorsal thalamus, pretectum, and M5 nucleus of Schober. IR was seen in the isthmic, trigeminal, and octaval levels of the ventrolateral neuropil and the vagal region of the caudal rhombencephalon. At this late prolarval stage, glycine was observed in the proptic nucleus, hypothalamus,

thalamus, pretectum and the nucleus of the medial longitudinal fascicle, mesencephalon, isthmus and rhombencephalon. Later in the true larvae stage, glycine IR was found in olfactory bulb, preoptic nucleus, oculomotor nucleus, dorsal isthmic gray and isthmic reticular formation. GABA and glycine appear to co-localize in the M5 nucleus, reticular formation, and dorsal column nucleus during larval stages (Villar-Cervino et al., 2009). In the lamprey retina, glycine IR is first seen in the inner plexiform layer during early metamorphosis stages (M1). Near-adult expression, including IR in the inner and outer plexiform layers, inner and outer nuclear layers and the horizontal cell layer, is seen by late transformation stages (M6) (Abalo et al., 2008). Many taxa and areas of the CNS remain to be assayed for glycine IR, making comparing expression patterns between organisms difficult. The data currently available in mice, chick and frogs suggests that glycine first appears in the spinal cord after GABA across all three taxa. In the retina, on the other hand, differences in the life cycles of mice and lamprey, the two species assayed for glycine IR in the retina, prevents much comparison. The lamprey undergoes a maturation process where they can remain functionally blind larvae for up to 8 years before adulthood (Villar-Cervino et al., 2009).

GLYCINE TRANSPORTERS

Given the somewhat problematic nature of distinguishing between the amino acid glycine present in all cells and the low levels of neurotransmitter-specific glycine at early stages of development using immunohistochemistry, detection of glycine transporter expression at the mRNA and/or protein level has served as a valuable tool for analyzing the development of the glycinergic phenotype. GlyT1 and GlyT2 immunostaining are observed earliest in the mouse and rat at E10 in the midbrain floor plate and E12, respectively. By E17 two areas of GlyT1 expression are visible at the borders between the thalamus and hypothalamus and the border between the dorsal thalamus and zona incerta. GlyT2 levels rise in the ventral white matter of the spinal cord at E14 and then in the dorsal funiculus, reticular formation, spinal trigeminal nucleus, thalamus, cerebellum, colliculi and central tegmental tract at E17 (Jursky and Nelson, 1996). A study focusing on the auditory system observed GlyT2 IR develop from no staining at E18 to positive staining in all brainstem nuclei by P4. Next perisomatic and increased punctate signal developed in all brainstem nuclei except the inferior colliculus and medial geniculate body. Immunostaining decreased across the auditory system after P10 and adult expression levels were achieved within 4 weeks of birth (Friauf et al., 1999). In the rat spinal cord GlyT1 is first observed at E11 in the ventral ventricular zone. Immunostaining for VIAAT (VGAT) in the mouse retina confirms that glycinergic neurons appear in a similar pattern to the rat retina. IR occurs in the inner retina by P1, the outer retina by P3 and P5 in mouse and rat respectively, and adult expression in both by P11 (Johnson et al., 2003).

In lower vertebrates glycine transporters have generally been analyzed at the mRNA rather than the protein level. Higashijima et al. (2004) first observed glycinergic neurons in the zebrafish *Danio rerio* via *in situ* hybridization against GlyT2 in the mediodorsal domain of the rostral spinal cord 20-h post-fertilization (hpf). These results are consistent with the first appearance of rhythmic firing in interneurons of the dorsal spinal cord believed to be caused by glycinergic neurons. Brain expression was not observed until 3-day postfertilization (dpf), where a stark line is observed in the rostral hindbrain near rhombomeres 1 and 2. Some very weak expression is also seen in the ventral midbrain at this stage. Near complete expression was observed by this group by four dpf.

The other glycine transporter, GlyT1, is expressed in the hindbrain and spinal cord of the developing zebrafish in a non-overlapping pattern. In Situ hybridization using GlyT1 specific probe reveals that the transporter is expressed in the rostral spinal cord by at least 18 hpf. By 24 hpf GlyT1 expression is observed in five dorsal stripes in the hindbrain and along the dorsal midline of the caudal hindbrain and spinal cord. By the next day (48 hpf) the transporter is observed along the dorsal midline of the midbrain, hindbrain and spinal cord and the floor plate cells of the hindbrain and spinal cord. At 72 hpf hindbrain expression spreads but remains mostly dorsal and superficial. The strongest signal in the hindbrain at this point is along the ventricle border. The medial, floor plate, and dorsal midline cells of the spinal cord also begin to express GlyT1 at this point (Cui et al., 2005).

Expression studies in *Xenopus laevis* have revealed the appearance of GlyT1 first in the proliferative ventricular zone and intermediate zone of the midbrain, the ventricular layer of the hind brain, and the intermediate region of the anterior spinal cord during early tailbud stages (stage 24). By the time the tadpole can swim GlyT1 appears in the forebrain, retina, somites and blood islands. GlyT2 and VIAAT are initially expressed earlier than GlyT1 in the anterior spinal cord of the late neurula (stage 21). By stage 33 GlyT2 is found in the lateral forebrain, medial midbrain and mid-dorsal region of the spinal cord. VIAAT is expressed more widely slightly earlier (stage 27). Expression for the vesicular transporter is observed in the ventral and lateral regions of the telencephalon, the dorsal, ventral, and midpoint of the dorsal–ventral axis in the diencephalon and the intermediate zone of the spinal cord. VIAAT mRNA was also detected in ventral Kolmer-Agduhr cells of the spinal cord (Wester et al., 2008). Other investigators have shown that GlyT1 and GlyT2 are expressed in the ganglion and inner nuclear layers of the frog (*Rana pipiens*) and rat retinas (Pena-Rangel et al., 2008).

GLYCINE RECEPTOR

While no systematic analysis of all four GlyRα subunits and the β subunit has been conducted, there are several reports using immunohistochemistry and *in situ* hybridization for various subunits in some model species. Early immunostaining for GlyR in the rat supports glycinergic expression elucidated through glycine IR. The α1 subunit is first seen in the rat spinal cord at E14, after which time mRNA signal steadily increases in the ventral and dorsal horns until leveling off at P15. In the brain α1 is detected at near adult by P5. At E15 the α2 subunit is expressed in the telencephalon, diencephalon, midbrain and the first layer of the cortex, and remains through early postnatal stages (P5). Layers I, II and IV are all strongly labeled by P5, and adult expression is achieved by P15 with the addition of staining to layer VI. The remaining GlyR subunit, α3, is not observed until relatively late in development (P5), but remains throughout life. Initially the colliculi of the reticular thalamic nucleus are significantly stained, followed much later by the cerebellum at P40. The α3 subunit is not heavily expressed in the spinal cord, but is seen in both ventral and dorsal horns between E19 and P20. The β

subunit of GlyR is first expressed at E14 in both the telencephalon of the brain and the ventral and dorsal horns of the spinal cord. Although adult expression is accomplished quickly after E14 in the spinal cord, brain expression of the β subunit is more dynamic. At E19 the first two layers of the cortex, septum, Ammon's horn of the hippocampal formation, thalamus and midbrain are stained. Adult expression, now including the VI layer of the cortex, entorhinal cortex, hippocampus and subiculum occurs by P15 (Malosio et al., 1991b). The postnatal rat retina shows GlyR expression in the neuroblastic layer, while GlyR in the adult is only observed in the inner nuclear layer (INL) (Sassoe-Pognetto and Wassle, 1997). Young and Cepko (2004) have also shown that GlyR and specifically GlyRα2 are expressed in retinal progenitor cells at birth. GlyR is differentially expressed in OFF cone bipolar cells, but not ON cone bipolar cells. GlyR with α1 subunits was observed uniformly on the dendrites of parasol and midget ganglion cells in the same study (Grunert, 2000).

In the developing chick retina immunoreactivity against GlyR does not appear in the IPL until E12. The adult GlyR subunit α1 is not observed until E16 when it is seen punctate at synapses, suggesting that subunit expression is variable over the course of development (Hering and Kröger, 1996).

Immunohistochemistry against GlyRα2 and GlyRα3 shows distinct expression in the adult *Xenopus laevis* retina, while GlyRα1immunostaining shows no expression. GlyRα2 expression is observed mostly in the IPL, consisting of a thin, horizontal distal band and a broader, proximal horizontal band. Some scattered puncta are also observed in the INL. Similar patterns are seen with GlyRα3 immunostaining in the IPL, although scattered puncta are also detected in the OPL (Vitanova, 2006).

Although no detailed immunohistochemistry or *in situ* hybridization studies have been conducted for GlyRβ in Zebrafish, Hirata et al. (2005) did perform a series of RT-PCR assays for GlyRβ1 and GlyRβ2 over the course of embryonic CNS development. They found that GlyRβ1 is first expressed by the end of the first day of development, while GlyRβ2 is expressed first at day 3. GlyRβ1 is expressed in repeating bilateral clusters of cells in the hindbrain extensively expressed in the spinal cord at the end of the first day, with expression increasing and expanding through the second day (Hirata et al., 2005). *In situ* hybridization for GlyRα2 mRNA revealed expression at day one, but no expression at day two in optical sections of the spinal cord. GlyRα1 is also expressed in the spinal cord during the first day of embryonic development, but then increases in intensity in the spinal cord by day two (McDearmid et al., 2006). mRNA for the GlyRα2 subunit was observed in the outer nuclear layer of the adult zebrafish retina, but studies have not been conducted for these mRNAs more widely throughout the developing organism (Imboden et al., 2001).

COLOCALIZATION AND COMPARATIVE EXPRESSION PATTERNS

The co-localization and comparative expression kinetics of glycine and GABA present an under examined dimension of glycine's role in development. In general, the expression of glycinergic markers mirrors that of GABAergic markers. GABA tends to appear earlier and reach mature expression earlier than glycine, while glycine occasionally replaces GABA as the predominant inhibitory neurotransmitter (Allain et al., 2006). Immunostaining in the rat brain for GABA and glycine, for example, revealed a stark transition from GABA to glycine neurotransmission in the lateral superior olive. Co-expression of both GABAergic and glycinergic markers also change during development. In the mouse hypoglossal nucleus, for instance, 30% of GlyR positive cells are also IR for γ2GABA$_A$R at E17, a statistic that increases to 50% by P30 (Muller et al., 2004). The opposite transition appears in the lamprey, presenting one of the most significant differences in expression yet observed among chordates (Villar-Cervino et al., 2009). Both glycinergic and glutamatergic neurons are associated with rhythmic firing patterns, and as expected are both observed in the same areas of the developing zebrafish spinal cord, albeit not lo-localized on the cellular level. Before 30 hpf GlyT2 and the GABAergic marker glutamic acid decarboxylase (GAD) are co-localized in the majority of IR positive neurons for either marker. After 30 hpf most neurons become either glycinergic or GABAergic (Higashijima et al., 2004). Glutamate appears to be dominant in immature P6 amacrine cells, while glycine IR is observed in most adult amacrine cells (Fletcher and Kalloniatis, 1997). In the rat spinal cord synaptic co-localization of GlyR and GABA receptors occur at numerous layers. Gao et al. (2001) suggest that this redundant expression, combined with the observation that these amino acids are stored in the same synaptic vesicles, indicates a synergistic function of the two neurotransmitters that could help fine tune synaptic integration. In the rat retina, GABA and glycine immunostaining reveals co-localization in a minority of amacrine cells (Fletcher and Kalloniatis, 1997). Todd et al. (1996) take the question of co-localization a step further and examined the relationship between presynaptic glycine and GABA, and postsynaptic GlyR and GABA receptors in the rat spinal cord. This group reports that the observed co-IR for presynaptic glycine and GABA and postsynaptic gephyrin (as a marker for GlyR) and GABA receptors supports GABA and glycine cotransmission. Studies on GABA receptors alone, however, reveal no relationship between presynaptic input and aggregation of GABA receptors in rat hippocampal cells or cerebellar granule cells (Nusser et al., 1996, 1998; Kannenberg et al., 1999; Rao et al., 2000).

Taken together, these numerous studies of the expression patterns of glycinergic neurons in the developing vertebrate nervous system demonstrate similar emergence across lineages (see **Table 1**). Glycinergic neurons tend to appear during embryonic development in the rostral spinal cord, followed by increased expression caudally down the spinal cord and rostrally into the hindbrain, midbrain and retina. Although there are indications of differential regulatory mechanisms even between closely related species (Jursky and Nelson, 1996), this general pattern is maintained. These observations suggest that, despite small changes in the mechanisms organizing the distribution of glycinergic neurons, these neurons likely perform similar functions during the formation of later neural networks.

GLYCINE IN INVERTEBRATES

It was long believed that glycinergic neurotransmission was an almost purely vertebrate phenomenon. Walker et al. (1996) reported that no evidence has been found for glycinergic transmission in the nematodes, arthropods or annelids. Until relatively recently it was thought that the cnidarians lacked the ability to use

Table 1 | Summary of embryonic and early postnatal expression patterns for glycinergic markers.

Region	Lamprey	Zebrafish	Frog	Chick	Mouse	Rat
Spinal cord	N/A	**GlyT2:** mRNA rostral spinal cord 20 HPF; rostral 2/3rds spinal cord 4 DPF	**Glycine IR:** rostal SC, caudal HB, St. 22 **GlyT2:** mRNA rostral SC, St. 21 **VIAAT:** mRNA rostral SC, St. 21	**Glycine IR:** Parikarya (E8); Increase at E10	**Glycine IR:** dorsal and ventral horn (E12.5); adult levels by E16.5 **GlyT1:** mRNA in ventral ventricular zone (E11)	**GlyT1:** mRNA ventral ventricular zone E11; adult levels soon after P0 **GlyRα1:** mRNA ventral+dorsal horns E14 **GlyRα3:** mRNA ventral+dorsal horn E19 through P20 GlyRβ: mRNA ventral+dorsal horns E14
Brain	**GlycineIR:** caudal rhombencephalon E5; prosencephalon P2; diencephalon and mesencephalon, P15; widespread in true larval through adult stages.	**GlyT2:** mRNA rostral HB and ventral MB 3DPF	**GlyT1:** mRNA MB+HB/HB St. 24; FB+MB+HB St.27 **GlyT2:** mRNA MB+HB/HB St.31; FB+MB+HB low levels St. 33 **VIAAT:** mRNA MB+HB/HB St. 24; FB+MB+HB St.27	N/A	**GlyT1:** IR midbrain floor plate E10; borders of thalamus/MB+HB E17; adult levels after P5 **GlyT2:** IR borders of thalamus/ MB+HB E17; all brainstem nuclei by P4; adult levels after P5	**GlyRα1:** mRNA near adult by P5 **GlyRα2:** mRNA telencephalon, diencephalon, MB, cortex P5 **GlyRα3:** mRNA colliculi of RTN P5, cerebellum P40 **GlyRβ:** mRNA FB, MB E19
Retina	**Glycine IR**: IPL, M1; ONL, OPL, HCL, INL, IPL, M6	**GlyRα1**: mRNA ONL of adult	**VIAAT:** mRNA INL St.27 **GlyT1:** mRNA INL, ONL **GlyT2:** mRNA INL, ONL	**GlyR:** IR IPL E12	**VIAAT:** IR inner retina P1; outer retina P3; adult by P7	**Glycine IR:** ventricular zone and RPE/RPC P0; neuroblastic layer P4; amacrine cells P6; adult levels P11 **GlyT1:** mRNA INL, ONL adult **GlyT2:** mRNA INL, ONL adult **VIAAT:** IR inner retina P1; outer retina P5; adult by P7 **GlyRα1:** IR ganglion cells adult **GlyRα2:** mRNA ventricular zone + RPE/RPC + IR neuroblastic layer P0; only INL in adult

Summary of embryonic and adult expression patterns of glycinergic markers.
SC, spinal cord; HB, hind brain; IR, immunoreactivity; RPE, retina pigment epithelium; ONL, outer nuclear layer; OPL, outer plexiform layer; HCL, horizontal cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; RTN, reticular thalamic nucleus; M, metamorphosis stage; HPF, hours postfertilization; DPF, days postfertilization; P, postnatal; E, embryonic.

glycine as a neurotransmitter (Grimmelikhuijzen et al., 2002). It is now suspected that many phyla from the simple hydra to our own primate lineage use similar classical neurotransmitters (including glycine) that first evolved 1000 million years ago (reviewed in Walker and Holden-Dye, 1991; Walker et al., 1996). Some notable exceptions include nematodes and insects, neither of which have functional glycine receptors and instead use GABA or glutamate to mediate inhibitory neurotransmission (Ui-Tei et al., 1995; Bargmann, 1998). Although evidence for functional GlyRs in these groups has yet to appear, *Drosophila* appear to possess 25–30 genes for GABA/glycine receptors and *C. elegans* appear to possess 10 receptor genes (Yoshihara et al., 2001). In 2001 the first cnidarian glycine receptors were identified and characterized via biochemical and behavioral studies in *Hydra vulgaris*. These receptors were found to mirror the function of vertebrate glycine receptors; they are sensitive to both glycine and taurine, which can displace previously bound strychnine (Pierobon et al., 2001). Tino et al. (2005) began the process of cloning and expressing *H. vulgaris* glycine receptors. Glycine also appears to play a role in the coelenterate pacemaker system, where

it functions to inhibit endodermal pacemakers through glycine receptor binding and excite the pacemakers through NMDA receptor interactions (Ruggieri et al., 2004). In the adult glycinergic neurotransmission has been implicated in mouth opening during feeding (Pierobon et al., 2001, 2004). Kehoe et al. (2009) used sequence data from *Aplysia* 2-cys-loop gated chloride ion channels from *Aplysia*, a member of the platyhelminthes lineage to suggest that glycinergic neurotransmission may occur in this group. Glycinergic neurotransmission among invertebrates overall is poorly understood, and many more phyla must be examined before a complete picture of the evolution of this neurotransmitter can be developed.

DETERMINATION OF THE GLYCINERGIC PHENOTYPE

While expression pattern data has provided essential information on when the glycincergic phenotype emerges during embryonic development, the molecular mechanisms governing the process remain poorly understood. The glycinergic neurotransmitter phenotype requires the expression of the transporters GlyT2 and VIAAT for proper neurotransmission (reviewed in Betz and Laube, 2006). In part this is attributable to the fact that the glycinergic phenotype is so closely associated with the GABAergic phenotype. Both types of neurons use the same vesicular transporter VIAAT to mediate accumulation of the neurotransmitter into synaptic vesicles. GlyT2, however, plays an important role in switching between specific neurotransmitter phenotypes. In fact, Aubrey et al. (2007) observed increased release of glycine in neuroendocrine serotonergic BON cells made to express one of the two glycine transporters in a series of experiments using a novel double sniffer patch clamp technique. The neural glycine transporter GlyT2 also appeared to be more efficient than the glial glycine transporter GlyT1. By using *Caenorhabditis elegans*, a species which does not use glycinergic neurotransmission, these investigators were able to show that increasing cytosolic glycine plays an important role in switching neurotransmitter specification.

The specificity of the GlyT2 for the neuronal glycinergic phenotype provides another approach for analyzing the molecular mechanisms mediating the specification of this neurotransmitter phenotype, namely "working backwards" employing a reverse genetic approach through analysis of the upstream regulatory regions of the gene. The regulatory mechanisms of these terminal differentiation genes have been partially elucidated in some model species. Most of the work on the transcriptional regulation of GlyT2 has been conducted in the mouse and rat (Adams et al., 1995; Ponce et al., 1998; Ebihara et al., 2004). Ponce et al. (1998) report three GlyT2 transcriptional isoforms in the rat and Ebihara et al. (2004) report three other GlyT2 transcriptional isoforms in the mouse, each driven by an alternative promoter. GlyT2a is five amino acids shorter than the GlyT2b isoform at the N-terminal, and is able to accumulate glycine in COS. The GlyT2b isoform appears to only be able to exchange glycine. Zeilhofer et al. (2005) have developed transgenic mice with EGFP expression glycinergic neurons by using a BAC vector with 105 kb of 5' flanking DNA and 21 kb of 3' flanking DNA from the GlyT2 gene. To date no detailed functional studies of the GlyT2 promoter have been conducted in any model organism.

Another promising approach for unraveling the mechanism of glycinergic specification has been an analysis of various transcription factors whose expression correlates with that of GlyT2, in particular Ptf1a, Lbx1 and Pax2 (**Figure 1**). Investigations of Ptf1a, Lbx1, and Pax2 by Huang et al. (2008) were used to generate a model of the relationship between these three transcription factors in the dorsal spinal cord. Experiments on null mice embryos for combinations of these factors show that all three factors are necessary for expression of the glycinergic phenotype. Developing dorsal horn neurons in Ptf1a null embryos fail to express GlyT2 from E12.5 through E16.5 and $Pax2^{-/-}$ mice also fail to express GlyT2 properly from E12.5 through E18.5. GlyT2 production was observed, albeit at reduced intensity in Lbx1 homozygous negative mice. While these transcription factors clearly play a powerful role in the developing dorsal horn they did not appear necessary in the ventral horn. Overexpression experiments of these transcription factors also resulted in the development of ectopic inhibitory neurons producing either glycine or GABA. These studies also showed that Lbx1 alone is able to induce switching from GABAergic to glycinergic specifications. According to these authors Ptf1a appears to act as a master regulator of developing inhibitory neurons of the dorsal spinal cord, and can act in conjunction with Lbx1 to activate Pax2 as a downstream target. Pax2, in turn, has been shown to activate transcription of GlyT2 and other differentiation targets. Ptf1a has also been shown to play a role in the development of GABAergic and glycinergic cells in other areas of the CNS, including horizontal and amacrine cells of the mouse retina and glycinergic neurons of the cochlear nucleus. Recombination-based lineage tracing suggests that the transcription factor is expressed in precursors of both retinal cell types. Inactivation of Ptf1a prevents differentiation of horizontal and amacrine cells during retina development (Nakhai et al., 2007). Ptf1a null mouse embryos fail to develop inhibitory GABAergic and glycinergic neurons and Atoh1 null embryos fail to develop excitatory glutamatergic neurons in the cochlear nucleus(Fujiyama et al., 2009).

Batista and Lewis (2008) have also examined the role of Pax2, including both of its isoforms (Pax2a and Pax2b) and Pax8 in glycinergic differentiation. They showed that expression of each of any of these three transcription factors leads most often to adoption of the glycinergic phenotype. Morpholino knockdown of these transcription factors supported the redundant role of Pax2a/b and Pax8, as the glycinergic specification is not lost unless all three transcription factors are knocked down. Lhx1 and Lhx5 are expressed in inhibitory interneurons of the dorsal spinal cord and have been shown to increase expression of Pax2. Knockdown of Lhx1 and Lhx5 results in decreased expression of VIAAT, although they do not appear to be obligatory determinant of inhibitory neural phenotypes and appear to play no direct role in determining GlyT2 expression (Pillai et al., 2007). Neuron restrictive silencing factor has been shown to act as a repressor of the GlyRα1 subunit through binding to the 5' region of the GlyRα1 mRNA in studies of small-cell lung cancer cell-derived cell lines (Gurrola-Diaz et al., 2003; Neumann et al., 2004).

Specification of glycinergic amacrine in the retina appears to involve the homeobox gene Barhl2. Immunostaining of the developing retina for the transcription factor by Mo et al. (2004) revealed positive IR in both postmitotic amacrine and ganglion cells. Forced

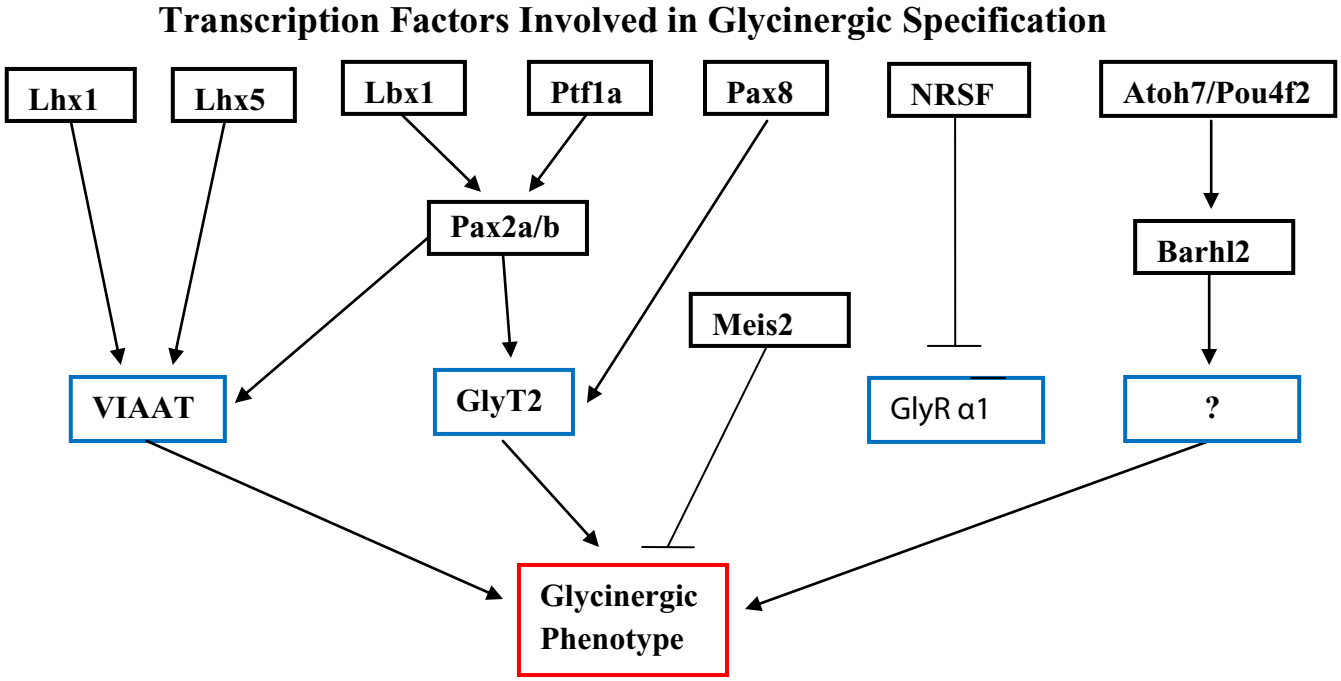

FIGURE 1 | A model for the roles of transcription factors Ptf1a, Lbx1, Pax2a/b, Pax8, Lhx1, Lhx5 and NRSF in regulating terminal differentiation genes of glycinergic neurons based upon current literature cited in text. Lbx1 and Ptf1a act together to control Pax2-dependent expression of VIAAT and GlyT2. Pax8 acts redundantly with Pax2 on GlyT2, while Lhx1 and Lhx5 also up-regulate VIAAT. NRSF down regulates the α1 subunit of GlyR and does not appear to act on either GlyT2 or VIAAT. Atoh7 and Pou4f2 both act upstream of Barhl2. The exact mechanism of this interaction is unknown, as is the target of Barhl2.

expression of Barhl2 resulted in more frequent differentiation of retinal progenitor cells into amacrine or ganglion cells, while dominant-negative studies by the same group yielded fewer amacrine and ganglion cells. No changes in GABAergic cell populations were seen during these experiments by Mo et al. (2004) but a later group (Ding et al., 2009) reported changes in GABAergic populations that mirrored changes in glycinergic populations when Barhl2 levels were experimentally modulated. Atoh7 and Pou4f2-null retina experiments caused down-regulation of Barlh2, suggesting these two factors act upstream of Barlh2 (Ding et al., 2009). Bumsted-O'Brien et al. (2007) demonstrate that another transcription factor, Meis2, may also be involved in glycinergic specification of mouse and human amacrine cells; cells that adopt a glycinergic fate repress Meis2 while GABAergic cells maintain high Meis2 levels, a result which suggests that Meis2 may be a repressor of glycinergic fates.

Over the past decade an increasing body of evidence has accumulated suggesting that neurotransmitter specification may be influenced by activity based mechanisms and environmental factors. It has been suggested that Ca^{+2} transients may influence the adoption of a neurotransmitter phenotype (reviewed in Spitzer, 2006). Certain patterns of Ca^{+2} spiking and frequency have been correlated with glutamate, glycine, GABA and acetylcholine neurotransmitter phenotypes. Increased calcium activity is associated with the inhibitory neurotransmitters, while high activity is associated with excitatory. Glycine receptors are found on embryonic *Xenopus laevis* muscle cells, but expression decreases during the course of normal development. If calcium transients are experimentally altered so as to increase the prevalence of glycinergic neurons, glycinergic currents can be detected on muscle cells (Borodinsky and Spitzer, 2006). There is even some evidence that calcium activity can override some other genetic differentiation signals, as HNK-1 or lim-3 positive cells could be induced to adopt the GABAergic or glycinergic phenotype by experimental inhibition of calcium transients (Spitzer et al., 2004).

THE ROLE OF NONSYNAPTIC GLYCINE DURING DEVELOPMENT

GlyR subunits α2 and β are expressed very early in the developing rat cerebral cortex (E14-P5) (Malosio et al., 1991b). While glycine levels in the cerebral cortex at this time are thought to be too low to allow normal neurotransmission (van den Pol and Gorcs, 1988; Zafra et al., 1995), the amino-sulfonic acid taurine is highly expressed and was hypothesized to act as a ligand for the receptor (Smith et al., 1992). Flint et al. (1998) found evidence supporting the hypothesis that taurine functions as a ligand for GlyR via nonsynaptic signaling in the early neocortex. Indeed, kittens deprived of prenatal taurine develop smaller brains that fail to differentiate normally (Palackal et al., 1986). Since 1998 evidence for nonsynaptic taurine-GlyR signaling has been found in the rat auditory cortex, hippocampus and dopaminergic neurons of the ventral tegmentum and most recently in the spinal cord (Mori et al., 2002; Wang et al., 2005; Scain et al., 2010). Mori et al. (2002) observed that pressure application of glycine onto hippocampal CA3 pyramidal cells exposed to antagonists for ionotropic

glutatmate and $GABA_B$ caused currents. These responses were associated with increased chloride conductance and could be halted by treatment with the glycine receptor antagonist strychnine. Together these results suggest the presence of functional GlyR in the early hippocampus, yet no evidence was found connecting these strychnine-sensitive chloride currents to synaptic stimulation. Mori et al. (2002) also found that the application of the β-alanine and taurine uptake inhibitor guanidinoethanesulfonic acid indicated that the modulation of these transporters could regulate tonic GlyR activation. Studies of rats aged 1–13 days found more evidence of nonsynaptic glycine signaling in dopaminergic neurons of ventral tegmentum acute brain slices. Currents were observed in current clamped neurons after the application of 0.01–0.03 mM taurine via interaction with non-synaptic GlyRs (Wang et al., 2005), providing additional support for non-synaptic or "preneural" glycinergic transmission. Outside of the brain, nonsynaptic glycine signaling was recently observed in the radial cells of the spinal cord using an outside-out sniffer technique measuring glycine release between E12.5 and E14. This signaling is attributed to glycine itself and not taurine, which is was not observed in immunostained radial cells at this stage of development. Radial cells were also observed to release glycine upon mechanical stimulation and to produce spontaneous rhythmic behavior in nearby immature neurons. To fully prove the existence of a nonsynaptic glycine signaling system endogenous glycine release must be demonstrated in addition to expression of functional GlyR (Scain et al., 2010).It must also be noted that the studies in support of nonsynaptic glycine signaling are limited due to the pharmacological tools used in the studies. Guanidinoethanesulfonic acid, for example, blocks taurine transporter and is a $GABA_A$ receptor agonist, while sarcosine blocks GlyT and serves as a co-agonist for NMDA receptor and a full agonist for GlyR (Mangin et al., 2002; Zhang et al., 2009a,b). While the role of non-synaptic glycine signaling remains somewhat unclear, if not controversial, there is compelling evidence demonstrating a determinative role for glycinergic signaling during the early stages of synapse and network formation in the spinal cord, brain, and retina.

THE ROLE OF GLYCINE IN NEURAL DEVELOPMENT

THE ROLE OF GLYCINE IN SPINAL CORD DEVELOPMENT

Glycinergic neurotransmission develops first in the spinal cord. Immature neural circuits of the developing spinal cord can spontaneously generate synchronous rhythmic activity patterns that can be classified as central pattern generators (CPGs) if they include multiple processes that act in an ascending and descending manor that successfully returns to a starting point. CPGs are often associated with rhythmic respiration, swallowing, and early locomotion. These spontaneous bursts can be caused by several neurotransmitters, including the normally inhibitory neurotransmitters GABA and glycine. Experiments using the GlyR antagonist strychnine and L-type Ca^{+2} blockers on spinal neurons suggest that GlyR activation leads to Ca^{+2} transients that in turn cause accumulation of the anchoring protein Gephyrin and GlyR. Taken together these results support an activity-driven model for synapse formation (Kirsch and Betz, 1998). Young neurons, which do not yet express the K+/Cl– co-transporter KCC2, typically have higher internal chloride ion concentrations when compared to the exterior of the cell, so activation of glycine dependent chloride channels results in depolarization instead of hyper-polarization observed in mature neurons (Reichling et al., 1994; Rivera et al., 1999). In some instances GABA and glycine have been shown to be active at the same synapse (Singer and Berger, 2000; Gao et al., 2001).

GlyR has been implicated in regulating interneuron differentiation in the zebrafish spinal cord. Targeted knockdown of the GlyR embryonic subunit α2 resulted in disruption of rhythm-generating networks and an increase in the number of mitotic cells (McDearmid et al., 2006). Studies using GlyR and $GABA_A$ receptor antagonists found that GABAergic and glycinergic synapses each have unique roles in modulating locomotion rhythms. Glycine functions to stabilize patterns of alternating rhythms (Hinckley et al., 2005). Although GABA may have a stronger role in the maturation of locomotor circuitry, glycine becomes the main neurotransmitter responsible for mediating reciprocal coupling between mature antagonistic motor centers in the mouse spinal cord (Sibilla and Ballerini, 2008).

Glycine transporters have also been shown to play an important role in CPG development. Hanson and Landmesser (2004) used strychnine to block or slow spontaneous rhythmic firing produced by glycinergic neurons. They showed that spontaneous bursting activity is necessary for motoneurons to make their first dorsal/ventral pathfinding decisions, perhaps due to differential expression of EphA4 and polysialic acid on NCAM. Later the same group (Hanson and Landmesser, 2006) used sarcosine to block GlyT1 activity and modulate spontaneous rhythmic firing. These investigators found that altering burst frequency disrupted dorsal/ventral and anterior/posterior pathfinding decisions of lumbosacral motoneurons. Mutant motor phenotypes such as mutant EphA4 and ephrinB3 mice are also being utilized to shed light on normal neural development in the spinal cord. Addition of sarcosine returned the mutant synchronous activation pattern back to normal alteration, thus indicating that the balance between excitation and inhibition over the midline was shifted toward excitation in mutants (Kullander et al., 2001, 2003).

One of the most therapeutically interesting aspects of glycine neurotransmission in the spinal cord is its role in the development of nociception. Proper formation of pain perception pathways requires $GABA_A$ receptors and GlyRs in the dorsal horn. It has been shown that reducing GlyR synaptic inhibition can lead to hypersensitivity to pain (Ahmadi et al., 2002; Coull et al., 2003). Harvey et al. (2004) have shown that GlyRα3, which is expressed in the superficial laminae of the mouse dorsal horn, plays a pivotal role in inflammatory pain sensitization by demonstrating that prostaglandin E_2-induced synaptic inhibition is completely abolished in GlyRα3–/– mice. Although most inhibitory synapses are GABAergic in rodent neonates, the balance shifts in favor of glycinergic synapses during maturation (Baccei and Fitzgerald, 2004). GlyR and the glycine transporters GlyT1 and GlyT2 are currently being investigated as pharmacological targets for the treatment of pain disorders (reviewed in Dohi et al., 2009).

THE ROLE OF GLYCINE IN BRAIN DEVELOPMENT

The role of glycinergic neurotransmission in the brain presents a more complex story that is far from fully understood. Although certain areas have seen heavy research, such as the lateral superior olive, many have yet to be pursued in depth. Development of

respiratory neural circuits has long been known to involve inhibitory neurotransmission and glycine in particular. Although it is unclear if glycine is absolutely required for the generation of basic breathing rhythms in neonate rodents, there is strong evidence that glycine plays a role in modulating and patterning. Respiratory rhythms are not abolished by application of glycine or GABA antagonists or alteration of extracellular chloride ion concentrations (Onimaru et al., 1990). Experimentally introduced exogenous glycine can, however, augment the frequency and amplitude of respiratory rhythms. Conversely, mice without functional GlyT1 are unable to breathe properly at birth and die within the first day. The strong connection between glial glycine and respiration may be mediated via suppression of NMDA receptor function, although experimental evidence in support of this hypothesis has yet to be gathered (Gomeza et al., 2004). The investigation of the exact role of glycinergic neurons in the development of this system has been confusing and contradictory. Some investigators have reported that glycine causes hyperpolarization and depression of respiration in the neonatal spinal cord and brainstem, which is surprising given the lack of KCC2 channel expression before E19 in rats to form the [K+] necessary to drive such activity (Brockhaus and Ballanyi, 1998; Ren and Greer, 2006). Others have found that glycine causes depolarization of neurons involved in respiration in the developing medulla (Ritter and Zhang, 2000). Ren and Greer (2006) have proposed that these discrepancies may be due to differences in $[K^+]_0$ used in each of these studies.

The developing hippocampus appears to utilize excitatory glycinergic signaling for proper neural network formation. The adult inhibitory neurotransmitters GABA and glycine have been implicated in giant depolarizing potentials (GDPs) in the hippocampus in studies on rats, rabbits and rhesus macaques. GDPs are associated with changes in intracellular Ca^{+2} transients, especially the CA3 region. Observation of GDPs in hippocampal slices from these species suggests that glycine may play an important role in hippocampal network formation across mammal lineages (Prida et al., 1998; reviewed in Ben-Ari, 2001).

The central auditory system is one of the best characterized examples of glycinergic neurotransmission in the developing mammalian brain. The lateral superior olive (LSO) is one of the first stations in the ascending auditory pathway. The LSO receives excitatory inputs from the cochlear nucleus and inhibitory inputs from the medial nucleus of the trapezoid body in the adult. From fetal stages through postnatal day 7 in gerbils and rats glycinergic transmission results in excitatory postsynaptic potentials. This period of dual excitatory input occurs during an important stage of neuronal growth and strengthening of dendritic arbors (Sanes and Friauf, 2000). A growing body of evidence is connecting this period of glycine induced depolarization with LSO network maturation via modulating intracellular Ca^{+2} concentrations (Malenka and Nicoll, 1993; Sanes and Friauf, 2000). Kandler et al. (2002) have demonstrated that glycinergic inputs of the LSO at this stage can induce increased Ca^{+2} concentrations and action potentials using calcium imaging techniques. More recently Kullmann and Kandler (2008) showed that subthreshold synaptic responses result in local changes in Ca^{+2} concentration, while suprathreshold responses elicit global Ca^{+2} modulations throughout the dendrites of the postsynaptic cell. Seven days after birth glycinergic neurons adopt adult inhibitory neurotransmission. This new stage again mirrors a change in the maturation of LSO neural networks; synaptic pruning. It is hypothesized that the change in the survivability of dendrite arbors is connected to the change in glycinergic transmission.

The theme of postsynaptic potential switching during neural circuit modeling is observed in numerous instances over the course of development. Glycinergic neurotransmission has been shown to influence neural maturation via similar mechanisms in the respiratory brainstem nuclei, hippocampus, and the LSO of the auditory system (Ben-Ari, 2001). It is unclear how many other neural pathways glycinergic signaling may influence (Young-Pearse et al., 2006).

THE ROLE OF GLYCINE IN RETINAL DEVELOPMENT

Glycinergic transmission and GlyR mediated taurine signaling have been shown to play important roles in embryonic and early postnatal retinal development. GlyR mediated taurine signaling has been implicated in directing the proliferation of rod photoreceptor cells, while glycinergic transmission has been shown to be important to light-dependent maturation of retinal ganglion cells and bipolar cells. Studies in chicks and rats have given varied results, indicating that the glycine's function in retinal development may not be well conserved between taxa (Berki et al., 1995; Sassoe-Pognetto and Wassle, 1997).

Overexpression of GlyR subunit α2 leads to the development of double the percentage of rod photoreceptors at the expense of Muller glial cells. Mutation studies of the gene for the GlyR β subunit had little effect on the outer retina, photoreceptors or bipolar cells. These results indicate the importance of the α2 subunit in taurine signaling. Inhibition of taurine uptake by guanidinoethylsulfate and introduction of exogenous taurine both resulted in an increased percentage of photoreceptor cells in the mature retina. It is unclear exactly how GlyR mediated taurine signaling directs rod photoreceptor cell differentiation. Since no increased cell death is observed in GlyRα2 over expression experiments, it is thought that GlyR mediated taurine signaling causes cells to exit the mitotic cycle, only to later adopt the rod photoreceptor phenotype. Taurine's ability to directly induce rod photoreceptor cells regardless of mitotic state via GlyR activation remains untested. In addition to taurine signaling, it has been shown that rod photoreceptor cell differentiation requires a combination of GABA and glycine. The pharmacology of this interaction is yet to be determined (Sassoe-Pognetto and Wassle, 1997; Young and Cepko, 2004).

The role of glycinergic transmission in the development of ganglion cells has been investigated using a line of mutant mice known as *spastic*. These mice possess an insertion of the transposable element (LINE1) in the 5th intron for the β subunit gene of GlyR, causing aberrant mRNA splicing (Kingsmore et al., 1994; Mulhardt et al., 1994). Although mutations in this gene appeared to have few effects on photoreceptors or bipolar cells, the β subunit appears to be important in the development of retinal ganglion cells. Xu and Tian (2008) found that retinal ganglion cells failed to form properly in the center through the sublamina *a* of the inner plexiform layer of the retina. These investigators also observed the same pattern of mis-expression in normal mice raised in light-deprived conditions.

Together these results suggest that the mechanism connecting optic stimulation to the maturation of retinal ganglion cell connectivity is mediated via GlyR synaptic transmission.

CONCLUSIONS AND FUTURE DIRECTIONS

As is apparent from the literature cited in the previous sections, the amino acid glycine, which serves as an essential inhibitory neurotransmitter in the adult nervous system, also plays a myriad of important roles in neural development. While significant progress has been made in elucidating the various roles of glycinergic signaling during embryogenesis as well as the mechanisms by which neurons adopt this important neurotransmitter phenotype, recent findings have raised new questions and opened fresh avenues of research.

For example, one area that is particularly ripe for future study and potentially very informative for the field of glycinergic neurotransmission is an integrative "evo-devo" approach that combines expression data with analyses of upstream regulatory regions from an array of different species. The detailed analyses of the developmental expression patterns on the individual glycinergic phenotype markers have provided much of the necessary groundwork for future comparative studies. Nevertheless, to close the gaps in our knowledge, a systematic and comparative analysis of glycine IR and GlyT2, VIAAT, and the GlyR on both the mRNA and protein level in each of the major model organisms would be informative. Additionally, to date, there has been little developmental analysis of glycinergic markers in non-model systems, an endeavor that would provide increased comparative richness. However, for an integrated understanding, expression studies from a variety of species should be combined with functional analyses of the upstream regulatory regions that govern the expression of the various domains of each of the glycinergic markers. Currently there are virtually no detailed analyses of the regulatory regions controlling the expression patterns of any of the glycinergic marker genes. The availability of sequenced genomes from a variety of species and programs such as PipMaker allows investigators to conduct powerful *in silico* analysis to identify regions of conservation in non-coding regions among divergent species, regions that may serve as important evolutionarily conserved regulatory control regions (Elnitski et al., 2003). While not a substitute for *in vivo* transgenic analysis which demonstrates that a given stretch of DNA drives a specific realm of expression, *in silico* analysis provides a means to conduct global comparative genomic analyses and can potentially identify important conserved regulatory regions, even hundreds of kilobases up- or downstream of the coding region. For example, 5 kb of DNA upstream of the *X. laevis* GlyT2 gene was compared to upstream sequences from *X. tropicalis*, zebrafish, mouse and human *in silico* using PipMaker software. Three conserved non-coding (CNC) regions were observed 4.7 kb, 2 kb, and 200 bp upstream of the start site (unpublished data), suggesting a possible regulatory role. Understanding the regulation of "terminal differentiation" genes such as GlyT2 or VIAAT is a particularly interesting problem given that many genes associated with the differentiated phenotype display profoundly similar expression patterns across species, yet do not share significant sequence similarity in their regulatory regions. Although exact expression for glycinergic markers obviously differs among these organisms, the general pattern of expression timing and location is maintained between these groups. Further analysis of control regions of the glycinergic genes may shed light on this important issue in transcriptional regulation as well as on the evolution of glycinergic signaling. Recent work has also questioned the previous view that glycinergic signaling is unique to the vertebrate lineage. Cloning and sequence analysis accompanied by careful expression data on both the mRNA and protein level from organisms representing a variety of different phyla should resolve this question and also address the broader evolutionary issue.

While analysis of regulatory regions has the potential to identify novel factors not yet known to be involved in glycinergic specification, recent work has already identified a number of factors known to be involved in this process. Additional analysis along the lines of Batista and Lewis (2008), Huang et al. (2008), and Joshi et al. (2009) on how these individual factors interact in a network will be essential; the use of chip and high throughput sequencing technologies to conduct more global analyses of gene expression during glycincergic specification and following the inactivation of putative glycinergic regulatory genes will provide a more complete picture of the gene networks involved. However, these approaches must still be complemented by gene expression analysis at the single cell level, the level at which specification is actually occurring. Indeed, one of the difficulties of glycinergic phenotype specification is that many, if not all, of the identified factors also serve as GABAergic specification factors. This intriguing feature may also present a valuable clue to the underlying mechanism of determination, namely that cells initially co-express GABAergic and glycinergic determination factors as well as differentiation markers prior to selecting one or the other system, a hypothesis supported by co-expression data (Aubrey et al., 2007) and electophysiological data (Muller et al., 2006, 2008). Interestingly, the developmental kinetics of glycine closely mirrors GABA in most of the phyla in which comparative expression studies have been conducted, with glycincergic neurons often, but not always, developing slightly later. Additional colocalization studies of glycinergic and GABAergic phenotype markers (as well as other neurotransmitter markers) throughout development on the single cell level are clearly indicated. These studies would be particularly useful if they are complemented with electrophysiological analyses to determine if and when the gene expression correlates with functional synaptic signaling.

The relative roles of "hard-wired" transcriptional regulatory control versus activity-dependent mechanisms remain an important issue in glycinergic specification and in neurotransmitter phenotype determination generally. Recent work has demonstrated that neurotransmitter specification has an activity-dependent component in which high levels of activity as evidenced by calcium spiking is associated with the development of inhibitory phenotypes and lower levels correlated with the formation of excitatory phenotypes. Whether activity-dependent mechanisms or hard-wired molecular switches are responsible for glycincergic determination requires additional research.

Equally important is the function of glycinergic signaling during development. Clearly the presynaptic role remains controversial and understudied. Elucidation will require careful and sensitive expression assays for each of the components to ensure that the "machinery" is present, followed by rigorous functional

perturbation experiments in multiple species. A recent study showing that inhibitory and excitatory phenotypes may be mediated by presynaptic GABA lend support to a possible presynaptic role for glycine (Root et al., 2008), however identification of the molecular mechanisms, that is, the presence of a receptor, is essential. While the presynaptic role remains problematic, the role of glycine and glycinergic neurons in circuit and network development has assumed a new importance. Continued functional studies employing conditional knockouts to identify new or early roles will continue to be informative. An integrated approach using detailed gene expression and electrophysiological methodologies as employing the tools of comparative genomics and "evo-devo" analysis combined with functional assays including novel gene swapping experiments (along the lines of those conducted by Aubrey et al. (2007)) will provide an encompassing picture of how this important phenotype is specified and its numerous roles in the development of the nervous systems.

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The glycinergic system in human startle disease: a genetic screening approach

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Human startle disease, also known as hyperekplexia (OMIM 149400), is a paroxysmal neurological disorder caused by defects in glycinergic neurotransmission. Hyperekplexia is characterised by an exaggerated startle reflex in response to tactile or acoustic stimuli which first presents as neonatal hypertonia, followed in some with episodes of life-threatening infantile apnoea. Genetic screening studies have demonstrated that hyperekplexia is genetically heterogeneous with several missense and nonsense mutations in the postsynaptic glycine receptor (GlyR) α1 subunit gene (*GLRA1*) as the primary cause. More recently, missense, nonsense and frameshift mutations have also been identified in the glycine transporter GlyT2 gene, *SLC6A5*, demonstrating a presynaptic component to this disease. Further mutations, albeit rare, have been identified in the genes encoding the GlyR β subunit (*GLRB*), collybistin (*ARHGEF9*) and gephyrin (*GPHN*) – all of which are postsynaptic proteins involved in orchestrating glycinergic neurotransmission. In this review, we describe the clinical ascertainment aspects, phenotypic considerations and the downstream molecular genetic tools utilised to analyse both presynaptic and postsynaptic components of this heterogeneous human neurological disorder. Moreover, we will describe how the ancient startle response is the preserve of glycinergic neurotransmission and how animal models and human hyperekplexia patients have provided synergistic evidence that implicates this inhibitory system in the control of startle reflexes.

Keywords: glycine, hyperekplexia, receptor, transporter, mutation

INTRODUCTION TO HYPEREKPLEXIA

Glycine receptors (GlyR) are heteropentameric ligand-gated chloride ion channels that facilitate fast inhibitory neurotransmission in the human central nervous system (CNS) (Lynch, 2009). In humans, GlyRs have four functional subunits, GlyR α1–α3 and β that exist in heteromeric αβ combinations although the exact stoichiometry is a matter of intense debate (Grudzinska et al., 2005). Dysfunction of inhibitory glycinergic neurotransmission causes startle disease/hyperekplexia in humans (OMIM 149400), characterised by neonatal hypertonia and an exaggerated startle reflex in response to tactile or acoustic stimuli. In some instances, this can result in life-threatening infantile apnoea episodes. The most common disease-causing genes are those encoding the postsynaptic GlyR α1 subunit (*GLRA1*) on chromosome 5q33.1 (Shiang et al., 1993, 1995; Rees et al., 1994, 2001) and the presynaptic glycine transporter GlyT2 (*SLC6A5*) on chromosome 11p15.2 (Eulenburg et al., 2006; Rees et al., 2006). Both disease loci have revealed dozens of pathogenic missense, nonsense and frameshift mutations that can be inherited in either a dominant or recessive manner, with several recurrent mutations in *GLRA1* (**Figure 1**).

The description of mutations in the GlyR α1 subunit gene by Shiang et al. (1993) was the first channelopathy associated with ligand-gated chloride channels, whilst the mutations in the GlyT2 gene reported by Rees et al. (2006) defined the first neurological disorder linked to a defect in presynaptic transporter for a classical fast-acting neurotransmitter. However, both studies owe a great debt to murine models of hyperekplexia which revealed key candidate genes for the human screening programme (Becker et al., 1992; Buckwalter et al., 1994; Kingsmore et al., 1994; Mülhardt et al., 1994; Ryan et al., 1994; Gomeza et al., 2003a). Screening of genes encoding additional postsynaptic proteins involved in GlyR-mediated transmission including the GlyR β subunit (*GLRB*; Rees et al., 2002), gephyrin (*GPHN*; Rees et al., 2003) and collybistin (*ARHGEF9*; Harvey et al., 2004) has revealed only single individuals with potential mutations in these genes. Although mutations in *GLRB* are a clear cause of exaggerated startle in humans and animals (Kingsmore et al., 1994; Mülhardt et al., 1994; Rees et al., 2002; Hirata et al., 2005), deletions in *GPHN* have been associated with molybdenum co-factor deficiency (Reiss et al., 2001), whilst gene rearrangements in *ARHGEF9*, encoding the RhoGEF collybistin are more commonly associated with X-linked mental retardation(Harvey et al., 2008b; Marco et al., 2008; Kalscheuer et al., 2009). Despite our successes in disease gene discovery, approximately 35% of the hyperekplexia patients we recruited to our cohort studies are devoid of mutations in genes encoding postsynaptic GlyRs, associated proteins and the presynaptic transporter GlyT2. Although our knowledge of the proteomics of glycinergic synapses is extremely limited, this suggests that other genes encoding presynaptic transporters or receptor/transporter-associated proteins are suitable candidates for mutation

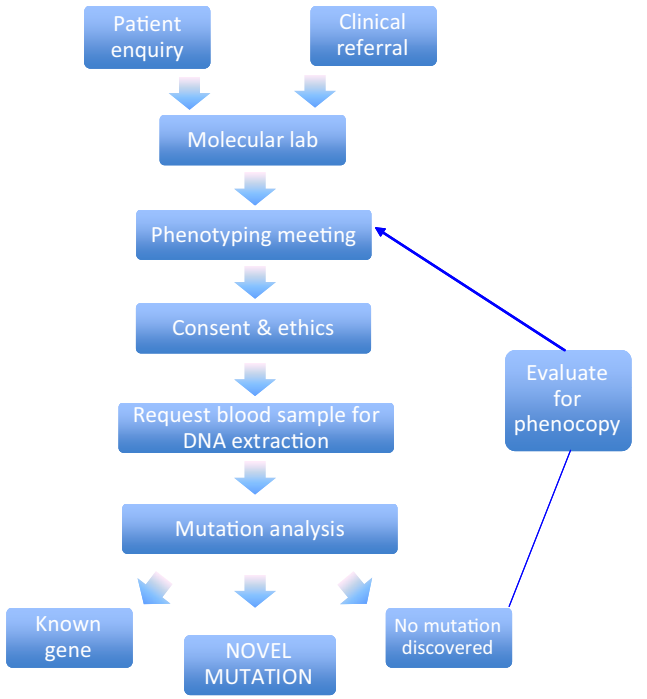

FIGURE 1 | Molecular genetic screening pipeline. This schematic diagram highlights the key stages involved in the clinical ascertainment and molecular genetic diagnosis.

screening in hyperekplexia. In this review, we describe the genetic screening/structure-function approaches utilised in our collaborative group to study hyperekplexia, and discuss future genetic screening approaches for the analysis of this genetically heterogenous neurological disorder.

CLINICAL ASCERTAINMENT OF HYPEREKPLEXIA PATIENTS

Since 1994, we have received over 260 referrals from clinicians located world-wide to perform genetic testing for hyperekplexia. This is currently performed at the Molecular Neuroscience Laboratory within the Institute of Life Science at Swansea University[1]. Once an anonymous patient clinical summary is received from the referring clinician, data is reviewed by a research committee with a view to assessing whether the patient phenotype fulfils the clinical criteria for mutation screening (**Figure 1**).

Alternative diagnoses could include paroxysmal extreme pain disorder, startle epilepsy or *acquired*(autoimmune) hyperekplexia. If the clinical data is suggestive of *inherited* hyperekplexia then a study information sheet, consent form and clinical pro-forma are issued to the referring clinician. If the patient and/or their family wish to pursue genetic testing on a research basis, then the referring clinician will obtain consent and collect a blood sample. The completed consent form and clinical pro-forma are then returned to the research team along with the blood sample, which is marked with a unique identifier and transferred for extraction of genomic DNA for mutation screening. Details from the clinical pro-forma are then incorporated into an anonymous dataset to allow the capture of standardised clinical information for each hyperekplexia sample received. The clinical pro-forma, along with evidence of consent, is stored securely on-site at ILS Swansea.

Since new UK-based ethical approval was confirmed in June 2006 (previous ethical approval in Cardiff University, UK, 1994–1999 and Auckland University, New Zealand, 2000–2006), 41 index patient samples have been accepted for hyperekplexia mutation screening and another 11 are currently undergoing the validation/ascertainment. An additional, 13 familial samples have been received for cascade screening – samples which require verification of identified mutations in close relatives. Testing of parental samples of index cases also enables confirmation of the origin of mutations in cases where compound heterozygosity is suspected.

There are potential risks associated with running an international genetic screening service in this way. Since clinical observations are essential to describe the phenotype of the patients referred to our laboratory, we are dependent upon the detail of information provided by the referring clinicians. Occasionally we receive messages from families, index cases and carers where they have received a clinical diagnosis of hyperekplexia. We strongly believe that giving a genetic diagnosis in the absence of genetic counselling is bad practice, so we encourage these individuals to make contact with us via a clinical referral.

CLINICAL PHENOTYPING OF HYPEREKPLEXIA PATIENTS

Deciding which patient samples to screen can be challenging. If entry criteria are too narrow then the phenotype is never expanded, too broad and the service can be overwhelmed and yet not produce valuable results (see **Table 1**). Hyperekplexia is almost certainly a rare condition, but of unknown prevalence and the true phenotype is complicated by cases which report association with co-morbid conditions such as sudden infant death (Giacoia and Ryan, 1994), epilepsy (Lerman-Sagie et al., 2004), abdominal herniae (Eppright and Mayhew, 2007) or developmental delay (Praveen et al., 2001). The early descriptions of hyperekplexia focussed on the shared clinical features (stiffness, startle and falls), the hereditary nature of the condition and the degree of phenotypic variability (which were qualified as 'major' and 'minor' variants) (Kirstein and Silfverskiold, 1958; Kok and Bruyn, 1962). Although many cases were autosomal dominant, this is not exclusively the case and this misconception represents a bias towards studying large families with many affected members (Rees et al., 1994; Masri and Hamamy, 2007). A useful sign particularly in neonatal hyperekplexia (occasionally seen in cerebral palsy) is the nose-tap response: the root of the nose is lightly tapped, provoking a brief and involuntary backwards-retraction of the head.

Neonatal apnoea episodes show an important and potentially life- threatening association with hyperekplexia. These are paroxysmal attacks of abdominal hypertonia with resulting hypoxia. It is important that parents are therefore taught the Vigevano manoeuvre (flexing of the head and limbs toward the trunk) which counteracts these attacks (Vigevano et al., 1989). Whether these hypoxic spells are related to simple intercostal muscle stiffness, brainstem abnormalities or developmental delay remains unknown.

[1]www.swan.ac.uk/ils

Table 1 | Diagnostic criteria for human hyperekplexia.

REQUIRED CRITERIA
Startle: Exaggerated startle reflex to unexpected auditory or tactile stimuli. The startle response can be prolonged and be present before birth. Consciousness is unaltered during startle episodes. Nose-tap test is positive and does not habituate
Stiffness: Generalized stiffness ***immediately after birth***, normalising during the first years of life. The stiffness can be predominantly truncal or lower limb, increases with handling and disappears during sleep. Short period of generalised stiffness following the startle response during which voluntary movements are impossible. This can result in falls in adults
Exclusion of mimics: Normal MR imaging, no dysmorphism or congenital deficits noted. Normal EEG during startle episode. Autonomic features of paroxysmal extreme pain disorder absent
SUPPORTIVE CRITERIA
Inguinal, umbilical, or epigastric herniae
Congenital dislocation of the hip
Hypoxic attacks in infancy

THE EFFECT OF PHENOCOPY

Hyperekplexia is (not unsurprisingly) most commonly confused with seizure disorders (see **Table 2**) such as benign neonatal convulsions. However, in hyperekplexia, consciousness is retained and there is no EEG correlate to either the startle or hypertonic posturing. False positive results are reported, since exaggerated limb jerking can result in a degree of artefact on the EEG trace. However, symptomatic improvement is often seen with benzodiazepines such as clonazepam and less frequently with other anticonvulsant drug therapies.

Despite the classical features being present from birth or even earlier (some reports suggest that exaggerated startle responses can be felt *in utero* (Badr El-Din, 1960; Dalla Bernardina et al., 1988; Leventer et al., 1995) the majority of definitive diagnoses are made during infancy. A positive test in an infant can result in older family members receiving an explanation for previously unexplained symptoms. Alternatively, a proportion of early neonatal referrals with primary hypertonia can sometimes develop into more sinister degenerative disorders, which is not typical of inherited hyperekplexia and these cases are invariably negative for GlyR and GlyT2 mutations. However, we are also eager to differentiate between these early onset congenital cases and adult onset cases of hyperekplexia. Since autoimmunity to GlyRs has recently been found in a single case of acquired hyperekplexia (Hutchinson et al., 2008) we can now process samples from such individuals for testing for anti-GlyR or GlyT2 antibodies, using immortalised human cell lines expressing recombinant GlyRs or GlyTs. Hypertonia (predominantly in the lower limbs) is the key presenting feature of stiff-person syndrome, which is associated with either autoimmunity to gephyrin (Butler et al., 2000) orglutamic acid decarboxylase 65 (GAD65; Duddy and Baker, 2009). Furthermore, attempts to definitively describe clinical symptoms and prevalence of hyperekplexia are challenged by potential referral bias. To our knowledge, hyperekplexia has not been diagnosed at the genetic level in people who are ethnically Slavic, South or Central American and very few cases have been reported from Polynesia (see **Figure 2**). This may be of importance, since there are certain cultural neuropsychiatric conditions such as the 'Jumping Frenchmen of Maine' and 'Latah Syndrome', which share some characteristics with hereditary hyperekplexia (Kurczynski, 1983). Whether these conditions represent the exaggerated startle described in certain anxiety conditions is unknown, since some investigators describe these cultural startle conditions as predominantly psychosomatic in nature (Bartholomew, 1994). Exaggerated startle may also be secondary to predominately pontine pathology: brainstem infarction, infection, haemorrhage or hypoxia can all produce hyperekplexia-like symptoms. In addition, individuals with paraneoplastic syndromes, multisystem atrophy and multiple sclerosis can all exhibit exaggerated startle responses (Bakker et al., 2006).

MOLECULAR GENETICS OF HYPEREKPLEXIA

The most common genetic causes of human hyperekplexia are mutations in the GlyR α1 subunit (*GLRA1*) and GlyT2 (*SLC6A5*) genes (see Harvey et al., 2008b for a review and comprehensive mutation data). Therefore, new patient samples that conform to our diagnostic criteria are analysed by PCR amplification of individual exons and dideoxy DNA sequencing on an Applied Biosystems 3100 capillary sequencing platform to detect sequence variants in *GLRA1* and *SLC6A5* (Rees et al., 2006). For all candidate genes, analysis includes exons and flanking splice branch points, donor and acceptor sequences, 5′ and 3′ untranslated regions and splice variants are derived *insilico*, using the Human Genome Browser at the University of California, Santa Cruz[2]. One future aim is also to capture sequence variants in the *GLRA1* and *SLC6A5* gene promoters, although only the former has been characterised in any detail (Morris et al., 2004), and the minimal promoter encompasses 5.4 kb of sequence. A robust readout of promoter activity is also required in order to assess any detrimental effect of potential sequence variations. By contrast, GlyT2 transcripts are extensively alternatively spliced at the N-terminus (Ponce et al., 1998; Ebihara et al., 2004), and it is possible that multiple tissue-specific or developmentally regulated promoters exist, complicating potential sequence analysis. Candidate genes with rare or as yet unknown mutation frequencies undergo medium-throughput gene variation detection by analysing PCR amplimers with a high-resolution melting (HRM) platform (LightScanner, Idaho Technologies, USA). HRM is a highly-sensitive method of analysing genetic variations in short PCR amplicons generated in the presence of the saturating double-stranded DNA binding dye LCGreen® Plus (Idaho Technologies). The technique allows the PCR amplicons to be distinguished based on their dissociation during rapid melting (**Figure 3**). HRM analysis has a published detection rate of 100% for heterozygous mutations (Lonie et al., 2006; Kennerson et al., 2007) and has the added advantages of high speed (~15 min per 96 well plate run), no post-PCR handling and greatly reduced costs (9 p per amplicon) compared to sequencing (~£5 per read from commercial services)[3]. Thus, HRM allows for rapid and cost-effective mutation discovery in dsDNA

[2]http://genome.ucsc.edu

[3]http://www.dnaseq.co.uk/

Table 2 | Clinical phenocopies of human hyperekplexia.

Phenocopy	Comparisons with human startle
ACQUIRED HYPEREKPLEXIA	
Sub-acute anti-glycine receptor antibody mediated condition that responds to immunosuppression and plasma exchange (Hutchinson et al., 2008)	***Similarities*** Truncal rigidity, muscle spasms and stimulus induced startle ***Differences*** Features not present from early life immunosuppression clearly efficacious. Apnoea attacks not described
PAROXYSMAL EXTREME PAIN DISORDER	
Autosomal dominant condition recently shown to be a sodium channelopathy involving *SCN9A* (previously known as familial rectal pain syndrome; Fertleman et al., 2007)	***Similarities*** Onset in neonatal period or infancy, persists throughout life. Dramatic syncopes with bradycardia and sometimes asystole. Tonic attacks are triggered by factors such as defecation, cold wind, eating, and emotion ***Differences*** Autonomic manifestations predominate initially, with skin flushing in all and harlequin colour change. Later attacks of excruciating deep burning pain often in the rectal, ocular, or jaw
JUMPING FRENCHMEN OF MAINE/LATAH SYNDROME	
Culturally bound neuropsychiatric syndromes thought to be an anxiety/somatisation disorder (Meinck, 2006)	***Similarities*** Excessive response to startle ***Differences*** Echopraxia (involuntary repetition of another's words or actions) and echolalia (repetitive vocalisations)
STARTLE EPILEPSY	
Startle epilepsy is a reflex epileptic seizure precipitated by a sudden stimulus; most patients are young and have infantile cerebral hemiplegia (Meinck, 2006)	***Similarities*** Surprising stimuli induce motor reactions – consciousness can be preserved in seizures ***Differences*** Neuro-imaging will almost certainly be abnormal
STIFF PERSON SYNDROME	
Progressive axial stiffness and intermittent spasms mainly evoked by unexpected stimuli; associated with auto-antibodies to either gephyrin (Butler et al., 2000) or GAD (Meinck, 2006)	***Similarities*** Stimulus induced hypertonia, startles and falls. Hypertonia can preferentially affect lower-limbs ***Differences*** Stiffness/hypertonia is much more prolonged than the paroxysmal attacks seen in hyperekplexia
TOURETTE'S SYNDROME	
Motor and vocal tics, associated with an exaggerated startle reflex, behaviour change and stereotypy (Bakker et al., 2006)	***Similarities*** Startle response, symptoms precipitate by stressors ***Differences*** Vocalisations and obsessive/compulsive behaviours. Motor tics can be complex and appear semi-purposeful
CRISPONI SYNDROME	
An autosomal recessive syndrome initially described in 12 different families in southern Sardinia; caused by mutations in the CRLF1 gene (Crisponi, 1996; Crisponi et al., 2007)	***Similarities*** Evident at birth. Marked muscular contraction of the facial muscles in response to tactile stimuli or during crying, contractions slowly disappear as infant calms. Generalised seizures (albeit rare) and mild psychomotor delay in some. Low GABA levels in CSF have been described ***Differences*** Abundant salivation simulating a tetanic spasm. Neck muscle hypertonia. Facial anomalies (large face, chubby cheeks, broad nose with anteverted nostrils and long philtrum). Bilateral camptodactyly. Hyperthermia
SYMPTOMATIC STARTLE AND MYOCLONUS	
Neuropsychiatric – anxiety states including generalised anxiety disorder, post traumatic stress disorder *Cerebral* – Children with cerebral palsy, post-traumatic or hypoxic encephalopathy, para neoplastic syndromes *Brainstem* – particularly pontine patholog yeg Multiple system atrophy (Bakker et al., 2006)	***Similarities*** Symptoms will be exaggerated by stressors. Stimulus sensitive (e.g. touch) can be seen following hypoxic brain injury. Children with cerebral palsy may have a positive nose-tap test ***Differences*** Acquired cause often clear, for example late adult onset of multi system atrophy. Hypertonia not a feature of anxiety syndromes

with improved sensitivity compared to other screening platforms. However, limitations include the optimisation of amplification conditions in the presence of LCGreen® Plus, limitations on the size of amplicons (<400bp) and non-specific amplicons or primer dimers significantly reduce HRM performance. For a comprehensive review of HRM see White and Potts (2006).

PCR products showing variant melting profiles suggestive of allelic heterogeneity undergo purification and direct sequencing to identify the genetic variation. Population studies of genomic variation are performed using agarose gel restriction fragment length polymorphisms (RFLPs) to confirm the absence of the mutation in the healthy population. This can also be confirmed

FIGURE 2 | Global origin of hyperekplexia referrals. Upper panel: A geographical representation of the global origin of hyperekplexia patients recruited to our laboratory (red) and other laboratories (blue – Information obtained from the NCBI PubMed search engine). Lower panel: Detail showing the origin of patients recruited from central Europe.

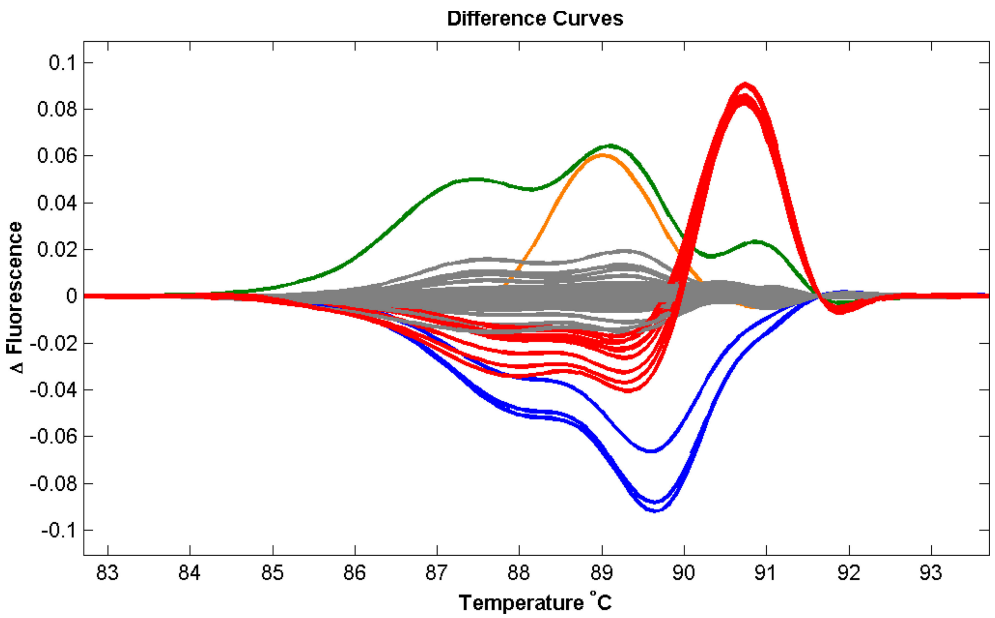

FIGURE 3 | Validation of the Light Scanner technology. To assess the application of this method we performed a series of validation assays utilising known genetic variations in the *GLRA1* gene in a population of hyperekplexia patients. This screening method accurately detected all heterozygous (red and orange) and homozygous (blue) missense mutations present. The most common *GLRA1* mutation associated with hyperekplexia, R271Q, is shown in red. The assay also detected a false-positive variant (green) generated due to the poor quality of amplified PCR product. Population control samples are shown in grey.

byassessing HRM profiles of control DNA samples alongside a mutation-positive sample. DNA sequencing chromatograms are then assessed using an automated mutation surveyor programme (MutationSurveyor, SoftGenetics®, USA) which is particularly useful for detecting homozygous changes in the sequence data. After this systematic analysis in our laboratory, 53 single, unrelated,

sporadic cases of hyperekplexia have proved gene-negative in screening approaches for *GLRA1*, *GLRB*, *GPHN*, *ARHGEF9* and *SLC6A5*.

Both dominant and recessive mutations in *GLRA1* are associated with sporadic and familial startle disease, whilst a proportion of sporadic hyperekplexia is accounted for by the homozygous inheritance of recessive or compound heterozygous *GLRA1* mutations (Rees et al., 2001). By contrast, the vast majority of mutations in *SLC6A5* are inherited as compound heterozygotes or show recessive inheritance of a single mutation from consanguineous parents. To date, there is only a single example of a dominant mutation in *SLC6A5* (Rees et al., 2006). Hence, the parental carriers of *SLC6A5* mutations are typically asymptomatic indicating that dominant negative effects are not a common mutational mechanism. Other explanations for a single mutation could include an undetected second-hit event, not easily detectable by PCR and sequencing. For example, promoter mutations, large deletions or intronic SNPs causing allelic dropout due to PCR primer mismatches are possible. Even synonymous SNPs that do not cause an amino acid substitution could cause creation of an ectopic splice donor or acceptor site, causing missplicing of gene transcripts. We have begun to assess these issues by using multiplex ligation-dependent probe amplification (MLPA) analysis in index cases, to assay for possible deletions in *SLC6A5* and *GLRA1*. MLPA provides the means for the quantitative analysis of various changes in gene structure and/or gene copy number of several dozens (40–50) of DNA targets in a single reaction containing small amounts (~20ng) of human chromosomal DNA. Binary probes containing a sequence-specific part and a universal part are hybridized to their DNA targets and ligated. Each ligated probe is then PCR amplified with a universal primer pair and gives rise to an amplicon of unique size. The relative amount of each amplicon reflects the quantity of the corresponding target that is present in the nucleic acid sample. While allelic drop-out can be assessed by designing an additional primer set, changes in the promoter sequences and splicing patterns of *GLRA1* and *SLC6A5* are more challenging. In particular, we are also limited by the lack of patient-specific RNA resource or the assurance that the genes are expressed in peripheral leukocytes. Affordable next generation re-sequencing (e.g. 454 Roche, Illumina Solexa, AB SOLiD) along with high-throughput splicing assays may resolve these current limitations. These resources would also allow us to assess changes in RNA editing of GlyR transcripts, an important consideration for studies of GlyR α2 and α3 subunit genes in health and disease (Meier et al., 2005; Eichler et al., 2009).

MOLECULAR ANALYSIS OF POTENTIALLY PATHOGENIC MUTATIONS

Potentially disease-causing mutations can also be subjected to functional and molecular modelling analysis to provide insight into the precise mechanisms underlying pathogenicity. In the case of *SLC6A5,* mutations were analysed by homology modelling of GlyT2 using the crystal structure of the bacterial leucine transporter (LeuT) (Yamashita et al., 2005). Simple sequence alignments of LeuT with GlyT1 and GlyT2 allowed us to identify residues potentially involved in coordinating glycine and Na^+ binding (Rees et al., 2006), which were later confirmed by assays on recombinant wild-type and mutant GlyT2 including [^{3}H]glycine uptake assays, sophisticated electrophysiological analyses in *Xenopus* oocytes and molecular modelling (Rees et al., 2006; Harvey et al., 2008a).

Structural modelling is carried out using a homology modelling pipeline built with the Biskit structural bioinformatics platform (Grunberg et al., 2007). Our pipeline workflow incorporates the NCBI tools platform (Wheeler et al., 2007), including the BLAST program (Altschul et al., 1990) for similarity searching of sequence databases. Protein sequences corresponding to the Protein Databank of protein structures were searched for homology with the gene of interest in order to identify putative structural homologues. T-COFFEE (Notredame et al., 2000) was used for alignment of the test sequence with the template, followed by 10 iterations of the MODELLER homology modelling program (Eswar et al., 2003) (**Figure 4**).

FURTHER HYPEREKPLEXIA CANDIDATE GENES

Our recent research into glycinergic transmission has clearly demonstrated the importance of both presynaptic (GlyT2) and postsynaptic (GlyR α1β) mechanisms in health and disease. This work has also suggested that our search for other suitable candidate genes for mutation analysis in human hyperekplexia should be broadened (**Table 3**). Potential target genes are discussed below in the context of their putative roles in glycinergic synaptic physiology.

GlyT1

Whilst inhibitory glycinergic neurotransmission is dependent upon agonist-mediated activation of postsynaptic GlyRs, this function is dependent upon having a sufficient pool of releasable glycine within the presynaptic terminal and prompt termination of glycinergic transmission. GlyT1, encoded by *SLC6A9* (1p34.1) fulfils the latter function, and is predominately expressed on glial cells. Studies on GlyT1 knockout mice (Gomeza et al., 2003b; Tsai et al., 2004) suggest that loss of function of GlyT1 results in a pathological accumulation of synaptic glycine, causing severe motor deficits and premature death as a result of respiratory failure. The phenotype of the GlyT1 knockout mouse resembles a devastating neurological disorder known as glycine encephalopathy (OMIM 605899) although no *SLC6A9* mutations have been found to date in this disorder in humans. However, it is possible that more subtle missense mutations could result in a gain of function of GlyT1, causing enhanced clearance of glycine from the synaptic cleft into neighbouring glial cells. This could cause depletion of glycine in neighbouring presynaptic neurones, which may result in hyperekplexia in humans (Harvey et al., 2008a).

GlyT2 INTERACTORS

Further presynaptic candidates for genetic analysis include proteins interacting with GlyT2, such as syntenin-1 and ULIP6. ULIP6 is encoded by *DPYSL5* (2p23.3) and is a brain-specific phosphoprotein of the Ulip/collapsing response mediator protein family. ULIP6 interacts with extended intracellular N-terminus of GlyT2 in a phosphorylation dependent manner (Horiuchi et al., 2005). Since ULIP6 has been implicated in GlyT2 endocytosis and recycling (Eulenburg et al., 2005; Horiuchi et al., 2005), it is plausible that mutations in ULIP6 could cause hyperekplexia by altering levels of presynaptic GlyT2. By contrast, the PDZ containing protein syntenin-1, encoded

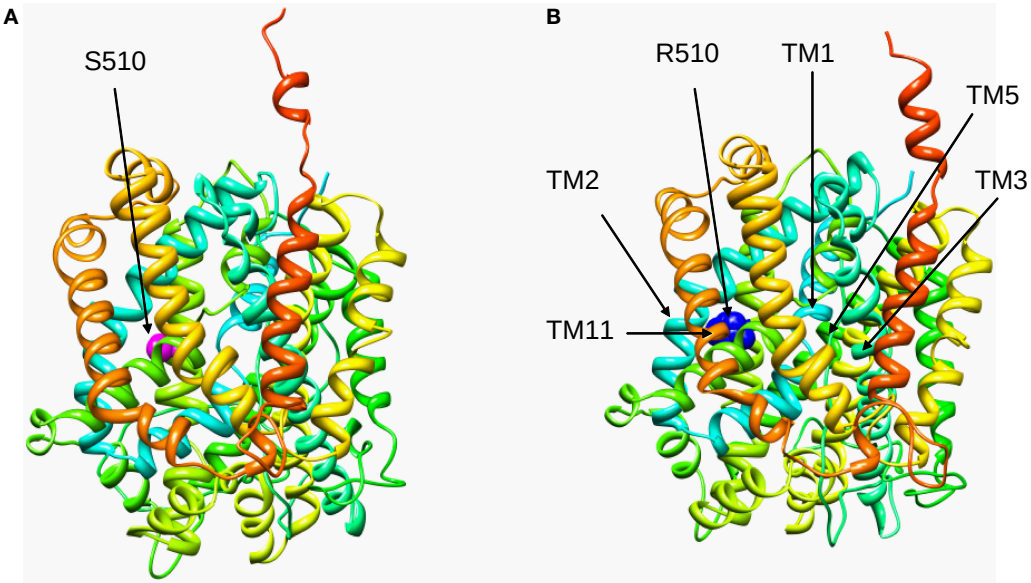

FIGURE 4 | Structural modelling of human GlyT2. The wild-type **(A)** and the S510R mutant **(B)** model were generated based on homology with the crystal structure of LeuT, a bacterial Na^+/Cl^--dependent neurotransmitter transporter homologue (PDB: 2A65). The models cover residues 191–754 of GlyT2 and show the position of the S510R mutation in TM7 along with the extensive re-arrangement of other transmembrane regions in the S510R that results in defective membrane trafficking of S510R and trapping of wild-type GlyT2 (see Rees et al., 2006). Models were visualized using the molecular graphics program Chimera (http://www.cgl.ucsf.edu/chimera/).

Table 3 | Candidate genes for mutation screening in hyperekplexia.
Chromosomal locations were obtained from http://ncbi.nlm.nih.gov/

Candidate gene	Location	Protein	Proposed role at glycinergic synapse
SLC6A9	1p34.1	GlyT1	Termination of glycinergic neurotransmission by uptake into glial cells
SLC32A1	20q11.23	VIAAT	Vesicular transport of GABA and glycine
SLC6A17	1p13.3	NTT4	Vesicular transport of glycine
DPYSL5	2p23.3	ULIP6	GlyT2 interacting protein
SDCBP	8q12.1	Syntenin-1	GlyT2 interacting protein

by the gene *SDCBP* (8q12.1) is thought to regulate the trafficking or presynaptic localisation of GlyT2 in glycinergic neurones (Ohno et al., 2004). Mutations that affect syntenin PDZ binding domains could cause mislocalisation of GlyT2 and other interacting partners, since GlyT2 localisation in the presynaptic terminal is dependent on the C-terminal PDZ binding motif (Armsen et al., 2007). However, due to the wide range of proteins interacting with syntenin-1, we consider it unlikely that defects in syntenin-1 will give rise to classical hyperekplexia (Harvey et al., 2008b).

VIAAT

Another obvious hyperekplexia candidate gene is the vesicular inhibitory amino acid transporter (VIAAT), encoded by *SLC32A1* (20q11.23). VIAAT is expressed in both GABAergic and glycinergic neurones (Chaudhry et al., 1998), and is responsible for the chloride-dependent loading of presynaptic vesicles with GABA and glycine (Jin et al., 2003; Juge et al., 2009). We suggest that certain mutations in VIAAT could lead to the specific loss of glycine (but not GABA) loading into synaptic vesicles, thus resulting in hyperekplexia. VIAAT was first identified as a mammalian homologue of the 'uncoordinated' *C. elegans* mutant *unc-47*, which was known to be defective in a presynaptic component of GABA release. Despite the fact that *C. elegans* do not appear to use glycine as a neurotransmitter, in a sophisticated cellular assay Aubrey et al. (2007) were able to show that UNC-47 is able to readily transport both GABA and glycine into vesicles. Importantly, the UNC-47 mutation G462R was shown to abolish GABA, but not glycine uptake. Since residue G462 is conserved at the equivalent position (G500) in rodent and human VIAAT sequences, mutations in *SLC32A1* could theoretically compromise GABA uptake into synaptic vesicles while leaving glycine uptake intact. Since it is clearly possible to separate GABA and glycine transport by a single missense mutation, our hypothesis is that other missense mutations could also result in a GABA-specific VIAAT, potentially resulting in hyperekplexia.

NTT4

The orphan transporter NTT4 (also known as Rxt1), encoded by *SLC6A17* (1p13.3) has recently been implicated as a vesicular transporter for glycine, proline, leucine and alanine (Parra et al., 2008). This novel finding suggests that NTT4, which is highly expressed in several brain regions, including the spinal cord (Liu et al., 1993), may have an important role in glycinergic transmission and possibly hyperekplexia.

THE FUTURE – PHENOTYPING, GENOMIC AND PROTEOMIC REVOLUTION

Despite the advances in the molecular genetics of hyperekplexia of the last 20 years, we are no closer to describing certain clinical aspects than the original pioneering study by Andermann et al. (1980). Hyperekplexia is rare, easily misdiagnosed and clonazepam is still the current treatment of choice. We have been unable to find a genetic basis to the original delineation – the so called 'major' and 'minor' variants. Collections of large cohorts, particularly containing sporadic cases may help us move away from the inherent bias caused by studying large families with a single gene mutation. Future challenges include improving testing turnaround times and accessibility, and collecting comprehensive clinical data to improve our understanding of possible differences in clinical phenotypes caused by mutations in *GLRA1* versus *SLC6A5*. This will also enable us to identify potential hyperekplexia-associated co-morbidities and identify potential phenocopy referrals.

On the horizon is the prospect of third generation sequence platforms which will facilitate automated re-sequencing of large genomic segments containing disease-causing genes and new candidate loci and possibly even whole genomes of affected individuals. These include the pyrosequencing-based platform of next generation 454 sequencing (see Rothberg and Leamon, 2008) which was recently used for rapid genome re-sequencing of an individual genome at a fraction of the cost of previous platforms (Wheeler et al., 2008). Such coverage will provide not only the sequence of coding exons and flanking spice sites, but information on intragenic DNA, SNP haplotype risk factors and copy number variables. Affordability is at present the main barrier and as a medium-term solution enrichment of individual chromosomal regions containing *GLRA1* and *SLC6A5* may provide an interim solution. For example, Zheng and colleagues have recently developed a method for high-throughput variant detection, utilising specific genomic regions for target amplification by capture and ligation (TACL), allele enrichment and array resequencing (Zheng et al., 2009). This platform has identified rare and novel variants, and will undoubtedly lead to improvements in our understanding of complex genetic disorders.

Molecular biology, animal models of glycinergic function and detailed proteomic studies will continue to provide further candidates for genetic screening in hyperekplexia and other potential disorders of glycinergic synapses. The new targets described above are being screened at present in patients lacking mutations in *GLRA1* and *SLC6A5*. Molecular genetic studies of unresolved ENU-induced mutations in zebrafish (Granato et al., 1996) may also tease out novel determinants of glycinergic function (Hirata et al., 2010). These leads suggest that new genes of major effect could shortly join *GLRA1* and *SLC6A5* in our molecular screening programme.

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Cellular transport and membrane dynamics of the glycine receptor

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Regulation of synaptic transmission is essential to tune individual-to-network neuronal activity. One way to modulate synaptic strength is to regulate neurotransmitter receptor numbers at postsynaptic sites. This can be achieved either through plasma membrane insertion of receptors derived from intracellular vesicle pools, a process depending on active cytoskeleton transport, or through surface membrane removal via endocytosis. In parallel, lateral diffusion events along the plasma membrane allow the exchange of receptor molecules between synaptic and extrasynaptic compartments, contributing to synaptic strength regulation. In recent years, results obtained from several groups studying glycine receptor (GlyR) trafficking and dynamics shed light on the regulation of synaptic GlyR density. Here, we review (i) proteins and mechanisms involved in GlyR cytoskeletal transport, (ii) the diffusion dynamics of GlyR and of its scaffolding protein gephyrin that control receptor numbers, and its relationship with synaptic plasticity, and (iii) adaptative changes in GlyR diffusion in response to global activity modifications, as a homeostatic mechanism.

Keywords: glycine receptor, gephyrin, transport, motor proteins, cytoskeleton, diffusion, SPT, neuronal activity

INTRODUCTION

Glycine receptors (GlyRs) mediate synaptic inhibition in brain and spinal neurons and locate either at glycinergic (Triller et al., 1985, 1987; Betz, 1991) or mixed glycinergic/GABAergic postsynaptic sites (Lévi et al., 1999; Dumoulin et al., 2000). GlyRs bind directly the scaffold protein gephyrin (Meyer et al., 1995) at different cellular compartments. GlyR molecules are associated with gephyrin in intracellular vesicles (Hanus et al., 2004), which apply gephyrin as a cargo adaptor and link the receptor to microtubule-dependent motor proteins that power long distance bidirectional transport between neuronal somata and distal neurites (Maas et al., 2006, 2009). In addition to its association with GlyR during intracellular transport, gephyrin stabilizes the receptor once inserted in the surface membrane, in particular at synaptic sites. The first evidence of a functional synaptic microdomain was the detection by light and electron microscopy of GlyR and gephyrin aggregates in front of the presynaptic bouton (Triller et al., 1985). Synaptic gephyrin clustering precedes the postsynaptic localization of GlyRs *in vivo* as well as *in vitro* (Kirsch et al., 1993b; Bechade et al., 1996; Colin et al., 1998; Dumoulin et al., 2000). The recruitment of GlyR by gephyrin within clusters depends on a functional receptor (Kirsch and Betz, 1998; Lévi et al., 1998) and requires the presence of the appropriate presynaptic innervation (Lévi et al., 1999). Disruption of the gephyrin scaffold by antisense oligonucleotides or after intracellular antibody capture prevents the formation of GlyR clusters (Kirsch et al., 1993a; Zacchi et al., 2008). The same consequence is observed in the gephyrin-deficient mouse (Feng et al., 1998). Actually, gephyrin molecules are able to trimerize and dimerize simultaneously via its G- and E-domains, respectively (Sola et al., 2001, 2004; Saiyed et al., 2007). It has been postulated that this arrangement leads to the formation of a hexagonal lattice in the postsynaptic density (Xiang et al., 2001; Sola et al., 2004), offering multiple binding sites for GlyRs and representing a structure for new gephyrin molecules to be added (see **Figure 1**).

Here we discuss active and passive parameters of GlyR and gephyrin dynamics at both intracellular and cell surface compartments. We particularly focus on whether and how changes in neuronal activity modulate these processes underlying the regulation of synaptic strength and/or plasticity.

TRANSPORT TO AND FROM SYNAPTIC REGIONS

KIF AND DYNEIN-RELATED TRANSPORT PROCESSES

Neurons are highly polarized cells with axons and dendrites. Many neuronal molecules are needed in one but not in the other compartment and require sorting and long-distance delivery into peripheral neurites (Hirokawa and Takemura, 2005). Within axons and dendrites, longitudinally oriented microtubules serve as rails for ATP-dependent molecular motors, which convert chemical energy into mechanical work and mediate intracellular transport of membraneous organelles and macromolecular complexes (Desai and Mitchison, 1997; Hirokawa and Takemura, 2005; Caviston and Holzbaur, 2006). Microtubules are unipolar structures, made of α- and β-tubulin subunits that lead to plus- and minus-ends within the polymer. In axons and distal dendrites, the fast growing plus-ends point away from the cell body (Baas et al., 1988). Motor proteins of the kinesin (KIF) and dynein superfamilies drive molecular cargo along microtubule tracks. Most KIFs are plus end-directed motors and participate in anterograde transport that selectively guides molecules from the soma into neurite processes. In contrast, cytoplasmic dynein motors are minus end-directed and mediate retrograde transport from the periphery toward the cell body (Hirokawa and Takemura, 2005; Caviston and Holzbaur,

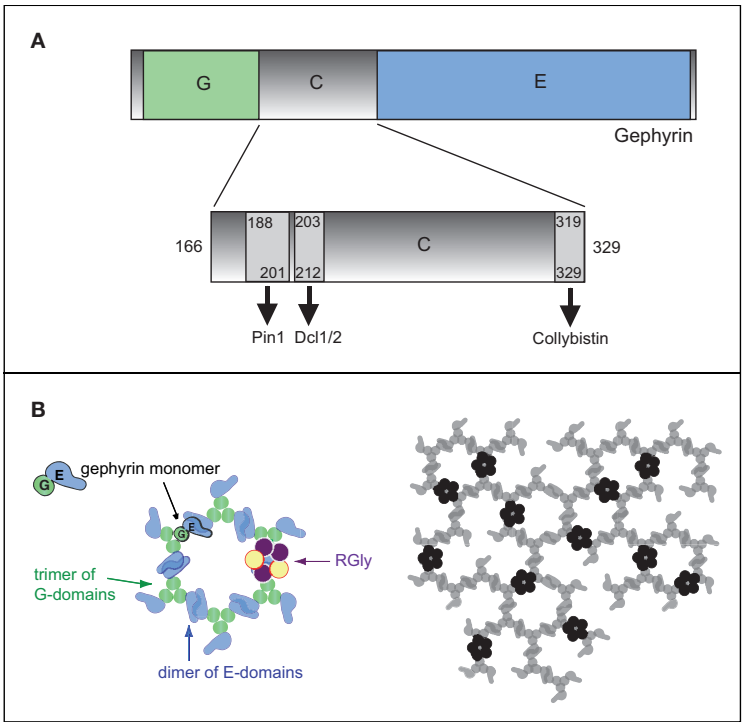

FIGURE 1 | Gephyrin domains and structural organization. (A) Schematic depiction of the three gephyrin domains (G, C, E): the N-terminal G-domain (G) and the -terminal E-domain (E) are separated by a central C-domain (C). The C-domain is magnified below. Sequences of the binding sites for Pin1 (Zita et al., 2007), Dlc1/2 (Fuhrmann et al., 2002) and collybistin (Kins et al., 2000) are depicted by arrows. Numbers represent amino acid positions within the gephyrin protein. **(B)** The gephyrin «hexagonal lattice» oligomerization model: E-and G-gephyrin domains are able to dimerize and trimerize, respectively (left panel). Combination of these two properties would lead to a hexagonal structure of gephyrin (right panel) underneath the postsynaptic membrane, where GlyR (in black) would anchor itself through the binding of the intracellular loop of the beta subunit with the E-domain of gephyrin.

2006), in which cargo molecules eventually undergo degradation. In close proximity to the plasma membrane, actin microfilaments also serve as rails for local molecular transport. Here, unconventional myosins often mediate the final steps of plasma membrane delivery or the initial steps of surface membrane internalization, respectively (Bridgman, 2004). In accordance with a distinct subcellular distribution of cytoskeletal elements, individual cargoes are thought to switch between actin- and microtubule-based transport (Radtke et al., 2006) to travel toward submembrane or intracellular compartments.

Most synaptic proteins including the GlyR are synthesized in the cell body. They require active long-distance vesicle transport into neurites and toward the plasma membrane to reach their functional destination, the synapse compartment. Molecular motors mediate intracellular cargo transport with velocities in the range of μm/sec on average (Hirokawa and Takemura, 2005) and live cell imaging identified mobile transport packets of GlyR fusion proteins that bidirectionally traveled through neurite processes (Maas et al., 2006). However, under conditions of synaptic plasticity that require the rapid delivery of newly synthesized material, long-distance transport might be limited in providing sufficient amounts of synaptic components on a fast time scale. Alternatively, neurons use RNA-protein granules (Kanai et al., 2004) to target individual messenger RNAs (mRNAs) into dendrites and apply local translation in close proximity to axo-dendritic contacts (Sutton and Schuman, 2006). Consistently, GlyR α-subunit mRNAs were found to localize in neuronal dendrites (Racca et al., 1997, 1998; Gardiol et al., 1999) and the GlyR binding protein gephyrin was shown to interact with RAFT1/mTOR (Sabatini et al., 1999), a critical signalling component in translational control (Ma and Blenis, 2009), suggesting that glycinergic synapses represent sites of local translation under certain conditions.

Originally, GlyRs (Bechade et al., 1996) and the GlyR-interacting protein gephyrin (Seitanidou et al., 1992; Colin et al., 1996) were identified at intracellular sites in neurons, and depolymerization of microtubules dispersed the subcellular accumulation of both proteins (Kirsch and Betz, 1995). A direct association of gephyrin with the light chains Dlc-1/Dlc-2, components of the microtubule-dependent dynein motor, suggested that microtubule transport might be involved in the subcellular localization of these factors (Fuhrmann et al., 2002) (**Figure 1**).

Neuronal coexpression of epitope-tagged gephyrin and GlyR α1 subunits confirmed that gephyrin indeed localizes to GlyR-containing intracellular vesicle structures (Hanus et al., 2004). In fact, gephyrin accelerated the accumulation of GlyRs at the cell surface and depolymerization of microtubules interfered with these targeting processes (Hanus et al., 2004). These data were complemented by functional evidence that active microtubule-dependent motor protein complexes interact, colocalize and comigrate with GlyR- and gephyrin-fusion proteins through neurite processes

over time (Maas et al., 2006, 2009) (**Figure 2**). For anterograde transport toward the plasma membrane, GlyR-gephyrin complexes were found to apply conventional kinesin (KIF5) as their driving force (Maas et al., 2009). Specific blockade of KIF5's motor function interfered with the delivery of gephyrin into peripheral neurites and the knockdown of gephyrin gene expression caused in turn a significant reduction in GlyR surface membrane delivery (Maas et al., 2009). In contrast, GlyR-gephyrin transport toward the cell center is mediated by the retrograde-directed dynein motor complex (Maas et al., 2006), known to participate in both receptor internalization processes downstream of the sorting endosome (Traer et al., 2007) and long distance retrograde trafficking through neurite processes (Caviston and Holzbaur, 2006). In general, mobile GFP-gephyrin transport packets in the synapse are continuously added to and removed from immobile postsynaptic gephyrin scaffolds in the minute range (**Figure 2**) and rapidly switch between neighbouring synapses over time (Maas et al., 2006). Notably and similarly to AMPA receptor (AMPAR) transport complexes (Setou et al., 2002), the postsynaptic anchoring protein (gephyrin) was found to act as a cargo adaptor that directly links the receptor (GlyR) to either its kinesin or dynein motor, respectively (Kneussel, 2005; Maas et al., 2006, 2009).

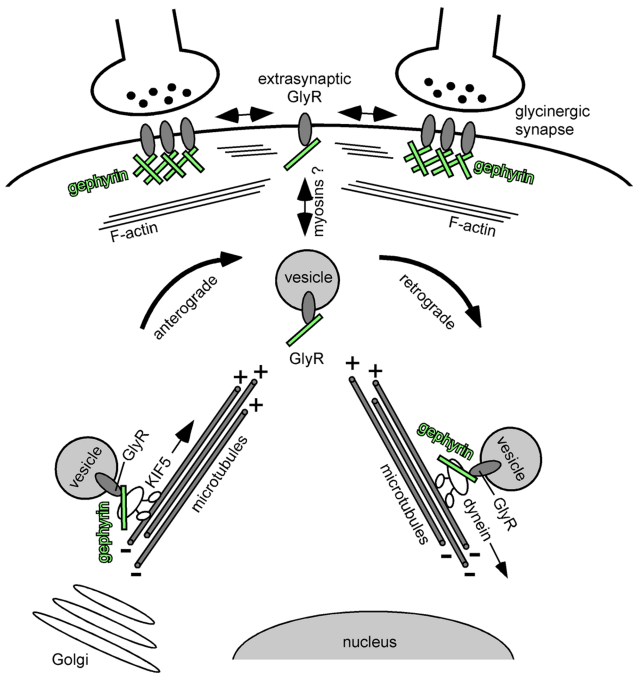

FIGURE 2 | GlyR-gephyrin intracellular cytoskeleton transport. Newly synthesized glycine receptors (GlyRs) that leave the Golgi compartment reach the plasma membrane through active transport mechanisms along cytoskeletal elements. KIF5 motor proteins connect to vesicular GlyRs via gephyrin (green) that serves as a cargo adaptor in the transport complex. The KIF5/gephyrin/GlyR complex moves in anterograde directions toward the plus-ends of microtubules. It is currently unclear whether myosins mediate the final steps of GlyR surface membrane delivery and the initial steps of plasma membrane internalization, respectively, to traverse the submembrane actin cortex. At postsynaptic sites, gephyrin (green) forms a submembrane scaffold and mediates GlyR clustering. Exo-/ and endocytosis of receptors is thought to occur at extrasynaptic sites. Upon GlyR internalization, a GlyR/gephyrin/dynein transport complex mediates retrograde minus end-directed microtubule transport to intracellular compartments. Cytoplasmic dyneins are thought to participate in endocytic processes downstream on the sorting endosome (e.g. delivery to multivesicular bodies and/or lysosomes). In analogy to the anterograde GlyR transport complex, gephyrin (green) serves as a cargo adaptor that connects the vesicular receptor with its motor.

Together, independent approaches have revealed that GlyR and gephyrin functionally associate already at the intracellular level prior to their role in receptor scaffolding at postsynaptic sites. Although both proteins were found to undergo long-distance intracellular transport in a microtubule-dependent manner, it is currently unclear whether myosin-type motor proteins participate in local GlyR-gephyrin transport at actin-rich compartments underneath the neuronal plasma membrane (**Figure 2**). GlyR-gephyrin intracellular transport in neurons resembles other receptor-motor systems heading to and from glutamatergic spine synapses (Kneussel, 2005). However, which functional parameters regulate transport and drive GlyR-gephyrin-complexes particularly to inhibitory shaft synapses is currently barely understood.

ACTIVITY-DEPENDENT MECHANISMS REGULATING MICROTUBULE STRUCTURE

Functional regulation of active intracellular transport could occur at least at three different levels. First, neurons apply the alternate use of individual cargo adaptors, which connect motors with selected cargoes and are thought to mediate transport specificity (Setou et al., 2000, 2002; Hirokawa and Takemura, 2005; Maas et al., 2006). In addition, cargo adaptors participate in the regulation of the trafficking direction, for instance whether transport complexes selectively move into axons or dendrites (Setou et al., 2002). Second, activity-dependent phosphorylation of motor proteins upon a Ca^{2+}-dependent activation of the kinase CaMKII, has been shown to regulate synaptic microtubule transport (Guillaud et al., 2008). In fact, phosphorylation of the KIF17 tail led to a local dissociation of an NMDA receptor motor-cargo complex, thereby releasing the cargo vesicle in close proximity to the synapse. Whether similar regulatory signals apply to all synaptic transport systems including the GlyR-gephyrin complex, requires further investigation. However, it is an attractive hypothesis to consider that a local slow-down or dissociation of intracellular trafficking complexes might increase the probability to exchange cargo between intracellular transport and surface membrane compartments. Notably, this model suggests that synaptic activation enables individual synapses to capture new molecules from a nearby flow of intracellular cargo.

A third way to regulate transport is to modify the structure of the tracks along which motors move. Different post-translational modifications (PTMs) of α- and β-tubulin have been described, which include phosphorylation, polyglutamylation, polyglycylation, tyrosination, methylation and acetylation (Verhey and Gaertig, 2007). Upon these modifications, microtubules create diverse arrays with specific cellular functions in neurons. The addition of post-translational tubulin signals generates subpopulations of microtubules that selectively affect downstream microtubule-based functions, such as for instance the binding of various microtubule-associated proteins (MAPs) that could in turn affect kinesin motility (Fukushima et al., 2009) (**Figure 3**). Posttranslational addition of elongated polyglutamyl side chains to tubulin had been previously shown to functionally regulate the transport of

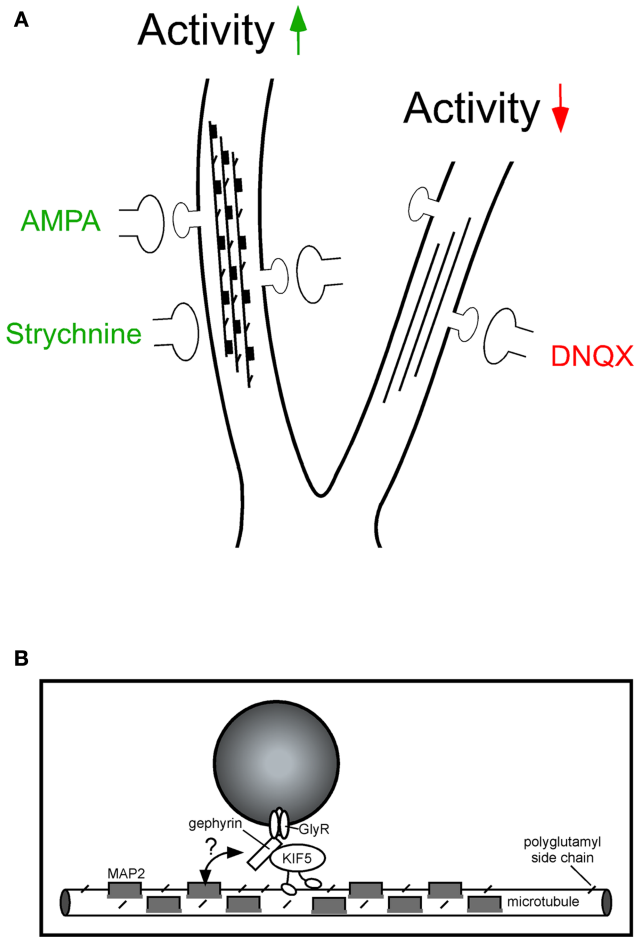

FIGURE 3 | Activity-dependent polyglutamylation of tubulin alters intracellular transport. (A) Model of microtubule track changes through polyglutamylation (diagonal lines) and MAP2 binding (dark squares) upon altered neuronal activity. Increased activity, as induced through GlyR blockade (strychnine) or AMPAR activation (AMPA), interferes with gephyrin delivery into distal neurites (left). This effect is not observed upon neuronal activity reduction through AMPAR blockade (6,7-Dinitroquinoxaline-2,3-dione, DNQX) and can be prevented through functional depletion of neuronal polyglutamylase (the respective enzyme that adds polyglutamyl side chains to tubulin). Although it is unclear which modification is dominant, both represent negative signals for cargo delivery. **(B)** The individual cargo adaptor in the motor-cargo complex (gephyrin) is thought to mediate specificity of transport, as individual motor proteins transport multiple cargoes. Notably, KIF5-mediated transport of gephyrin is significantly reduced under strychnine conditions, whereas KIF5-mediated transport of GRIP1 (another cargo adaptor driven by the same motor) remains unaltered. The individual cargo adaptor within the transport complex (gephyrin) is therefore a candidate factor to sense modifications at the microtubule track surface (double arrow, question mark). Modified after Maas et al. (2009).

synaptic vesicles, a cargo of KIF1A (Ikegami et al., 2007). A recent study further revealed that tubulin polyglutamylation in neurons is significantly enhanced upon increased neuronal activity, induced through either AMPAR activation or blockade of the inhibitory GlyR with its antagonist strychnine (Maas et al., 2009) (**Figure 3A**). With respect to GlyR-gephyrin intracellular transport, increased tubulin polyglutamylation negatively interfered with gephyrin delivery into peripheral neurites and led to protein accumulation in the cell soma. Notably, these effects could be prevented upon gene expression knockdown or functional inactivation of the respective enzyme, known as neuronal polyglutamylase. This indicates that an activity-dependent signalling cascade crosstalks to enzymes involved in microtubule modification (Maas et al., 2009). Polyglutamylation further regulates the binding of MAPs to microtubules (Bonnet et al., 2001) and MAP2 is known to negatively influence kinesin transport (von Massow et al., 1989; Lopez and Sheetz, 1993). Consequently, strychnine blockade of GlyRs over 8h significantly increased MAP2 binding to microtubules and reduced KIF5C particle mobilities, the actual motor involved in anterograde GlyR-gephyrin transport (Maas et al., 2009) (**Figure 3B**). It is therefore likely that synaptic transmission induces intracellular signalling that regulates the PTMs of transport tracks. These PTMs in turn determine in an activity-dependent manner, how much cargo may arrive at synaptic sites at a given time. Interestingly, live cell imaging revealed that strychnine-mediated GlyR blockade altered the percentage of mobile gephyrin, but not of GRIP1 particles over time, although both proteins act as cargo adaptors of the same motor protein (KIF5) and couple this motor to either GlyRs (Maas et al., 2009) or AMPARs (Setou et al., 2002), respectively. The currently available data therefore suggest that the actual cargo adapter, but not the motor itself, represents a critical factor that senses surface modifications at microtubule transport tracks, as induced through neuronal activity changes (**Figure 3**).

Regulatory mechanisms of this kind would be suitable to determine the intracellular transport direction of cargoes in a complex dendritic tree. If synapses in a local branch of a dendrite were to be highly active, cargo delivery into this region could be compromised due to microtubule PTMs that act as negative traffic signs or stop signals. In contrast, cargo transport into neurites, where reduced synaptic activity occurs, would be promoted. It will remain a future challenge to identify the intermediate components that mediate signalling between synaptic surface membranes and microtubules. Furthermore, it will have to be identified whether other posttranslational tubulin modifications, as for instance tubulin tyrosination (Konishi and Setou, 2009), undergo activity-dependent regulation in neurons. In summary, intracellular transport critically participates in the steady-state process of synaptic molecule turnover in neurons and can be tuned by synaptic activity at different molecular levels, including cargo adaptor, motor protein and cytoskeletal track levels. However, it should be noted that the lateral diffusion of surface membrane receptors, also known to undergo activity-dependent regulation (Lévi et al., 2008), might apply independent signalling pathways.

MEMBRANE INSERTION OF GlyR

In theory, exocytosis of the GlyR-gephyrin complex could happen either at specific sites (such as the postsynaptic density), or at random locations of the plasma membrane, followed by subsequent incorporation in the synapse. There is a lack of data regarding this question, and only indirect evidence suggests that delivery of GlyR does not happen at synaptic sites (Rosenberg et al., 2001). Regarding other receptors, $GABA_AR$ exocytosis occurs exclusively at extrasynaptic sites (Thomas et al., 2005; Bogdanov et al., 2006), and studies on the AMPAR GluR1 subunit showed that it is inserted

in somatic and dendritic locations (Adesnik et al., 2005; Yudowski et al., 2007) and in the latter case, in the spine membrane (Park et al., 2004). However, there can be variations among different receptors and among subunits of the same receptor, since the AMPAR GluR2 subunit has been shown to be inserted directly at synapses (Passafaro et al., 2001).

DYNAMICS OF GlyR AND GEPHYRIN IN THE PLASMA MEMBRANE

GlyR DIFFUSION IN THE PLASMA MEMBRANE

Receptors are transmembrane proteins and, as for any other protein inserted in the plasma membrane, their movements undergo physical constraints. The fluid mosaic cell membrane model established more than thirty years ago by Singer and Nicolson (1972) predicted "lateral and rotational freedom and random distribution of the components in the membrane." Since then, it has been deeply remodelled and a new concept emerged where diffusion is far from being unrestricted (see Vereb et al., 2003 for a review). We now know that the plasma membrane is dynamic and structured, containing proteins that act as transient traps for other proteins ("pickets": individual or multimolecular complexes, and lipid rafts microdomains) and obstacles that restrict their diffusion ("fences", such as submembraneous filaments of cytoskeleton) (Dietrich et al., 2002; Kusumi et al., 2005). In addition, one should bear in mind that inhibitory postsynaptic membranes are highly viscous and crowded, more than excitatory ones (Renner et al., 2009).

Once inserted in the plasma membrane, how does GlyR behave? In spite of what was known about the fluidity of the membrane, for a long time only immunocytochemistry of fixed tissue or cells could be used to visualize receptors and synapses. A static view of the synapse prevailed, revealing only the amount of receptors clustered in front of the presynaptic bouton at a given moment. This was also true for the putative receptors located in extrasynaptic regions, whose presence was suggested by electron microscopy observations and electrophysiological recordings. A more refined picture is now available, that takes into account both plasma membrane intrinsic features and time. Progress in videomicroscopy techniques and in particular the improvement of the CCD camera sensitivity, along with the use of fluorescent probes, made the study of the dynamics of living cells material possible. A study by Rosenberg et al. (2001) provided the first evidence that surface membrane GlyRs, as located outside synaptic sites, had a dynamic behaviour. The authors followed the temporal sequence of GlyRα1 insertion on the plasma membrane and observed the initial insertion of GlyRs at the somatic membrane level. GlyR diffused from there to dendritic sites, at an estimated linear diffusion rate of 5×10^{-2} μm s^{-1}. In a different approach, Meier et al. (2001) used optical tweezers to direct a 0.5-μm-latex bead, coupled to antibodies against GlyR, and observed the trajectories of the bead moving on the surface of spinal cord neurons. This demonstrated for the first time that individual receptors were able to diffuse within the plasma membrane.

A real breakthrough on the study of membrane GlyR behaviour came later on from the use of antibodies coupled to quantum dots (QDs) (Dahan et al., 2003). QDs are nanometer-sized probes that provide long-lasting fluorescence emission (Bawendi et al., 1990; Bruchez et al., 1998). Because of this property, they can be used to track identified molecules (single-particle tracking, SPT) for periods much longer than organic fluorescent dyes (20 min vs. 10 s). Trajectories of QDs recorded on living neurons revealed that, at the cell surface, GlyRs exchanged rapidly between extrasynaptic and synaptic compartments (Dahan et al., 2003) (**Figure 4A,B**). In extrasynaptic regions, QD-labelled GlyRs had characteristic Brownian, free-diffusing molecules trajectories. In the membrane context, these are passive random movements of proteins within the lipid bilayer that give a characteristic linear function of the mean square displacement (MSD) versus time (**Figure 4C**). The mean diffusion coefficient can be inferred from the MSD curves, and revealed that GlyR explored an extrasynaptic area of 1×10^{-1} μm^2 s^{-1}.

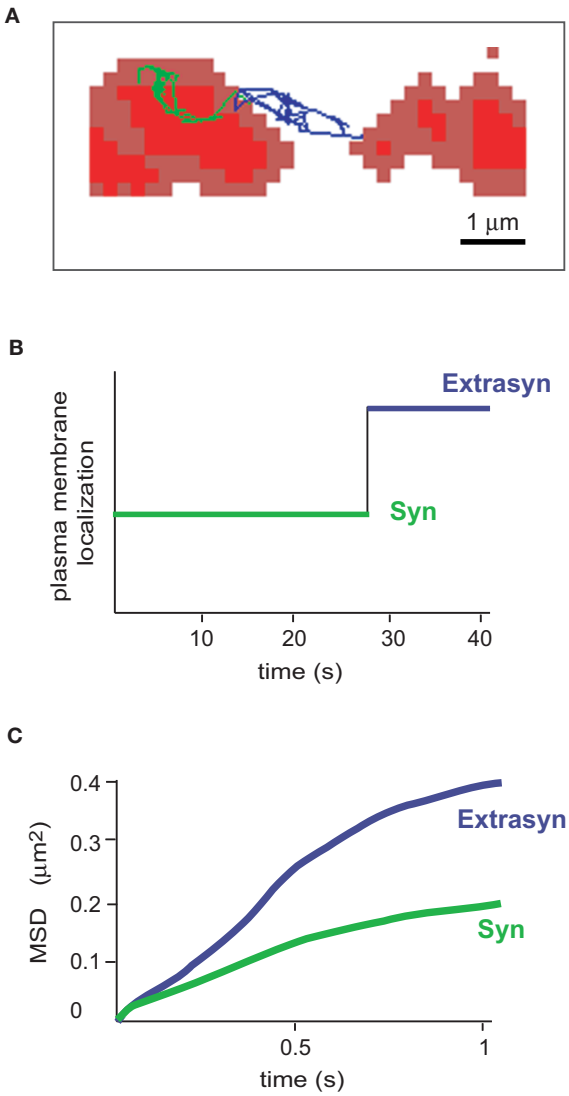

FIGURE 4 | Diffusion properties of the glycine receptor. (A) Example of an individual GlyR-QDot trajectory exchanging between a synaptic (trace in green) and an extrasynaptic location (trace in blue). FM4-64-stained synapses are in red. **(B)** Time spent by the GlyR-QDot in the different compartments over a 40-s recording (same colour code). **(C)** Time-averaged MSD function of the QDot shown in **(A)**. The two curves represent synaptic (green) and extrasynaptic (blue) portions of the trajectory. Curves are typical of confined (negatively bent) and free-diffusing molecules, respectively.

Within the synaptic compartment, two receptor populations could be distinguished: "rapid"-diffusing receptors (mean diffusion value of 7.3×10^{-2} μm^2 s^{-1}, about 20% of synaptic receptors) and "slow"-diffusing ones (1×10^{-3} μm^2 s^{-1}). The latter showed a biphasic MSD curve, typical of movements limited by other proteins inserted in or associated with the plasma membrane, and also called *confined diffusion* (**Figure 4C**). Thus, the spontaneous trajectories of GlyRs showed that one receptor molecule can change from one diffusive state to another as it travels through distinct functional compartments, and that each behaviour has its own characteristics.

The same swapping behaviour between synaptic and extrasynaptic domains was observed for other receptors, namely the inhibitory $GABA_A$ receptor (Lévi et al., 2008; Bannai et al., 2009) and glutamatergic AMPA, NMDA and mGluR receptors (Sergé et al., 2002; Tardin et al., 2003; Groc et al., 2004). For these receptors, diffusion rates in the different membrane domains were within similar ranges to those of GlyRs. A general pattern has thus emerged for both inhibitory and excitatory synapses. Receptors can be trapped by and released from an anchoring domain within seconds to minutes, leading to a rapid supply/depletion of receptor molecules at the synapse. This behaviour could account for the diffusion-trap model of receptor accumulation during synapse formation, driven by the progressive recruitment of scaffolding proteins from extrasynaptic to synaptic locations (Kirsch et al., 1993a; Colin et al., 1998; Rao et al., 1998; Cottrell et al., 2000; Dumoulin et al., 2000; Borgdorff and Choquet, 2002; Choquet and Triller, 2003; Bellone and Nicoll, 2007). Diffusion dynamics could also be responsible, along with changes in exocytosis and endocytosis rates, for the rapid regulation of receptor numbers required in synaptic plasticity events such as long-term potentiation (Shi et al., 1999; Lu et al., 2001; Earnshaw and Bressloff, 2006; Lisman and Raghavachari, 2006; Zhao et al., 2008) and long-term depression (Carroll et al., 1999; Luscher et al., 1999; Earnshaw and Bressloff, 2006) (see Newpher and Ehlers, 2009, for a review). Finally, it has been demonstrated that the homeostatic regulation of the network activity itself was mediated at the cellular level through the lateral diffusion of receptors, as discussed in Section "Regulation of GlyR diffusion by neuronal activity" in this review.

ROLE OF THE SCAFFOLDING MOLECULE GEPHYRIN IN GlyR DIFFUSION

The previous results showed that the receptors have confined trajectories whenever located at synaptic sites. To assess whether there was a link between diffusion of GlyR and interaction with the scaffolding protein gephyrin, Meier et al. (2001) transfected neurons with Venus-tagged Gephyrin (Ve-Ge) and a modified GlyRα1 construct able to bind gephyrin (GlyRa1bgb; Meier et al., 2000). Trajectories were followed thanks to a latex bead coupled to antibodies against GlyR. This approach showed that GlyRα1βgb clusters exhibited lateral diffusion along the plasma membrane and, in the absence of gephyrin, trajectories were typical of free diffusing molecules (diffusion coefficient 2.5×10^{-2} μm^2 s^{-1}). In neurons co-transfected with Ve-Ge, particles alternated between fast (1.1×10^{-2} μm^2 s^{-1}) and slow (1.1×10^{-3} μm^2 s^{-1}) diffusion rates, depending on the absence or presence of gephyrin clusters, respectively. Interaction between the receptor and the submembraneous protein accounted for the confined movements recorded. When compared with endogenous receptor diffusion results from Q-Dot trajectories (Dahan et al., 2003), values for slow-diffusing receptors were similar in the two experiments. However, extrasynaptic endogenous receptors diffused ten times faster than GlyRα1βg non associated with gephyrin clusters (Meier et al., 2001). This discrepancy could be due to the difference in methodology (Q-Dot vs. 500 nm latex bead). Alternatively, it could be explained by a difference in the membrane composition of spinal cord neurons, since Dahan et al. used mature neurons, while Meier et al. used immature ones (2–3 DIV). Indeed, changes in lipids have been documented throughout maturation of neurons in culture (Prinetti et al., 2001), and cholesterol depletion was demonstrated to change the diffusion rates of $GABA_A$R in hippocampal neurons in culture (Renner et al., 2009).

A complementary study further investigated the role of gephyrin in GlyR diffusion, in particular outside synaptic locations (Ehrensperger et al., 2007). As in the previous paradigm, neurons were co-transfected with Ve-Ge and GlyRα1βgb constructions, and this time trajectories of GlyRα1βgb were tracked by use of QDots. The diffusion coefficient found for GlyRα1βgb associated with gephyrin confirmed the slow diffusing rates from Meier et al. (2001). Two new findings arised from this study. First, the diffusion of a native form of GlyRa1 (not binding gephyrin) was 20 times faster than that of GlyRα1βgb in cells cotransfected with Ve-Ge, indicating that, even outside *visible* gephyrin clusters, gephyrin restricts GlyR dynamics. This is coherent with the fact that GlyR and gephyrin associate early after synthesis and during trafficking (Hanus et al., 2004; Maas et al., 2006), and with the presence of their complex at the plasma membrane outside synaptic locations. Second, receptors associated with Ve-Ge could either be stable (high confinement, slow diffusion), or swap between different Ve-Ge clusters during the 40-s recordings. This observations lead to the concept that receptor stabilization by clusters of gephyrin is only transient. However, as receptors can escape from a given gephyrin domain, the diffusive behaviour suggested the existence of multiple association states between the two (Ehrensperger et al., 2007). Transient stabilization by scaffolding proteins also appear to be the rule for other receptors. Jacob et al. (2005) demonstrated that $GABA_A$R diffusion properties also relied on the presence of gephyrin clusters, and reversible interactions in a short time-scale between receptors and scaffolds have also been shown for AMPA receptors with PSD-95 and stargazin (Bats et al., 2007) and for mGluR5 with Homer (Sergé et al., 2002).

DO SYNAPTIC SCAFFOLDS ALSO SHOW DYNAMIC BEHAVIOUR?

Glycine receptor exchanges between synaptic and extrasynaptic compartments, and interaction with the scaffolding protein gephyrin stabilizes receptor movements. But how stable is the scaffold itself? To address this issue, Hanus et al. (2006) recorded the movements of gephyrin in spinal cord neurons transfected with Ve-Ge. Synaptic clusters of Ve-Ge displayed submicrometric lateral motion around a central position, with a diffusion rate of 7.1×10^{-4} μm^2 s^{-1}. This value is within the same range of that of the "slow" endogenous synaptic receptors, but very different from the "fast" synaptic ones (7.3×10^{-2}μm^2 s^{-1}, Dahan et al., 2003). Thus, movements of receptors and movements of gephyrin should be considered as distinct but simultaneous phenomena. Fluorescence recovery after photobleaching (FRAP) experiments proved valuable to further investigate the behaviour of populations of gephyrin molecules within scaffolds. Clusters of Ve-Ge or mRFP-gephyrin were bleached (Calamai et al.,

2009) and in the two cases, 40 % of the bleached molecules were replaced by non bleached ones within 30 min (reviewed in Specht and Triller, 2008). Taken together, these results show that gephyrin clusters move and that, while doing so, molecules of gephyrin exchange between different pools. Molecules being added and removed in a regular fashion to/from the structure formed by gephyrin underneath the synapse could explain that receptors swapping from one domain to another would still be attached to gephyrin molecules even outside synaptic locations, as demonstrated by Ehrensperger et al. (2007). In excitatory synapses, scaffolding proteins also exchange in a dynamic fashion. In particular, CamKII, Homer, GKAP and Shank have an important mobile pool, while PSD95 is relatively stable at the PSD, as shown by FRAP experiments (Gray et al., 2006; Kuriu et al., 2006; Sharma et al., 2006).

However, the question whether gephyrin dynamics could influence the dynamics of GlyR remained to be addressed. The studies cited previously were undertaken with the full-length isoform of gephyrin (Ge, corresponding to the p1 clone in other publications). Still, other splice variants of gephyrin exist in the CNS that can or cannot bind GlyR, and have oligomerization properties different than those described in **Figure 1** (Bedet et al., 2006; Saiyed et al., 2007). Calamai et al. (2009) investigated how changes in gephyrin dynamics, through the oligomerization of different variants and deletion mutants, influenced GlyR clustering and diffusion. At extrasynaptic regions, analysis of SPT trajectories of endogenous GlyR showed that the diffusion rates in neurons transfected with the variants that lacked optimal polymerization properties were significantly higher than in neurons transfected with full-length Ve-Ge ($1–2 \times 10^{-2}$ μm^2 s^{-1} vs. 6.3×10^{-3} μm^2 s^{-1}, respectively). Thus, gephyrin–gephyrin association dynamics do influence the lateral difffusion of GlyR outside the synapse. Such a modulation could not be assessed at synaptic locations since the variants seem to be excluded from mixed synaptic clusters in neurons co-transfected with Ge and the variants (Calamai et al., 2009). However, a direct implication of the integrity of the multimolecular stargazin-PSD95-AMPAR complex on the residency time of AMPA receptors at the synapse has been demonstrated by SPT, in a model of a mutant mouse, deficient for the stargazin-PSD95 interaction (Bats et al., 2007; see Newpher and Ehlers, 2008).

ROLE OF CYTOSKELETAL ELEMENTS IN GlyR AND GEPHYRIN DYNAMICS

Native GlyRs bind to gephyrin through a direct interaction between the GlyRβ subunit M3-M4 loop and the E domain of gephyrin (Kirsch and Betz, 1995; Kneussel et al., 1999; Kim et al., 2006), and gephyrin in turn associates with microtubules. Gephyrin also interacts indirectly with the actin microfilament cytoskeleton through proteins like profilin and Mena/Vasp (Mammoto et al., 1998; Giesemann et al., 2003), and with GTPases through collybistin (Xiang et al., 2006). Actin and microtubules appear thus as good candidates to modulate gephyrin and/or GlyR diffusion, as they do for gephyrin trafficking (Maas et al., 2009). A number of studies indicate that these two components are indeed involved in the regulation of synaptic components density. In particular, a reduction in size and immunoreactivity intensity of gephyrin and GlyR clusters was observed after microtubule depolymerization, correlated with a decrease in the amplitude of glycinergic mISPCs (Kirsch and Betz, 1995; van Zundert et al., 2004; Charrier et al., 2006). A similar response was observed by immunocytochemistry after actin network disruption (Charrier et al., 2006).

The effects of actin filament and microtubule depolymerization on the lateral diffusion of GlyRs (Charrier et al., 2006) and gephyrin (Hanus et al., 2006) were studied by use of the drugs latrunculin and nocodazole, respectively. SPT analysis revealed that, after addition of latrunculin or nocodazole, GlyR diffusion was significantly increased. The receptor explored larger areas of extrasynaptic membrane and exchanged more frequently between extrasynaptic and synaptic compartments. Within synapses, an increase in GlyR diffusion coefficients was seen after latrunculin treatment only (1×10^{-2} μm^2 s^{-1} vs. 1×10^{-3} μm^2 s^{-1} for control). Regarding gephyrin, the synaptic clusters diffused less after microfilament depolymerization, but showed increased MSD values after microtubules disruption. Taken together, these data suggest that, at synaptic locations, actin contributes simultaneously to the confinement of the receptor and to the mobility of gephyrin, regulating the organization of confinement sites (also see Renner et al., 2009). The interactions between gephyrin, GlyR and the cytoskeleton that regulate diffusion appear to be complex and not fully understood. A simpler view emerges from excitatory synapses, probably because mostly actin is present in spines -even though recent data suggest a role for microtubules in spine morphology plasticity (Jaworski et al., 2009). Allison et al. (2000) showed that actin depolymerization reduced the number of AMPAR clusters at both synaptic and extrasynaptic locations, while a reduction was only observed for synaptic NMDAR aggregates. Receptor diffusion was not assessed by SPT in these conditions, but the mobile fraction of scaffolding proteins GKAP, Shank and Homer, that exchanged in a dynamic fashion as seen by FRAP experiments, disappeared after lantrunculin administration (Kuriu et al., 2006). No effect was observed in PSD 95 distribution, which confirms the results obtained by Usui et al. (2003).

REGULATION OF GlyR DIFFUSION BY NEURONAL ACTIVITY

Since receptor diffusion mechanisms appear to be controlled by a range of interacting factors, an important issue is whether activity of the network itself can regulate its behaviour. This question was assessed for GlyR dynamics by SPT in spinal cord neurons, where modifications were induced by administration of tetrodotoxin, alone or in combination with GlyR, $GABA_AR$, AMPAR and NMDAR antagonists (Lévi et al., 2008). Synaptic transmission was shown to control GlyR lateral diffusion via activation of the NMDAR, leading to a greater confinement of synaptic and extrasynaptic receptors and slower diffusion rates. This was correlated with increased levels of GlyR in synaptic clusters and increased amplitude of glycinergic mIPSCs. Thus, global excitatory activity directly controls efficiency of transmission through receptor lateral diffusion and clustering, and suggests an implication of GlyR diffusion in homeostatic regulation (i.e. the mechanisms through which a neuron adapts its inhibition when the excitation level is modified). In this case, changes in diffusion rates could be a very early step in network homeostasis. A study by Bannai et al. (2009) on $GABA_AR$ in hippocampal neurons revealed that upon pharmacological increase of excitatory activity, the synaptic and extrasynaptic diffusion coefficients of $GABA_AR$ were increased, and that they were correlated with reduced confinement areas and decreased amplitude of the recorded mIPSCs. These major findings demonstrate that network excitatory activity regulates GlyR and GABAR diffusions in opposite directions, highlighting a functional regulatory difference between the two inhibitory receptors. Interestingly, in mixed

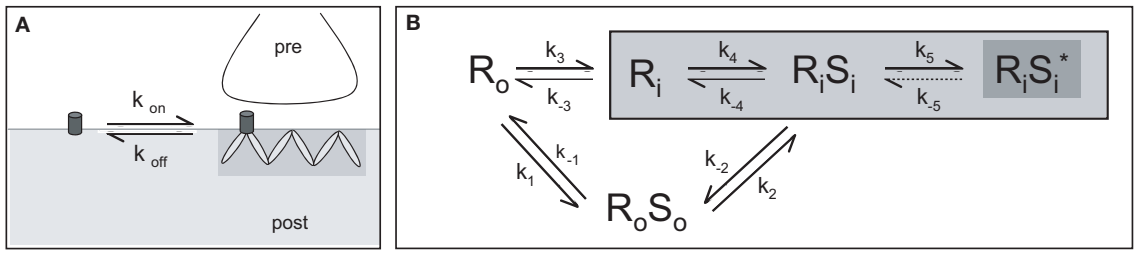

FIGURE 5 | Model of receptor diffusion and stabilization at synapses. **(A)** Receptor exchanges between extrasynaptic and synaptic domains. The rates of entry and exit from gephyrin clusters define the k_{on} and k_{off}, respectively. **(B)** Representation of the different paths leading to the stabilization of GlyR by gephyrin clusters. Association of receptor (R) and its scaffolding protein gephyrin (S) can occur outside (equilibrium 1) or inside (equilibrium 4) synaptic sites. Once within clusters, receptor-scaffold complexes may reach a higher level of stabilization (equilibrium 5, dark gray). The index "i" indicates the inside and the index "o" the outside of the synaptic domain (light gray area) (modified from Ehrensperger et al., 2007).

inhibitory synapses (containing both GlyR and $GABA_AR$) of spinal cord neurons (Lévi et al., 1999; Dumoulin et al., 2000), no effect of excitatory activity mediated by NMDAR was observed on the lateral diffusion of $GABA_AR$ (Lévi et al., 2008). Finally, among the glutamatergic receptors, AMPAR but not NMDAR diffusion dynamics were found to be activity-dependent (Tardin et al., 2003; Groc et al., 2004, 2006; Ehlers et al., 2007). In conclusion, homeostatic adaptation by receptor diffusion regulation represents a mechanism used by several neurotransmitter systems to drive quick changes in the distribution of receptor molecules between extrasynaptic and synaptic compartments and tune individual synaptic strength to the network activity. Noteworthy, receptor diffusion regulation by global activity has been shown to rely on calcium signalling (Borgdorff and Choquet, 2002; Tardin et al., 2003; Lévi et al., 2008; Bannai et al., 2009).

THEORETICAL MODEL OF RECEPTOR DIFFUSION REGULATION

Analysis of receptor trapping and release events from postsynaptic gephyrin-containing scaffolds led to the view of receptor dynamics as an equilibrium state where scaffolds behave simultaneously as acceptors and donors of receptors. This interaction can be chemically characterized by association (k_{on}) and dissociation constants (k_{off}) (**Figure 5A**). Multiple association states exist between the two proteins, which can be summarized in the equilibrium representation in **Figure 5B** (Choquet and Triller, 2003; Holcman and Triller, 2006; Ehrensperger et al., 2007; Triller and Choquet, 2008). Receptors can associate/dissociate with the scaffold molecule within the synapse or outside of it, and diffuse together on the cell membrane. However, from the results presented here, we can expect the receptor forms non associated with gephyrin (either at extrasynaptic or at synaptic sites, and defined as Ro and Ri in **Figure 5**, respectively) to represent only a small proportion of the receptor pool. Once present in an associated form at synaptic sites (RiSi), a higher degree of stabilization of the complex could be reached (equilibrium 5, asterisk). Such a dynamic equilibrium starts unravelling the apparent paradox in which the function of a synapse requires it to be stable in time (metastability), and still its receptors units show instability by quickly getting in and out of it. This model, obtained through results of GlyR diffusion, is also supported by and can be generalized for other receptors.

Based on the previous model, Sekimoto and Triller (2009) developed a new general mesoscopic model, considering the highly compartmentalized structure of the synapse. The postsynaptic domain was spatially defined as a three-layer (membrane, sub-membrane, cytoplasm), two-zone (synaptic, extrasynaptic) model where all interactions between receptors and scaffolding proteins occurred. Within this model, the authors considered both the concentrations and chemical potentials of receptor and scaffolding protein. This resulted in a highly cooperative thermodynamic model of postsynaptic domain stability. Changing the concentrations of receptor and scaffolding molecules in a given compartment, or modifying the interaction between them, led to discrete modifications of receptor numbers at synapses. Another important issue arising from this work is that stabilization is a reciprocal mechanism: receptors are stabilized by their interaction with the scaffold, but the opposite is also true. This notion can be particular relevant during synapse formation and plasticity, since no player on its own could be responsible for synapse construction and adaptive modifications.

In conclusion, we have focused on the review and discussion of data regarding the trafficking of the GlyR inside the neuron and at the plasma membrane. GlyRs associate intracellularly after synthesis with gephyrin, and the complex travels to the membrane applying the microtubule-dependent motor protein KIF5. Transport can be regulated by neuronal activity through phosphorylation of motor proteins or through PTMs of tubulin. Once inserted in the membrane, the GlyR-gephyrin complex is able to diffuse to synaptic sites where it is stabilized. However, stabilization is transient, since molecules can exchange rapidly between different compartments, and relies on the integrity of the cytoskeleton. Theoretical models indicate that the transient stabilization of the receptor by gephyrin and the turnover of the latter is however compatible with a "dynamic stabilization" of the postsynaptic domain. Finally, network activity influences both intracellular transport and the diffusion dynamics of GlyRs, which adapts its numbers at synapses to match activity changes in a homeostatic fashion.

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Glycinergic synapse development, plasticity, and homeostasis in zebrafish

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The zebrafish glial glycine transporter 1 (GlyT1) mutant provides an animal model in which homeostatic plasticity at glycinergic synapses restores rhythmic motor behaviors. GlyT1 mutants, initially paralyzed by the build-up of the inhibitory neurotransmitter glycine, stage a gradual recovery that is associated with reductions in the strength of evoked glycinergic responses. Gradual motor recovery suggests sequential compensatory mechanisms that culminate in the down-regulation of the neuronal glycine receptor. However, how motor recovery is initiated and how other forms of plasticity contribute to behavioral recovery are still outstanding questions that we discuss in the context of (1) glycinergic synapses as they function in spinal circuits that produce rhythmic motor behaviors, (2) the proteins involved in regulating glycinergic synaptic strength, (3) current models of glycinergic synaptogenesis, and (4) plasticity mechanisms that modulate the strength of glycinergic synapses. Concluding remarks (5) explore the potential for distinct plasticity mechanisms to act in concert at different spatial and temporal scales to achieve a dynamic stability that results in balanced motor behaviors.

Keywords: glycinergic synapse, synaptic plasticity, GlyT1 mutant, glycine encephalopathy, motor behavior, zebrafish, glycine receptor, glial glycine transporter

The zebrafish, *Danio rerio*, provides a vertebrate animal model of inherited human diseases that impact glycinergic synapse function and plasticity (Oda et al., 1998; Cui et al., 2005; Hirata et al., 2005; Downes and Granato, 2006; Rigo and Legendre, 2006; Mongeon et al., 2008). As the predominant inhibitory neurotransmitter in vertebrate brain stem and spinal cord, glycine is critically important for the generation of rhythmic motor behaviors (Moss and Smart, 2001). Indeed, in humans, rhythmic motor behaviors are disrupted by mutations that either reduce glycinergic signaling in the case of the startle syndrome hyperekplexia (Bakker et al., 2006; Harvey et al., 2008), or augment glycinergic signaling in the case of glycine encephalopathy (Applegarth and Toone, 2006). In patients with glycine encephalopathy, elevated glycine disrupts respiratory circuits so that babies with the disease require a ventilator to breathe (Applegarth and Toone, 2004). With time however, many affected infants recover the ability to breathe on their own, and a small subset of these infants recover normal neurological functions (Boneh et al., 2008), suggesting compensatory or homeostatic mechanisms can reduce the severity of the disease. Variable outcomes in human patients with glycine encephalopathy stand in contrast to 100% motor impairment in the mouse, or 100% motor recovery in the zebrafish models of the disease (Gomeza et al., 2003a; Luna et al., 2004). Zebrafish, therefore, are ideal models for studying homeostatic mechanisms that can restore motor behaviors. In zebrafish, gradual motor recovery is mirrored by reductions in evoked glycinergic post-synaptic potentials. This gradual reduction in the strength of glycinergic signaling culminates with the down-regulation of glycine receptor RNA and protein (Mongeon et al., 2008). Still, there are outstanding questions, such as how motor recovery is initiated, and how sequential plasticity mechanisms orchestrate behavioral recovery. We discuss these questions in the context of what is known about synaptogenesis and plasticity at glycinergic synapses.

GLYCINERGIC SYNAPSES IN PATTERNED MOTOR BEHAVIORS

Patterned motor output is generated in the spinal cord by neuronal circuits made up of excitatory (mostly glutamatergic) and inhibitory (mostly glycinergic) interneurons that synapse both with each other and with motor neurons. The balance between excitatory and inhibitory synapses onto interneurons and motor neurons underlies normal functioning of locomotor circuits that produce rhythmic motor output (Grillner et al., 1995; Hultborn and Nielsen, 2007). In mammals, for example, individual motor neurons coordinate between 20,000 to 50,000 synaptic inputs (Gelfan, 1963; Hochman, 2007). These pre-synaptic inputs align with post-synaptic receptors to determine the duration and frequency with which post-synaptic neurons fire action potentials. Genetic disruption of glycinergic synapses onto both interneurons and motor neurons results in locomotory dysfunction (Kingsmore et al., 1994; Mulhardt et al., 1994; Gomeza et al., 2003a, b; Cui et al., 2005; Hirata et al., 2005). Such locomotory dysfunction is exhibited by two human genetic diseases, hyperekplexia and glycine encephalopathy. Hyperekplexia is characterized by insufficient glycinergic inhibition leading to an excessive startle response (Harvey et al., 2008). Glycine encephalopathy, in contrast, is marked by excess glycinergic inhibition leading to hypotonia and subsequent difficulties in breathing (Applegarth and Toone, 2006).

As a model for genetically inherited diseases of the nervous system, zebrafish have significant advantages that compliment existing mammalian models. In particular, compared to mammals, zebrafish develop externally over a compressed time scale, facilitating both observations and experimental manipulations to understand

underlying mechanisms by which genetic mutations manifest themselves in stereotyped behaviors (Granato et al., 1996; Gahtan and Baier, 2004; Burgess and Granato, 2007; Fetcho et al., 2008; McLean and Fetcho, 2008). At the interface of behavior and genetics lie neuronal circuits that in zebrafish are accessible to both *in vivo* electrophysiological and imaging analyses (Ali et al., 2000; Saint-Amant and Drapeau, 2001; Ibanez-Tallon et al., 2004; Wen and Brehm, 2005; McLean et al., 2008; Wyart et al., 2009). Taken together, the ability to visualize development *in vivo* as well as to study genetic correlates of human disease make zebrafish an ideal and accessible model to address the ontogeny of inherited nervous system deficits.

The accessibility of the zebrafish nervous system makes it feasible to directly record inhibitory glycinergic responses in motor neurons. Specifically, inputs onto primary motor neurons from morphologically and genetically identified glycinergic inhibitory CoLo (Commissural Local) interneurons can be recorded. The ability to record from these interneurons *in vivo* has revealed their specific contribution to escape behaviors in zebrafish (Liao and Fetcho, 2008; Mongeon et al., 2008; Satou et al., 2009). Escape behavior in zebrafish larvae consists of a strong contra-lateral bend away from the stimulus (Faber et al., 1989). To ensure that *only* the side of the fish contralateral to the stimulus responds, the glycinergic CoLos inhibit motor neurons ipsi-lateral to the stimulus (Satou et al., 2009). Therefore, recordings of evoked CoLo post-synaptic potentials in motor neurons can reveal the contribution of particular synaptic proteins such as GlyT1 to glycinergic synapse function (Mongeon et al., 2008).

In the GlyT1 mutant, CoLo/motor neuron synaptic recordings can help explain the progression of GlyT1 mutant motor behaviors (Mongeon et al., 2008). As embryos and early larvae, GlyT1 mutants exhibit reduced movements that correspond to augmented glycinergic potentials (Mongeon et al., 2008). Elevated glycinergic inhibition throughout the nervous system would globally reduce excitability and motor output. This period of reduced excitability transitions to motor recovery at which point glycinergic potentials are dramatically reduced (Mongeon et al., 2008). This reduction in glycinergic synaptic responses reflects a global increase in nervous system tolerance to glycine that underlies motor recovery (Mongeon et al., 2008). Although the zebrafish spinal cord is less complex than the mammalian spinal cord both in terms of the gross neuron populations as well as the diversity of functional classes of interneurons (Goulding, 2009), zebrafish and mammalian spinal cords have proven similar in the physiological basis for mutant phenotypes (Tropepe and Sive, 2003; Ingham, 2009).

PROTEINS THAT DETERMINE GLYCINERGIC SYNAPTIC STRENGTH

The proteins of the glycinergic inhibitory synapse work together to:(1) package glycine into pre-synaptic vesicles, (2) concentrate post-synaptic glycine receptors so that receptors are juxtaposed with the pre-synaptic terminal, and (3) remove and recycle unbound glycine from the synaptic cleft to achieve temporal control of glycinergic synaptic transmission (Dresbach et al., 2008).

GLYCINERGIC PRE-SYNAPSE

Many pre-synaptic proteins localized at glycinergic synapses function to package and/or recycle glycine. Packaging glycine involves concentrating glycine into synaptic vesicles. A central player in this process, the Vesicular Inhibitory Amino Acid Transporter, VIAAT, utilizes a proton concentration gradient to transport glycine (as well as GABA) into synaptic vesicles. Because VIAAT binds glycine with low affinity, glycine must be concentrated in the synaptic terminal to millimolar levels by yet another transporter, the neuronal Glycine Transporter 2, GlyT2, expressed in the plasma membrane of the axon terminal. GlyT2 couples transport of one molecule of glycine to the cotransport of three Na^+ ions and one Cl^- ion, supporting the uni-directional transport of glycine into the axon terminal (Chen et al., 2004; Rees et al., 2006). Pre-synaptic release of glycine into the synaptic cleft then sets into motion several glycine uptake mechanisms that help to determine the time-course of glycinergic signaling. GlyT2 is critical to recycling glycine into the pre-synaptic axon terminal (Gomeza et al., 2003b).

GLIA

Like GlyT2, the glial glycine transporter type 1, GlyT1, maintains glycine levels by effectively removing glycine from the synapse and terminating glycinergic transmission (Eulenburg et al., 2005). GlyT1 is structurally similar to the 12-membrane spanning GlyT2 but differs in its expression domain and the stoichiometry of its transport mechanism. Like GlyT2, GlyT1 also employs sodium and chloride gradients to transport glycine, but unlike GlyT2, GlyT1 can both import and export glycine thus playing an important role in setting the levels of glycine bathing the nervous system (Supplisson and Roux, 2002; Eulenburg et al., 2005). While GlyT1 is predominantly expressed in glial cells, GlyT2 expression is specific to the pre-synaptic terminal (Zafra et al., 1995; Eulenburg et al., 2005; Betz et al., 2006).

GLYCINERGIC POST-SYNAPSE

In the glycinergic post-synapse, gephyrin bridges glycine receptors and the cytoskeleton (Prior et al., 1992; Charrier et al., 2006) and serves as a docking site for multiple regulatory proteins (**Figure 1**; Fritschy et al. 2008). Microtubules serve as highways along which glycine receptor/gephyrin complexes are delivered to the plasma membrane by dynein light chain 1 and 2 motors (Maas et al., 2006), while the sub-synaptic actin cytoskeleton provides a gephyrin-mediated anchor for the glycine receptor and is especially important for synaptic localization (Kirsch and Betz, 1995; Fritschy et al., 2008).

DISEASES OF THE GLYCINERGIC SYNAPSE

The inherited human diseases glycine encephalopathy and hyperekplexia result from defects in glycinergic signaling. Glycine encephalopathy is caused by tonic activation of glycinergic synapses, and is often a fatal disease as affected infants require artificial ventilation immediately after birth (Applegarth and Toone, 2004). With ventilation aids, some infants that exhibit glycine encephalopathy recover balanced patterned motor output (Boneh et al., 2008). Like their human counterparts, phenotypes displayed by GlyT1 mutant mice and zebrafish reflect increased inhibition as a result of inadequate glycine clearance from the synapse (Lopez-Corcuera et al., 2001; Eulenburg et al., 2005; Betz et al., 2006). In contrast, Hyperekplexia, caused by mutations that reduce the strength of glycinergic signaling, is defined by an exaggerated startle response: acoustic or tactile stimuli induce hypertonia

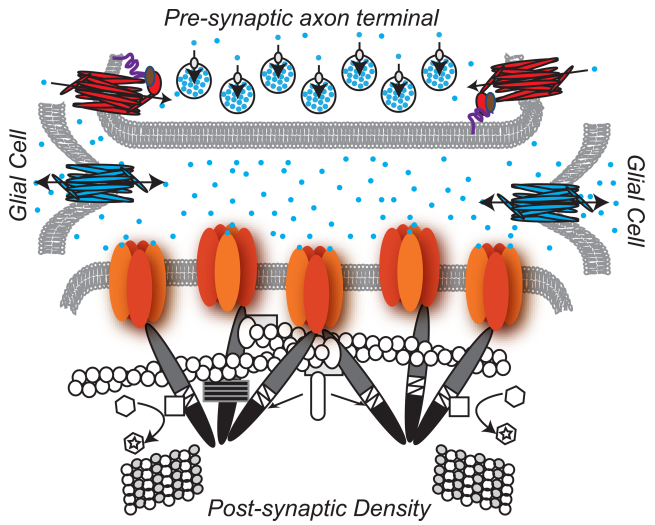

FIGURE 1 | The glycinergic synapse. Pre-Synaptic: Glycine is the predominant inhibitory neurotransmitter in the spinal cord. GlyT2-neuronal Glycine Transporter 2 functions in glycine re-uptake from the synaptic cleft (Betz et al., 2006) and is responsible for neurostransmitter recycling in the pre-synaptic terminal. GlyT2 is localized at the axon terminal through its carboxy-terminal, PDZ, protein-interaction domain. The GlyT2 PDZ domain anchors the GlyT2 to the PDZ protein syntenin 1, which in turn binds syntaxin, a member of the SNARE complex that governs fusion of vesicles with the plasma membrane (Geerlings et al., 2000; Geerlings et al., 2001; Ohno et al., 2004; Armsen et al., 2007). VIAAT-the Vesicular Inhibitory Amino Acid Transporter loads glycine into synaptic vesicles (Gasnier, 2004). Synaptic vesicles accumulate at the active zone of the axon terminal due to the actions of the SNARE complex, a large set of proteins, some of which are expressed on the vesicular membrane, and others of which are expressed on the plasma membrane (Rizo and Rosenmund, 2008). Glia: GlyT1-glial Glycine Transpoter 1 regulates the amount of glycine available to bind the glycine receptor and, at some synapses, has been shown to play a key role in terminating glycinergic synaptic transmission (Betz et al., 2006). Post-synaptic: Enriched in the post-synaptic membrane directly across from the pre-synaptic terminal, glycine receptors are chloride ion channels (Lynch, 2004). Five subunits, two alpha and three beta, associate to form the functional channel (Grudzinska et al., 2005). At least four independent genes (Glra1-4) encode alpha subunits in vertebrates. The α subunit genes encode the glycine binding site that when bound to the substrate, glycine, gates the channel. One gene, Glrb, encodes the β subunit. β subunits link the glycine receptor to the cytoskeleton through high affinity associations with the post-synaptic scaffold protein gephyrin. Gephyrin (Greek for bridge) links the glycine receptor to the cytoskeleton, forming a submembranous, hexagonal lattice (Bechade et al., 1996; Fritschy et al., 2008). The carboxy-terminal E domain of gephyrin dimerizes and interacts with the β subunit of the glycine receptor. The E domain also interacts with profilin 0 and Mena/VASP, both proteins involved in actin microfilament polymerization. The amino terminal G domain of gephryin forms trimers. A proline rich C domain is located between the E and G domains. This C domain is a highly-regulated platform for possible interactions between gephyrin and several other proteins. These proteins include: microtubules; GEFs Guanine nucleotide Exchange Factors (collybistins at GABAergic synapses; as yet unknown at glycinergic synapses) that, by activating CDC42, remodel the actin cytoskeleton; and RAFT1 that regulates localized protein translation. Finally, Pin-1 Peptidyl-prolyl isomerase 1 alters the configuration of gephyrin by inducing proline isomerization in a phosphorylation-dependent manner (Zita et al., 2007).

and difficulty breathing (Fritschy et al., 2008; Harvey et al., 2008). Mutations in either pre-synaptic genes, e.g. GlyT2, or post-synaptic genes, e.g. glycine receptor subunits, are known to cause the disease in humans (Harvey et al., 2008). Animal models, including GLRA1: *spasmodic* (Ryan et al., 1994; Findlay et al., 2003), GLRB: *spastic* (Kingsmore et al., 1994; Mulhardt et al., 1994), and GlyT2 knockout mice (Gomeza et al., 2003b) have paved the way to our understanding of the physiological basis of this genetic disease. Unlike the case in humans, the hypertonic phenotype in mice is lethal. Like mouse models, the zebrafish glycine receptor β mutant *bandoneon* mirrors the hyperekplexia phenotype, but unlike mouse is accessible at earlier developmental stages (Hirata et al., 2005). There are patients that present with hyperekplexia symptoms but have no mutations in known culprit genes (Rees et al., 2006; Harvey et al., 2008; Kalscheuer et al., 2009). Some potential candidate culprits include the host of other proteins enriched at the glycinergic synapse (Harvey et al., 2008).

FORMATION OF GLYCINERGIC SYNAPSES

Our understanding of the process of synaptogenesis has been transformed by *in vivo* studies in zebrafish (Niell et al., 2004; Meyer and Smith, 2006). These studies demonstrate that synaptogenesis occurs coincident with neuronal morphogenesis. *In vivo* synaptogenesis models also confirm the requirement for neuronal activity during synapse establishment (Kirsch and Betz, 1998). Synaptic glycine receptors exchange with extra-synaptic clusters or individual receptors within or outside the synapse (Meier et al., 2000). Because differences in glycinergic synaptic strength often rely on receptor density, plasticity mechanisms would depend on the ability of receptors to accumulate at or dissociate away from the synapse (Legendre et al., 2002).

In the nascent nervous system, synapses form during a time of several fundamental transitions: young neurons extend axons and dendrites to build circuits (Niell et al., 2004; Meyer and Smith, 2006); neuronal communication transitions from predominantly gap-junction mediated to predominantly synapse mediated (Saint-Amant and Drapeau, 2001; Brustein et al., 2003); and glycine- and GABA-evoked chloride conductances are depolarizing (Reynolds et al., 2008). Both maturing glycinergic and GABAergic synapses switch from initial excitatory- to inhibitory-signaling due to the expression of the potassium-chloride co-transporter 2, KCC2 (Rivera et al., 1999). Prior to maturation, both the ligand-gated receptors that underlie synaptic transmission and the voltage-gated ion channels that underlie endogenous firing properties are distinct from their mature counterparts (Ali et al., 2000). Immature neurons tend to express relatively few channels that underlie qualitatively different, low-frequency, spontaneous forms of electrical activity (Moody and Bosma, 2005) that trigger neurotransmitter release from growing axons during early steps in synapse formation (Zhang and Poo, 2001).

Many models of activity-mediated glycinergic synapse formation were derived from neuronal tissue culture (Bechade et al., 1996; Meier, 2003). Kneussel and Betz (2000) proposed the membrane activation model, suggesting that after glycine binds the receptor, post-synaptic depolarization activates calcium channels and triggers the specialization of the post-synaptic density. As the

pre-synaptic axon terminal matures, expressing VIAAT, gephyrin clusters become localized to the post-synaptic membrane. Specifically, glycine receptors are clustered when gephyrin anchors itself to the cytoskeleton, and binds the β subunit of glycine receptors (Kneussel and Betz, 2000; Luscher et al., 2000). Gephyrin can aggregate independent of synapse formation (Colin et al., 1996; Dumoulin et al., 2000), but receptors fail to cluster when gephyrin expression is reduced by antisense oligonucleotides (Kirsch et al., 1993). Moreover, initial synaptic receptor clustering also requires receptor activation (Levi et al., 1999): when glycine receptors are blocked with strychnine, the receptors no longer form clusters (Kirsch and Betz, 1998). Although activation of the receptor appears to be essential for specialization of the post-synaptic density, high levels of glycine in culture media are not sufficient to trigger receptor clusters, suggesting that other factors, such as cell adhesion molecules, associated with the pre-synaptic terminal are essential for glycine receptor clustering (Legendre, 2001).

Because the process of synaptogenesis establishes the template upon which mature behaviors are formed, how synaptogenesis is impacted by mutations in synaptic genes is likely to inform our understanding of mutant phenotypes. The accessibility of the zebrafish nervous system to both physiological recordings and imaging make this organism especially well-suited to addressing how mutations affect the process of synaptogenesis.

PLASTICITY MECHANISMS AT THE GLYCINERGIC SYNAPSE

In order to produce appropriate responses to varying stimuli, neuronal synapses must be plastic, having the capacity to change physiological connection strength (Nelson and Turrigiano, 2008). When subsets of synapses in a neuronal circuit are selectively altered (long-term potentiation or depression), synaptic plasticity alters behavior (Oda et al., 1998). However, when synapses undergo more global remodeling during development or in response to either environmental or genetic perturbation, synaptic plasticity underlies homeostasis that stabilizes behavior (Levi et al., 2008; Mongeon et al., 2008). Thus, behaviors can be either stabilized or changed by different types of neuronal plasticity. Because of the multi-component nature of the synapse, changes in connectivity can take place at multiple levels, including pre-synaptic, glial, and post-synaptic (Gaiarsa et al., 2002). Here we discuss examples of glycinergic synaptic plasticity in the context of first potentiation and then homeostasis.

That glycinergic synaptic strength can underlie behavioral change has been elegantly demonstrated at glycinergic synapses onto zebrafish Mauthner neurons (Korn et al., 1992; Charpier et al., 1995; Oda et al., 1998). Mauthner neurons are crucial for the speed of the teleost escape behavior (Liu and Fetcho, 1999). Two, bilateral Mauthner cell bodies in the hindbrain, extend axons that cross the midline before projecting caudally the full length of the spinal cord, exciting contra-lateral motor neurons and causing a rapid bend away from the stimulus (Faber et al., 1989). In response to sound, for example, the VIII cranial nerve stimulates both the ipsi-lateral Mauthner cell body and a glycinergic interneuron. This glycinergic interneuron synapses onto both bilateral Mauthner cell bodies. The resulting glycinergic inhibition ensures that only the Mauthner neuron ipsi-lateral to the stimulus fires a single action potential because the ipsi-lateral cell receives strong excitation just prior to inhibition. In response to repeated VIII nerve stimulation (sub threshold for inducing an escape response), glycinergic synapses onto both Mauthner cell bodies experience a persistent (more than 5 h) potentiation (Korn et al., 1992). This long-term potentiation is specific to glycingergic inhibition elicited by the VIII nerve, as recurrent inhibition mediated by a collateral of the Mauthner axon is not similarly potentiated. Most significant, VIII nerve/Mauthner glycinergic synaptic potentiation has a behavioral correlate: After auditory conditioning, fish are less likely to escape in response to stimuli of identical intensity (Oda et al., 1998). Thus, plasticity at glycinergic synapses has clear implications for altering behavior in response to changing environmental stimuli.

In contrast to long-term potentiation, homeostatic synaptic plasticity stabilizes behavioral output not only as circuits are reconfiguring during development but also in response to environmental and genetic perturbation (Turrigiano and Nelson, 2004; Davis, 2006; Mongeon et al., 2008). Homeostasis at the glycinergic synapses can be mediated by diverse mechanisms. For instance, at GABAergic synapses, pre-synaptic mechanisms involving the expression level of VIAAT have been shown to alter quantal content (the amount of neurotransmitter loaded into the pre-synaptic vesicle) in accordance with the amount of neuronal activity (De Gois et al., 2005; Erickson et al., 2006). At glycinergic synapses, two transporter proteins, VIAAT and GlyT2, have been shown to influence quantal content. Depending on the level of VIAAT expression, more or less glycine will be loaded into the synaptic vesicle. Likewise, when GlyT2 expression is reduced, glycinergic synapses lose strength because the amount of cytoplasmic glycine available to the VIAAT transporter becomes limiting (Lopez-Corcuera et al., 2001; Gomeza et al., 2003b).

The ability to directly measure *in vivo* receptor dynamics (membrane area explored per unit time) has identified glycine receptor diffusion dynamics as a post-synaptic mechanism underlying glycinergic synapse homeostasis (Levi et al., 2008; Renner et al., 2008). In response to electrical activity and specifically NMDA receptor activation, rapid homeostatic adjustments are triggered in the strength of glycinergic signaling by changing the dynamics of glycine receptor lateral diffusion (Levi et al., 2008). Receptor transit between synaptic, peri-synaptic, and extra-synaptic domains occurs within minutes, much faster than the rate of receptor turnover: receptor half-life at the plasma membrane is 14 hours (Rasmussen et al., 2002). Moreover, each domain is associated with a unique mobility with the highest diffusion coefficients associated with extra-synaptic receptors and the lowest diffusion coefficients associated with synaptic receptors (Dahan et al., 2003). Diffusion of receptors into and out of the post-synapse is regulated by the cytoskeleton (Charrier et al., 2006). When either actin or microtubules are depolymerized, dwell time at the synapse is decreased and diffusion away from the synapse is increased implicating receptor interactions with the cytoskeleton as crucial to glycine receptor diffusion dynamics.

COORDINATION OF PLASTICITY MECHANISMS

> "The living being is stable. It must be so in order not to be destroyed, dissolved or disintegrated by the colossal forces, often adverse, which surround it. By apparent contradiction it maintains its stability only if it is excitable and capable of modifying itself according to external

stimuli and adjusting its response to the stimulation. In a sense it is stable because it is modifiable—the slight instability is the necessary condition for the true stability of the organism." (Richet, 1900).

In zebrafish, a glial glycine transporter (GlyT1) mutation triggers a gradual homeostatic plasticity at glycinergic synapses that restores rhythmic motor output (Mongeon et al., 2008). Enabling rhythmic motor output in high glycine, sequential homeostatic mechanisms function together over a time-course of 2 days to reduce glycinergic post-synaptic potentials (**Figure 2**; second panel). Reduced glycinergic potentials are associated with reduced expression of both glycine receptor (Mongeon et al., 2008) and GlyT2 transcripts (Mongeon et al., 2008). Both the glycine receptor and GlyT2 have been previously implicated in reducing the strength of glycinergic synapses by their association with the human startle disease hyperekplexia. Although transcriptional mechanisms that reduce the expression of glycine receptor transcripts in mutants are likely to contribute to behavioral recovery, the timing of the reductions in glycine receptor expression occurs after post-synaptic potentials are already significantly reduced, implicating additional mechanisms in the recovery process.

Additional homeostatic mechanisms in the GlyT1 mutant could involve changes in spinal cord cell fates. The eventual fate of neural precursors is influenced by environmental stimuli that include neurotransmitters like glycine (Nguyen et al., 2001). In zebrafish, knocking down the α2 glycine receptor subunit reduces the number of interneurons that differentiate (McDearmid et al., 2006).

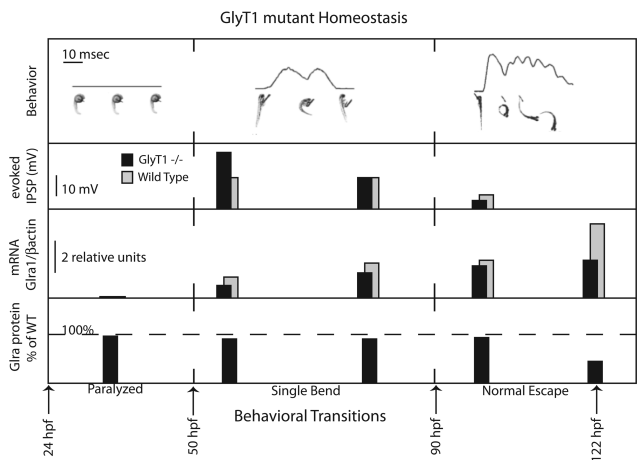

FIGURE 2 | GlyT1 mutant neuronal homeostasis. Top Row: GlyT1 mutants exhibit two behavioral transitions, from paralysis to a single bend at 50-h post fertilization and from single bend to full recovery at 90-h post fertilization. Line graphs are produced by motion detection software that tracks larval pixel displacement over time. Below line graphs till images of the embryos and larvae are placed at corresponding timepoints. Second row: Maximum amplitude in millivolts (mV) of evoked motor neuron glycinergic inhibitory post-synaptic potentials (IPSPs) recorded in GlyT1 mutants versus wild type larvae at three timepoints. Third Row: Relative quantitation of mRNA expression, a1 subunit of the glycine receptor (Glra1) normalized to bactin, using quantitative polymerase chain reaction to compare GlyT1 mutants versus wild type. Fourth Row: Comparison (% of wild type staining) of maximum antibody labeling of motor neuron glycine receptor alpha subunits in GlyT1 mutants.

Consistent with environmental plasticity in cell fate, changes spinal cord neuron excitability in frogs results in homeostatic changes in the ratio of neurons that become excitatory versus inhibitory: when spontaneous electrical activity is elevated by global over-expression of a sodium channel, more inhibitory neurons form at the expense of excitatory neurons; conversely, when spontaneous electrical activity is reduced by global over-expression of a potassium channel, more excitatory neurons form at the expense of inhibitory neurons (Borodinsky et al., 2004). In the case of the zebrafish glycine transporter 1 mutant, the early paralytic phenotype can be relieved by reducing nervous system glycine or by blocking the glycine receptor with strychnine (Cui et al., 2005; Mongeon et al., 2008). Therefore, it is likely that glycine reduces nervous system activity by tonic activation of glycine receptors, making it more difficult for neurons to reach threshold for an action potential. Perhaps the early reductions in glycinergic potentials reflect such changes in numbers of different types of spinal cord neurons.

Such a shift in neuronal cell type would impact synaptic inputs and have the potential to restore excitability in the glycine-inundated nervous system. With this in mind, it is interesting to speculate how changes in cell fate would impact motor behavior. Rhythmic motor behaviors can be mathematically modeled with a segmentally reiterated module of eight neurons: four neurons on the left side of the spinal cord are mirrored by another four neurons on the right side of the spinal cord (Goulding, 2009). These neurons include (1) the motor neuron that receives excitatory drive from (2) ipsi-lateral glutamatergic and (3) glycinergic interneurons. The fourth neuron type is (4) a glycinergic interneuron that crosses the spinal cord so that when one side of the spinal cord is activated, the other side is inhibited. While many more classes of interneurons are present in the spinal cord (Hale et al., 2001), the robustness of the simplified mathematical network suggests that if the four basic types of neurons remain, the motor circuit could tolerate changes in cell fate without necessarily losing rhythmicity (Kozlov et al., 2009).

Other forms of plasticity that could contribute to reduced glycinergic potentials are post-translational modifications of glycine receptor function, including those mechanisms that impact the rate of exchange of glycine receptors from synaptic to extra-synaptic sites (Levi et al., 2008). Future experiments will test the role of cell fate changes and post-translational regulation of the glycine receptor in GlyT1 mutant synaptic homeostasis. Both establishing the mechanisms that contribute to synaptic homeostasis at glycinergic synapses and determining how these mechanisms are regulated will contribute to our understanding of and hopefully the ability to treat diseases that result from glycinergic synapse dysfunction.

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High throughput techniques for discovering new glycine receptor modulators and their binding sites

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The inhibitory glycine receptor (GlyR) is a member of the Cys-loop receptor family that mediates inhibitory neurotransmission in the central nervous system. These receptors are emerging as potential drug targets for inflammatory pain, immunomodulation, spasticity and epilepsy. Antagonists that specifically inhibit particular GlyR isoforms are also required as pharmacological probes for elucidating the roles of particular GlyR isoforms in health and disease. Although a substantial number of both positive and negative GlyR modulators have been identified, very few of these are specific for the GlyR over other receptor types. Thus, the potential of known compounds as either therapeutic leads or pharmacological probes is limited. It is therefore surprising that there have been few published studies describing attempts to discover novel GlyR isoform-specific modulators. The first aim of this review is to consider various methods for efficiently screening compounds against these receptors. We conclude that an anion sensitive yellow fluorescent protein is optimal for primary screening and that automated electrophysiology of cells stably expressing GlyRs is useful for confirming hits and quantitating the actions of identified compounds. The second aim of this review is to demonstrate how these techniques are used in our laboratory for the purpose of both discovering novel GlyR-active compounds and characterizing their binding sites. We also describe a reliable, cost effective method for transfecting HEK293 cells in single wells of a 384-well plate using nanogram quantities of plasmid DNA.

Keywords: Cys-loop receptor, chloride channel, pharmacology, high throughput, drug discovery, transfection

SUBUNIT COMPOSITION, DISTRIBUTION AND FUNCTION

The inhibitory glycine receptor (GlyR) is a member of the Cys-loop ligand-gated receptor family that also includes the nicotinic acetylcholine receptor (nAChR), the GABA type-A receptor ($GABA_AR$) and serotonin type-3 receptor ($5\text{-}HT_3R$). Receptors of this class comprise five subunits arranged symmetrically around a central ion-conducting pore. Each subunit comprises four α-helical transmembrane domains and a large extracellular amino-terminal domain that harbours the ligand binding sites and the signature 'Cys-loop' (Unwin, 2003; Hilf and Dutzler, 2008). A total of five GlyR subunits have been identified so far, namely α1–α4 and β (Betz and Laube, 2006; Lynch, 2009). The α4 gene is a pseudo-gene in humans (Simon et al., 2004). All α subunits can form functional homomeric receptors but β subunit is functionally expressed only as a heteromer with the α subunit in a putative 2α:3β stoichiometry (Grudzinska et al., 2005).

In the adult rat, α1 subunit mRNA is abundant in brain stem, spinal cord and retina but is also found in superior and inferior colliculi, thalamus and hypothalamus (Malosio et al., 1991). Expression is low prenatally but increases from birth to reach to a maximum by the end of the third postnatal weak. The distribution and developmental profile of the α3 subunit is similar but occurs at lower levels, except in retina and spinal cord as discussed below (Malosio et al., 1991; Watanabe and Akagi, 1995). In contrast, the α2 subunit is present at high levels prenatally but declines postnatally to low levels in the adult (Malosio et al., 1991; Watanabe and Akagi, 1995). The β subunit, which shows a surprisingly widespread distribution throughout the spinal cord and the brain, also increases with age in parallel with the α1 and α3 subunits.

Inhibitory synaptic transmission in motor reflex circuits of the spinal cord is mediated by the α1β GlyR. This is perhaps best evidenced by the fact that hereditary mutations to the α1 GlyR that reduce the charge transfer rate of synaptic α1β GlyRs cause human startle disease, a disorder characterized by a temporary increase in muscular rigidity but no alteration in cognitive state (Bakker et al., 2006).

The retina expresses a diversity of GlyR subunits. Synaptic GlyRs comprising α1β, α2β and α3β GlyRs are all postulated to be present in anatomically discrete locations, although their individual contributions to visual processing are yet to be elucidated (Balse et al., 2006; Ge et al., 2007; Grunert and Wassle, 1993; Grunert and Ghosh, 1999; Haverkamp et al., 2003, 2004; Jusuf et al., 2005; Majumdar et al., 2007; Veruki et al., 2007). The α2 GlyR subunit also plays a crucial role in rod photoreceptor development (Young and Cepko, 2004). Glycinergic synapses also are found in several brainstem nuclei, particularly those of the central auditory pathways (Caspary et al., 2008), where the large fast glycinergic synaptic currents are important for the precise timing associated with directional hearing (Kandler, 2004). Extrasynaptic GlyRs are widely

distributed throughout the adult nervous system in areas such as the cortex (Flint et al., 1998), hippocampus (Mori et al., 2002) and basolateral amygdala (McCool and Farroni, 2001) where they are thought to mediate tonic inhibition. GlyR expression is not limited to neurons but is also found in sperm where GlyRs contribute to the acrosomal reaction that fuses sperm to the egg (Meizel, 1997). GlyRs have also been identified in macrophages and leucocytes where they are thought to mediate the anti-inflammatory effects of glycine (Gundersen et al., 2005).

THERAPEUTIC POTENTIAL OF GlyRs

MOVEMENT DISORDERS

As α1β GlyRs control the excitability of spinal motor neurons, agents that increase current flow through α1-containing GlyRs should decrease the activity of these neurons. Thus, α1-specific enhancing agents may be useful as treatments for startle disease and as muscle relaxants for hypertonic movement disorders such as spasticity. Such movement disorders are currently treated with $GABA_A$R-enhancing benzodiazepines which have substantial side-effects due to the widespread distribution of target $GABA_A$Rs throughout the brain. In contrast, their relatively limited distribution renders α1β GlyRs a more promising therapeutic target.

ANTI-INFLAMMATORY ANALGESIA

GlyR α1 and α3 subunits co-exist in individual glycinergic inhibitory synapses on nociceptive sensory neurons in the spinal cord dorsal horn (Harvey et al., 2004). An inflammatory mediator, prostaglandin E_2 acting at the EP_2 receptor, specifically inhibited both glycinergic inhibitory postsynaptic currents (IPSCs) in pain sensory neurons (Ahmadi et al., 2002; Harvey et al., 2004) and glycine-gated currents in recombinantly expressed α3 (but not α1) GlyRs (Harvey et al., 2004), implying that inflammatory pain sensitisation is mediated by inhibition of glycinergic IPSCs. Consistent with this, knockout of either the α3 GlyR subunit or the EP_2 receptor in mice abolished pain sensitisation induced by peripheral inflammation (Harvey et al., 2004; Reinold et al., 2005). Thus, inflammatory pain sensitization is caused by a PGE_2-mediated down-regulation of α3-mediated glycinergic IPSCs in nociceptive neurons. This in turn implies that molecules that can specifically potentiate or prolong IPSCs in spinal nociceptive neurons offer promise as anti-inflammatory analgesic lead compounds. Although it should be possible to compensate for the effects of inflammatory mediators on α3-containing GlyRs by pharmacological enhancement of charge transfer through synaptic α1-containing GlyRs, the risk of side-effects is higher given that α1 subunits are more widespread outside the dorsal horn (Lynch and Callister, 2006; Zeilhofer, 2005).

IMMUNOMODULATION

Functional GlyRs are present in immune cells including neutrophils (Wheeler et al., 2000), macrophages and leucocytes (Froh et al., 2002; Ikejima et al., 1997). There is mounting evidence that GlyRs in these cells mediate the anti-inflammatory and cytoprotective actions of glycine by antagonising the intracellular calcium increases that mediate the activation of these cells (Froh et al., 2002; Ikejima et al., 1997). Thus, by inhibiting the activity of immune cells, the systemic administration of glycine or GlyR potentiating agents could limit the damage inflicted by the systemic inflammatory immune response on essential biological molecules, cells and organs (Gundersen et al., 2005).

TEMPORAL LOBE EPILEPSY

RNA-edited high affinity α2 and α3 GlyR transcripts were found to be upregulated in patients suffering from temporal lobe epilepsy (Eichler et al., 2008, 2009). In the same patients, transcript levels of the potassium chloride co-transporter type-2 (KCC2) were found to be downregulated. To determine whether these alterations may lead to temporal lobe epilepsy, the effects of changes in expression levels of the respective transcripts were investigated using a neuronal culture system. As expected, upregulation of high affinity GlyRs and downregulation of KCC2 resulted in depolarising tonic GlyR activity which shunted excitatory inputs, and rendered GABAergic synapses excitatory. This in turn led to an increased glutamatergic to GABAergic synapse ratio and a reduction in dendrite lengths and excitotoxicity, thus providing a possible mechanism for the increased neuroexcitability associated with temporal lobe epilepsy (Eichler et al., 2008).

THE CASE FOR DISCOVERING NOVEL GlyR MODULATORS

Novel GlyR subtype-specific potentiating agents are required as therapeutic lead compounds for epilepsy, movement disorders, chronic inflammatory pain and immunomodulation. On the other hand, antagonists specific for high affinity α2 and α3 GlyRs may be useful as therapeutic leads for temporal lobe epilepsy. In addition to clinical leads, subunit-specific pharmacological probes are also needed for establishing the presence and establish the roles of different GlyR isoforms in central nervous system regions (e.g., retina and spinal cord dorsal horn) that express a diversity of GlyR subtypes. Often the simplest way of identifying the presence and physiological role of a particular ion channel subtype is to use an isoform-specific inhibitor to eliminate its contribution to the complex suite of conductances active at any one time in a neuron.

Unfortunately, almost all known GlyR modulators also have potent effects on other receptor types (Lynch, 2009) which limit their utility as probes for establishing the physiological role of different GlyR subtypes. Importantly, there are no compounds known to potentiate α3 GlyRs while having no effect on α1 GlyRs. Thus, the development of novel GlyR subunit-specific pharmacological probes is long overdue. To date there have been few, if any, published investigations describing systematic attempts to identify novel GlyR subtype-specific modulators.

Given the unusually high (>90%) amino acid sequence identity of α1 and α3 GlyR subunits in their extracellular and transmembrane receptor domains, it is relevant to consider whether pharmacological agents are likely to be found that discriminate strongly between these isoforms. Fortunately, there are two indications to suggest that such ligands may eventually be identified. First, using chimeras of α1 and α3 subunits, we identified the structurally divergent M4 transmembrane segment as a specific determinant of the large agonist efficacy difference between these two subtypes (Chen et al., 2009b). This suggests that the lipid-exposed M4 domain may be a promising region for subtype-specific modulators to bind. Second, anandamide,

N-arachidonyl-glycine and several synthetic cannabinoid agonists exhibit distinct pharmacological actions on α1 and α3 GlyRs (Yang et al., 2008). For example, WIN55,212-2 has no effect on α1 GlyRs but potently inhibits α3 GlyRs. Although the location of the cannabinoid binding site is not yet known, these findings bode well for the existence of pharmacologically distinct modulatory sites on α1 and α3 GlyRs.

TECHNIQUES FOR DISCOVERING NEW GlyR-ACTIVE DRUGS

AUTOMATED PATCH-CLAMP ELECTROPHYSIOLOGY

Patch-clamping is the definitive means of assaying ion channel function. It not only offers an unparalleled signal-to-noise ratio but also tracks the effects of compounds in real time. This later feature permits complex effects of compounds (e.g., fast potentiation followed by slow inhibition) to be resolved. Several automated patch-clamp technologies have been successfully implemented (Finkel et al., 2006; Lepple-Wienhues et al., 2003; Mathes et al., 2009; Milligan et al., 2009). Although the technology is advancing steadily in terms of reliability, throughput and cost, it is still limited by high cost and modest throughput (Dunlop et al., 2008). Thus, automated patch-clamp is probably best deployed as a means of confirming and quantitating the activity of compounds discovered by fluorescent assays (see below). Because automated patch-clamp technologies select cells at random for screening, it is generally necessary to create stable cell lines where all cells express the ion channels of interest in order to optimize data acquisition. Stably expressing cell line creation can be a slow, painstaking process, especially when one is interested in screening multimeric receptors.

RADIOACTIVE ASSAYS

These can be separated into radioligand displacement assays and radiotracer flux assays. In the case of GlyRs, radioligand displacement assays involve displacing ^{3}H-strychnine with unlabelled test compound (Young and Snyder, 1973). There are several limitations to this technology. First, it provides no information about the mode of action (if any) of a compound. Second, it may not detect compounds that modify receptor function without displacing the radioligand. Finally, long incubations with agonists may promote a desensitised state with different pharmacology to the normal resting closed or activated states (Changeux and Edelstein, 1998). Radioactive tracer flux assays detect the transmembrane flux of the radioactive anions, $^{125}I^-$ or $^{36}Cl^-$ (Kardos, 1993; Venglarik et al., 1990). I^- is highly permeant through GlyRs (Fatima-Shad and Barry, 1993). Being an assay of receptor function rather than binding, this technique overcomes some of the limitations of radioligand displacement assays. In general, radioactive assays are not optimal for high throughput (HT) drug discovery due to long incubation times and multiple processes (e.g., tracer loading, cell-wash and cell lysis steps) that limit HT, the high cost of materials, and the inconvenience associated with safe handling and disposal of radioactive materials.

COLORIMETRY

Transmembrane I^- influx can be measured via a change in colour of a non-fluorescent cytoplasmic indicator (Tang and Wildey, 2004). The rate of accumulation of intracellular I^-, and hence cell colour, will depend on the degree to which the channels are activated. Because all reactions will eventually proceed to steady-state regardless of the agonist concentration, it is necessary to control the length of time that the reaction is allowed to proceed. This assay has the advantage of low cost and avoids the safety and environmental issues associated with radioactive materials. However, as with the radioactive tracer flux assay, its utility is limited by the requirement for tracer loading, cell wash and lysis steps. An analogous approach, in which Cl^- influx precipitated an excess of intracellular Ag^+ ions leading to a colour change (Gill et al., 2006), may suffer the same limitations.

VOLTAGE SENSITIVE DYES (VSDs)

A wide range of VSDs is commercially available and the choice generally involves a trade-off between speed of response and dynamic range. In HT screening (HTS) applications, dynamic range is the prime consideration. A fluorescent membrane potential dye (marketed by Diagnostic Instruments Inc.) has successfully been employed to screen compounds against α1 GlyRs stably expressed in HEK293 cells (Jensen and Kristiansen, 2004). In that study, ionic gradients were established such that GlyR activation induced a depolarisation and a consequent increase in fluorescence. The dye response was relatively slow, taking at least 30 s to reach steady-state, but it offered an excellent dynamic range.

High response speed and dynamic range can be simultaneously achieved using fluorescence resonance energy transfer (FRET). This technique involves the non-radiative transfer of energy from a donor fluorophore to an acceptor fluorophore provided the donor emission spectra and acceptor absorption spectra overlap and the respective fluorophores are in close proximity. When the donor is excited at its absorption wavelength, FRET causes the acceptor fluorescence to increase and donor fluorescence to decrease. It has been shown that a donor, CC2-DMPE, and an acceptor, DiBac2, produce a robust FRET at negative membrane potentials when both lie at the external membrane surface (Gonzalez and Tsien, 1997). Upon depolarisation, DiBac2 crosses the membrane thereby increasing its distance from CC2-CPMPE and decreasing FRET efficiency. The subsequent fluorescence change occurs rapidly over a large dynamic range. This method has successfully been used to screen $GABA_A$Rs (Adkins et al., 2001) and GluClRs (Hamelin et al., 2005). In both cases, cells were bathed in low-Cl^- buffer so that $GABA_A$R activation induced a Cl^- efflux leading to depolarisation. This technique provides a rapid and sensitive indication of anionic receptor activation. However, the technique is expensive as it requires two exogenous fluorescent indicators. Also, because the plasma membrane concentrations of the respective fluorophores must be optimised individually for maximum FRET efficiency, this technique may require two dye incubation steps.

As a general point, exogenously applied dyes have several limitations. First, they constitute a significant recurrent expense and second, they require dye loading and (sometimes) cell-wash steps that limit HT. Third, and perhaps most seriously, lipophilic dyes are taken up by all cells regardless of whether or not they express the ion channel of interest. If, for example, only half the cells express the ion channel of interest then the dynamic range of the averaged fluorescence response is halved. Thus, to achieve its full dynamic range, all cells must respond to the dye. This requirement

means that techniques involving exogenously applied indicators are suited primarily to homogeneous assays such as those involving stably expressing cell lines.

GENETICALLY ENCODED ANION INDICATOR

Yellow fluorescent protein (YFP), an engineered variant of green fluorescent protein, is quenched by small anions and is thus suited to reporting anionic influx into cells. A random mutagenesis approach identified two mutations, I152L and V163S, each of which greatly enhanced YFP anion sensitivity (Galietta et al., 2001a). The I152L mutation confers a particularly high sensitivity to I^- quench ($K_i \sim 3$ mM). YFP-I152L has proved useful in screening compounds against the anion-conducting cystic fibrosis transmembrane regulator (Galietta et al., 2001b; Yang et al., 2003). It has also been applied to the GlyR and $GABA_AR$ (Kruger et al., 2005). This assay has several advantages over the VSD assay. First, although both assays have similar signal-to-noise ratios and dynamic ranges, the YFP assay is faster by a factor of at least three in achieving a given percentage fluorescence change (Jensen and Kristiansen, 2004; Kruger et al., 2005). Second, as a propagable substance, YFP cDNA is cheaper. Third, the simultaneous transfection of YFP and ion channel cDNAs in separate plasmid vectors results in a high rate of transient co-expression of YFP and functional recombinant channels (Kruger et al., 2005). Thus, the YFP-based assay does not require stably expressing cell lines to achieve its full dynamic range. Finally, as a genetically encoded probe, YFP is also suited to screening heterogeneous populations of isolated neurons where the YFP is transgenically expressed in a defined subset of cells. If an imaging-based detection system is used, this assay would also be suitable for 'high-content' screening (i.e., the correlation of multiple variables in the same cell). As an example of this, we have recently demonstrated the feasibility of screening a single compound against numerous GlyR receptor subtypes simultaneously via the YFP assay by using cell-permeant dyes to provide cells expressing a given receptor with a unique optical identifier (Gilbert et al., 2009c).

Although genetically encoded fluorescent detectors of membrane potential are steadily being improved, at this stage their maximum voltage-induced fluorescence changes are typically <10% of the resting fluorescence level (Baker et al., 2008; Perron et al., 2009). This narrow dynamic range may be too small to yield robust readouts during HTS, and may explain why these probes have yet to be broadly implemented as drug discovery tools.

GlyR DRUG DISCOVERY IN OUR LABORATORY

YFP-BASED PRIMARY SCREENING

Our laboratory employs YFP for primary HTS of large compound libraries or natural product fractions. For this purpose we have constructed a custom built robot comprising an inverted fluorescence microscope, an automated stage, an autosampler for liquid handling and a digital camera all under the control of Labview (National Instruments Corp, Austin, TX, USA) programs. The components of this device have previously been described in detail (Kruger et al., 2005). Experiments are performed on adherent HEK293 cells in 384 well plates. The autosampler can deliver up to three different solutions (with or without removing the previous solution) from different sources to a single well, thus providing a wide range of liquid handling options. Although multiple solution exchanges in single wells is useful for assay development, we avoid it for HTS as it slows the screening process too much. The CCD captures images like those in **Figure 1A** and image analysis is automated using customized DetecTiff© software (Gilbert et al., 2009b). VSD and YFP screening technologies have also been successfully employed with commercial fluorescent plate readers (Jensen and Kristiansen, 2004; Kruger et al., 2005).

To prepare cells for experiments, we simultaneously transfect HEK293 cells with cDNAs for YFP-I152L plus the GlyR clone of interest. We have recently published a detailed comparison of five transient transfection methods employed routinely in the laboratory for this purpose (Gilbert et al., 2009a). Once transfection is completed and the transfection reagent has been removed, around 2000 cells, suspended in 40 µl culture media, are plated into each well of a 384-well plate. Plates are then returned to the incubator and used for experiments 24–72 h later. Based on the proportion of fluorescent cells, transfection efficiency varies from 10 to 50%. As only those cells near the centre of the well are imaged, we are typically restricted to analysing 100–500 fluorescent cells per well (~1000 cells total per image). As noted above, we routinely achieve an excellent correlation between cells expressing YFP and cells expressing GlyRs.

Sample images of cells expressing YFP-I152L and α1 GlyRs are shown in **Figure 1A**. Imaged cells in the top and bottom rows were exposed to NaCl and NaI solutions, respectively. The images in the bottom rows were taken after an 8 s exposure to either NaI alone (bottom left panel) or NaI plus 1 mM glycine (bottom right panel). **Figure 1B** quantitates the percentage fluorescence change produced by the addition of glycine. Percentage fluorescence change is defined as $[(F_{final}/F_{init}) - 1) \times 100$ where F_{init} and F_{final} are the initial and final values of fluorescence, respectively. The experiment demonstrates that the YFP assay provides a robust indication of GlyR activation. To underscore this point, a sample experimental analysis output screen of a glycine dose–response experiment is shown in **Figure 1C**. The green histograms indicate the number of fluorescent cells in each of 24 wells in a single row of a 384-well plate. The blue columns show the number of cells that displayed a >20% quench in response to the addition of control NaI plus glycine at the indicated concentrations. Each concentration was added to two adjacent wells, with concentrations increasing from left to right as indicated in **Figure 1C**. This panel shows that almost all fluorescent cells displayed >20% quench at saturating glycine concentrations, supporting previous evidence for a strong correlation between YFP and GlyR expression. **Figure 1D** shows an alternative analysis of the results presented in **Figure 1C**, this time plotting mean percentage fluorescence change of all fluorescent cells against glycine concentration.

To demonstrate the robustness of this assay, **Figure 1E** shows a sample outcome of an actual screening experiment. In this experiment, cells were pre-incubated with either no compound ('negative control' in wells 1 and 2), 10 µM of the antagonist strychnine ('positive control' in wells 23 and 24) or a natural product fraction with each fraction plated into two adjacent wells (wells 3–22). A control image was taken prior to glycine addition and a test image was taken 8 s after glycine addition. The mean percentage fluorescence change for all cells in each well was calculated as described above and translated into a colour according to the scale shown in **Figure 1E**. Thus, the 'warmer' the colour, the greater the antagonist activity. This experiment yielded two strong hits as shown in

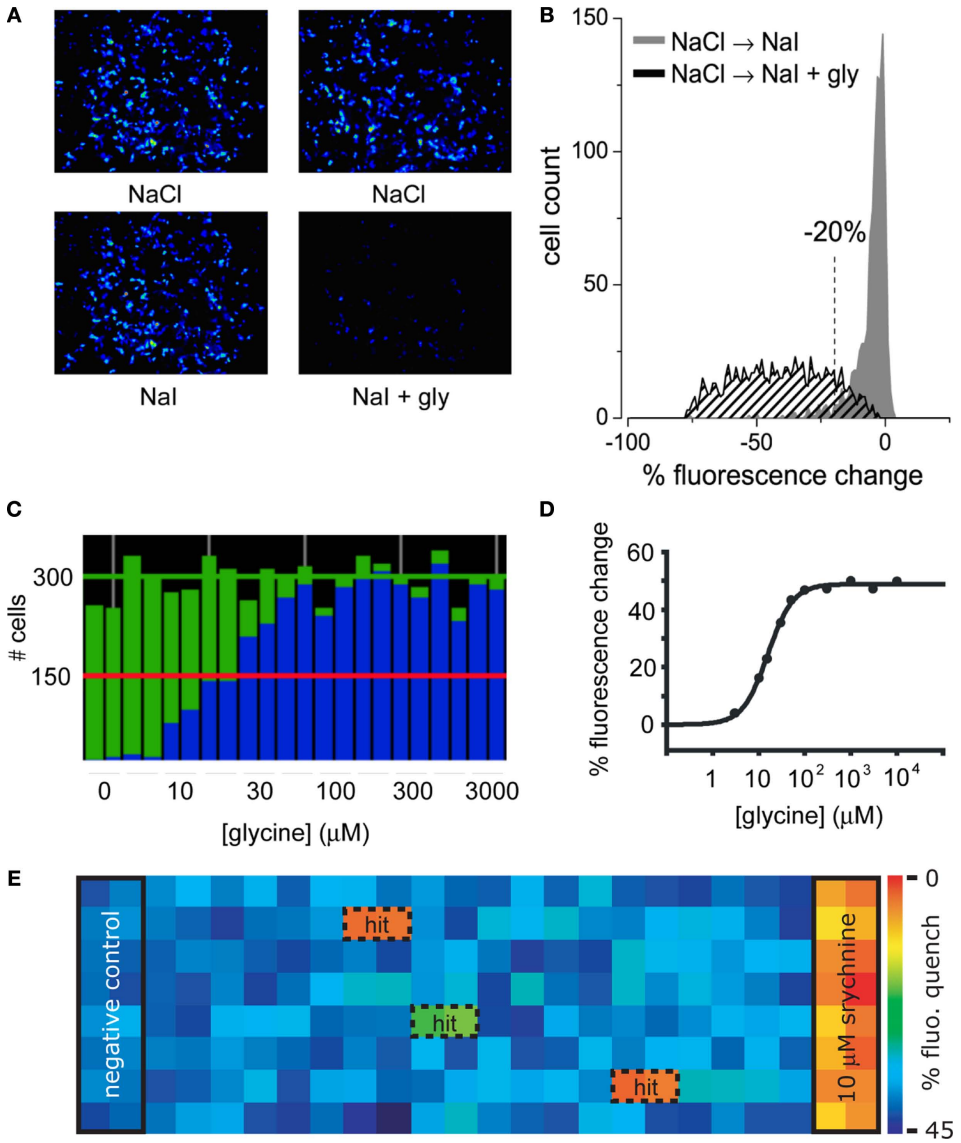

FIGURE 1 | Screening GlyRs using YFP -I152L. (A) Images in the upper row were recorded in NaCl control solution whereas images in the lower row were recorded from the same wells 8 s after replacement with NaI alone (left image) or NaI + 1 mM glycine (right image). **(B)** Cell fluorescence distribution for experiments shown in **(A)**. The '% fluorescence change' is defined in the text. **(C)** Glycine dose–response as revealed by YFP fluorescence change. Each green histogram represents the total number of imaged fluorescent cells in a single well of a 384-well plate. Blue histograms represent the number cells per well that quenched by >20% upon glycine addition. From left, glycine concentrations were (in μM): 0, 3, 10, 15, 30, 50, 100, 300, 1000, 3000. Each was applied to two wells and every second value is shown. **(D)** Mean percentage fluorescence change of all fluorescent cells in **(C)** plotted against glycine concentration. Fitted EC_{50} = 22 μM. **(E)** 'Heatmap' of an actual screening experiment comprising eight rows of a 384-well plate, with 'hits' (i.e., fractions producing antagonistic activity) boxed and labelled. Experimental design is described in the text.

orange, and a weaker hit as shown in green. We initially screen natural product fractions as antagonists as the dynamic range of an antagonist fluorescence response is typically much larger than it is for a potentiator response. Once a high affinity antagonist is identified, we isolate and screen analogues of this compound by automated patch-clamp electrophysiology to find subunit-specific positive and negative modulators.

Using this assay we have screened around 3000 natural product fractions against α1 GlyRs, yielding 27 active fractions. From these fractions we have identified at least four compound families with strong GlyR subunit-specific potentiating and inhibitory effects. From active fractions, we have so far identified 28 compounds with potent (nanomolar – low micromolar) effects on GlyRs as follows:

- 12 compounds that specifically potentiate α1 GlyRs,
- 1 compound that specifically potentiates α3 GlyRs,
- 2 compounds that potentiate both α1 and α3 GlyRs,
- 1 compound that specifically inhibits α1 GlyRs,
- 4 compounds that specifically inhibit α3 GlyRs and
- 8 compounds that inhibit both α1 and α3 GlyRs.

GENERATING STABLY EXPRESSING HEK293 CELL LINES

As automated patch-clamp electrophysiology requires stable cells lines expressing the channels of interest, we generated cell lines that stably express either α1 or α3 GlyRs. For this purpose, both human α1 and α3 GlyR subunit cDNAs were cloned into the pcDNA3.1 plasmid vector. Following transfection with linearised vector DNA, HEK293 cells were maintained for 14–21 days in selection medium containing 1 mg/ml of the selection antibiotic, G-418, and 10 μM strychnine. The strychnine was required to prevent the glycine in the culture medium from activating the GlyRs. The surviving cells were trypsinised, counted and diluted, so as to yield a suspension that was used to plate (on average) one individual clone into each well of several 96-well plates. Individual clones were then grown in the same selection medium for about 2 weeks. All wells identified as containing monoclonal colonies were screened for a functional response to 1 mM glycine using the YFP assay. In this screening several cell clones transfected with either α1 or α3 GlyR subunits exhibiting a significant response to glycine exposure were identified and then the selection process described above was repeated using these clones. Finally, after further testing, cells were split into 24-well plates, 6-well plates, 6 cm dishes and T75 flasks successively. All remaining clones were frozen at −80°C for later use. Clones of each subunit were then selected for further characterization in the patch-clamp and YFP assay.

AUTOMATED PATCH-CLAMP ELECTROPHYSIOLOGY

These recordings were performed using a Nanion Patchliner automated planar chip patch-clamp device (NPC-16A, Nanion Technologies GmBH, Munich, Germany). This device employs expendable planar glass 'chips' each consisting of a glass plate with 16 apertures embedded into a perspex microfluidic system which directs solutions to the internal and external sides of each aperture. The tiny volume of this microfluidic system is a major advantage of this system as it allows experiments to be conducted with extremely small quantities of compounds. For example, the external solution bathing the cells can be completely exchanged using 25 μl of new solution. Although chips permit 16 single cell recordings, only eight can be made simultaneously. The first step in forming the whole cell configuration is to inject a suspension of stably expressing cells into the microfluidic system thereby placing them at the external aperture surface. Then, by application of negative pressure to the opposite side of the aperture, a single cell is attached at random to the aperture. Contrary to the classical patch-clamp technique, it is the cell that is moved to the aperture and not the pipette that is moved to the cell. As with conventional patch-clamp, a seal is obtained by the application of suction in an automated manner and the membrane is then ruptured for whole cell access. The result is a low resistance electrical pathway to the inside of the cell allowing for whole cell patch-clamp recordings. Full experimental details of these standardized procedures, including compositions of internal and external solutions, are provided in recent reviews (Bruggemann et al., 2008; Farre et al., 2009; Milligan et al., 2009).

Sample glycine dose–response relationships from HEK293 cells stably expressing either α1 or α3 GlyRs recorded using the planar chip device are shown in **Figure 2**. The current recordings are comparable in quality to those generated by conventional patch-clamp and the glycine EC_{50} values generated using the planar chip device are comparable with those recorded in our laboratory using conventional electrophysiology (**Figure 2**, legend). This suggests that both the stably expressing cell lines developed in our laboratory and the planar chip recording system are appropriate for quantitating the sensitivity of α1 and α3 GlyRs to compounds of interest. Indeed, we have used this device to generate averaged dose–response relationships for a total 36 compounds of interest at α1 and α3 GlyRs, each averaged from 3 to 8 cells. In broad agreement with a previous study (Milligan et al., 2009), we typically experience a whole-cell recording success rate of around 80% of all cells sucked onto the aperture, with around 50% of recordings lasting for 10 min or more.

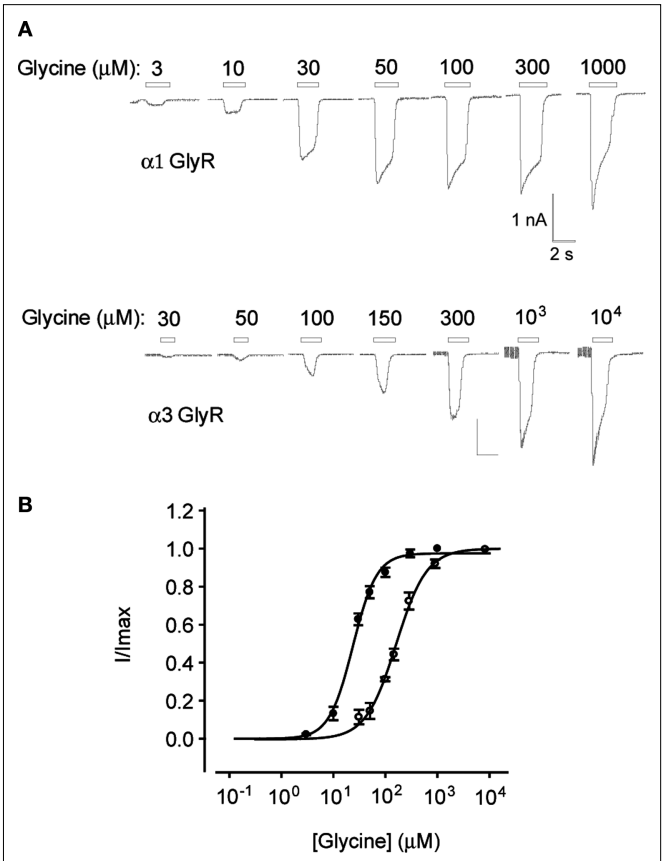

FIGURE 2 | Planar whole-cell patch-clamp data from HEK293 cells stably expressing α1 or α3 GlyRs. (A) Current responses in cells expressing α1 and α3 GlyRs. Indicated glycine concentrations were applied for 2 s at 1 min intervals. **(B)** Averaged glycine dose–responses averaged from five cells expressing either α1 (filled symbols) or α3 GlyRs (unfilled symbols). Error bars represent standard errors and curves are Hill equation fits to the averaged data. Individual α1 GlyR dose–response curves were fitted a mean glycine concentration of 24 ± 2 μM and a Hill coefficient of 1.9 ± 0.2 (n = 5 cells). Both values were not significantly different to their respective means (30 ± 1 μM and 1.7 ± 0.2, n = 5) recorded by conventional patch-clamp of HEK293 cells transiently expressing α1 GlyRs (Chen et al., 2009a). The α3 GlyR dose–responses were fitted with a mean glycine concentration of 166 ± 9 μM and a Hill coefficient of 1.4 ± 0.1 (n = 5 cells). In contrast, the respective mean values for α3 GlyRs recorded by patch-clamp of transiently transfected HEK293 cells were 309 ± 20 μM and 2.0 ± 0.1 (n = 5) (Chen et al., 2009a).

DISCOVERY OF NOVEL GlyR DRUG BINDING SITES BY SCREENING RANDOM MUTANT LIBRARIES

CREATION AND SCREENING OF A GlyR RANDOM MUTANT LIBRARY

It is not always possible to identify novel drug binding sites using site-directed mutagenesis. For example, at the GlyR it has not yet proved possible to identify the binding site for the non-glycinergic agonist, ivermectin. We are interested in identifying its binding site because, (1) it is commonly used as an anti-parasitic in agriculture, veterinary and human medical practice (Omura, 2008) and (2) it activates the GlyR by a mechanism distinct from glycinergic agonists (Pless et al., 2007) and thus may prove a useful tool for probing GlyR activation mechanisms. One way of discovering this and other intractable binding sites is to use HT methods to screen the drug of interest against a large library of randomly mutated GlyRs.

Random mutagenesis methods generally involve error-prone PCR and a range of strategies for implementing this approach have been developed (An et al., 2008; Emond et al., 2008; Fujii et al., 2006). The approach we used was to create a library of randomly mutated α1 GlyR-pcDNA 3.1 clones using the GeneMorph II Random Mutagenesis Kit (Stratagene). Following manufacturers' instructions, fragment PCR reaction conditions were optimized (750 ng template and 30 cycles of PCR) to yield a mutation frequency of one to two mutations per 1000 base pairs (15–20 clones from each PCR reaction were sequenced, covering more than 10,000 bp). The observed mutational spectra, which closely resembled that predicted for Mutazyme II, corresponded to one to three amino acid changes per receptor. The observed amino acid changes were scattered throughout the entire coding sequence with no evidence of clustering. Approximately 20–25% of the sequenced clones contained no mutations. A similar mutation rate was successfully employed in a similar screen of the TRPM8 channel (Bandell et al., 2006). As this was deemed an appropriate mutation rate, we then used a commercial service (Australian Genome Research Facility, Brisbane, QLD, Australia) to grow 1536 clones derived from the same PCR reaction in 384-well format and then extract plasmid DNA of sufficient quality. A glycerol stock of each clone was also made. This process resulted in an average yield of around 100 ng plasmid DNA per clone.

Since all transfection procedures of which we are aware use at least 100 ng plasmid DNA regardless of cell number or culture media volume, it was necessary to develop a technique of reliably transfecting much smaller amounts of plasmid DNA into single wells of a 384-well plate. This technique is presented in the Supplementary Material. In brief, we scaled down a standard calcium phosphate transfection procedure and tested its transfection efficiency with differing amounts of YFP plasmid DNA. As shown in **Figure 3**, the YFP transfection efficiency was found to be maximal at 10 ng plasmid DNA per well.

In a search for mutations that affect ivermectin sensitivity, we first added NaI containing 30 nM ivermectin to each well. This was followed by an application of 10 μM ivermectin and then one of 10 mM glycine. The rationale for this approach was that 30 nM ivermectin is subthreshold at the wild type (WT) α1 GlyR, whereas 10 μM is barely saturating (Shan et al., 2001). Thus, quantitating variations in receptor responses to both of these concentrations should permit the detection of mutations that substantially increase or decrease ivermectin sensitivity. The final saturating glycine application was a control to ascertain the functional expression of GlyRs in the event that a mutation completely eliminated ivermectin sensitivity.

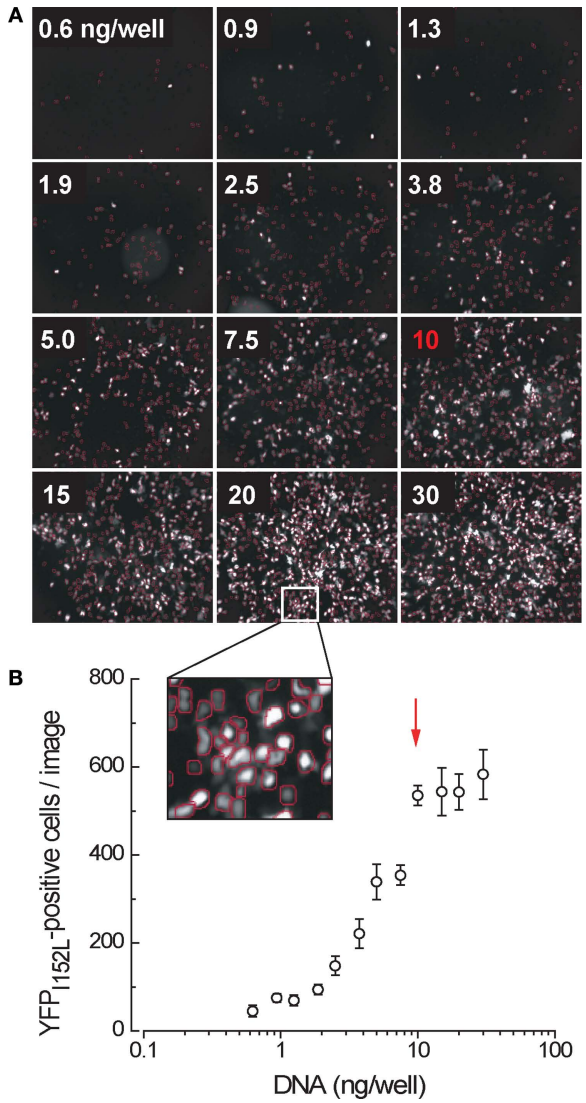

FIGURE 3 | Yellow fluorescent protein expression in cells transfected in single wells of a 384-well plate as a function of the amount of cDNA transfected. (A) Sample results from a single experiment. All wells contained an equivalent number (~5000) of cells. **(B)** Mean number of fluorescent cells per image, averaged from four independent experiments. Arrow denotes point of optimal transfection efficiency.

SAMPLE RESULTS OF RANDOM MUTANT SCREEN

After a first round screen of a total of 1566 clones (1536 random mutants plus 30 site directed mutants previously generated in the laboratory), we identified 44 mutant clones that significantly changed ivermectin sensitivity. The effects of 20 of these clones were confirmed by a second round of fluorescence-based screening. From this pool of clones, we have so far confirmed by nucleotide sequencing and electrophysiology a total of four α1 GlyR

mutations that significantly change ivermectin sensitivity. One of the mutations identified by this process was the highly conserved Y279F substitution in the extracellular M2-M3 linker domain. Examples of currents activated by the indicated concentrations of ivermectin and glycine in the WT and Y279F mutant α1 GlyRs are shown in **Figure 4A**. Averaged ivermectin dose–responses for both receptors, with current magnitudes normalised to those activated by glycine in the same cell are presented in **Figure 4B**. An averaged ivermectin dose–response for the Y279C mutant GlyR is also shown to indicate the specificity of the Y–F substitution in disrupting ivermectin sensitivity. Because this substitution involves simply the removal of a hydroxyl group, we hypothesise that Y279 is specifically

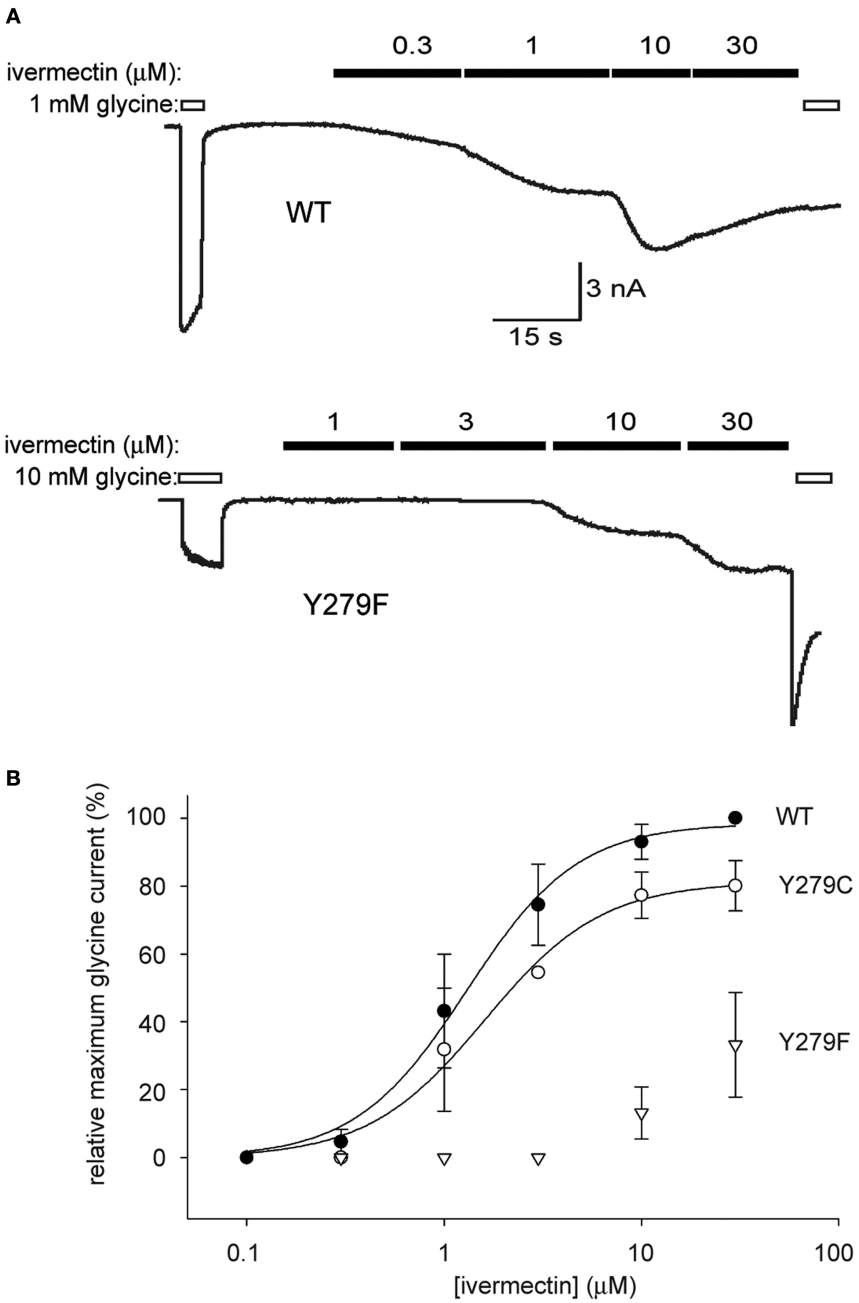

FIGURE 4 | The Y279F mutation dramatically reduces α1 GlyR sensitivity to ivermectin. (A) Conventional whole-cell patch-clamp recordings from HEK293 cells transiently expressing unmutated or Y279F mutant α1 GlyRs (upper and lower panels, respectively). The indicated concentrations of glycine and ivermectin were applied as indicated by the unfilled and filled bars, respectively. Because ivermectin activation is irreversible, dose–responses were generated by successively applying increasing ivermectin concentrations. **(B)** Averaged ivermectin dose–responses each averaged from five cells expressing either WT α1 GlyRs (filled circles), Y279C mutant α1 GlyRs (unfilled circles) or Y279F mutant α1 GlyRs (triangles). Error bars represent standard errors of the mean and curves represent fits to the averaged data with the Hill equation. The Y to F mutation causes an approximately 50 fold reduction in ivermectin sensitivity.

involved in either the binding or gating mechanism of ivermectin. Experiments are currently underway to resolve the molecular basis of this effect. We expect these experiments may provide crucial insights into the binding site or mechanism of action of this important pharmaceutical agent.

CONCLUSION

Despite GlyRs emerging as potential drug targets for therapies aimed at movement disorders, chronic inflammatory pain, temporal lobe epilepsy and immunomodulation, we are not aware of any systematic, published attempts to discover new drugs active at these receptors. The first aim of this review was to consider various HTS-amenable methods that may be applicable to these receptors. We conclude that an anion sensitive YFP is an optimal method for first round screening and that automated electrophysiology of cell stably expressing GlyRs is a useful means of confirming activity and quantitating the actions of identified compounds. The second aim of this review was to demonstrate how these techniques are used in practice in our laboratory for the purpose of both discovering novel GlyR-active compounds and establishing their mechanisms of action.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/molecularneuroscience/paper/10.3389/neuro.02/017.2009/

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A selective role for α3 subunit glycine receptors in inflammatory pain

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GlyR α3 has previously been found to play a critical role in pain hypersensitivity following spinal PGE_2 injection, complete Freund's adjuvant (CFA) and zymosan induced peripheral inflammation. In this study, although all models displayed typical phenotypic behaviours, no significant differences were observed when comparing the pain behaviours of *Glra3*$^{-/-}$ and wild-type littermates following the injection of capsaicin, carrageenan, kaolin/carrageenan or monosodium iodoacetate, models of rheumatoid and osteoarthritis, respectively. However, clear differences were observed following CFA injection ($p < 0.01$). No significant differences were observed in the pain behaviours of *Glra3*$^{-/-}$ and wild-type littermates following experimentally induced neuropathic pain (partial sciatic nerve ligation). Similarly, *Glra3*$^{-/-}$ and wild-type littermates displayed indistinguishable visceromotor responses to colorectal distension (a model of visceral pain) and *in vivo* spinal cord dorsal horn electrophysiology revealed no differences in responses to multimodal suprathreshold stimuli, intensities which equate to higher pain scores such as those reported in the clinic. These data suggest that apart from its clear role in CFA- and zymosan-induced pain sensitisation, hypersensitivity associated with other models of inflammation, neuropathy and visceral disturbances involves mechanisms other than the EP2 receptor – GlyR α3 pathway.

Keywords: glycine α3 subunit, prostaglandin E_2 inflammation, visceral pain, arthritis, electrophysiology

INTRODUCTION

Despite recent developments in the understanding of pain mechanisms and analgesia, the relief of pain, particularly that which lasts over long periods, remains a principal concern to healthcare professionals. Chronic pain can be broadly categorised into three types: inflammatory, neuropathic and dysfunctional. Inflammatory pain, arising from tissue damage caused by trauma or infection, is associated with conditions such as arthritis. Neuropathic pain, arising from trauma to, or pathological changes in, the peripheral or central nervous systems can occur as a consequence of stroke, ischaemia, diabetes or nerve infection (e.g. HIV, shingles, etc.). The third type of pain is underpinned by neural dysfunction and is thought to be associated with conditions such as irritable bowel syndrome (IBS), fibromyalgia and migraine which manifest as non-localised diffuse pain unaccompanied by either inflammation or nerve damage.

Tissue damage precipitates the release of multifarious endogenous pro-inflammatory mediators at sites in both the periphery and the CNS (mainly at the level of the spinal cord). Peripheral release of these mediators, namely prostaglandin E_2 (PGE_2), leads to the sensitization of primary afferents, thereby lowering thresholds for neuronal activation, and increasing nociceptor responsivity. This increased peripheral activity contributes to spinal hypersensitivity (central sensitisation) resulting in increased responsivity to noxious stimulation termed hyperalgesia, and allodynia where normally non-noxious stimuli such as cooling, gentle touch, movement and pressure are now perceived as being painful. Central prostanoid release can induce spinal disinhibition, relieving dorsal horn neurones from inhibitory control via spinal interneurones, thus facilitating the transmission of nociceptive information (Ahmadi et al., 2002; Reinold et al., 2005). One such mechanism of spinal disinhibition occurs through the postsynaptic activation of G-protein-coupled EP_2 receptors, and subsequent protein kinase A induced inhibition of α3 subunit containing glycine receptors, present in the superficial laminae of the spinal cord (Harvey et al., 2004).

GlyR α3 subunit knockout mice (*Glra3*$^{-/-}$) allow us to probe the function of this ligand-gated ion channel in the absence of any subtype-selective ligands. Previously, a role for GlyR α3 in inflammatory pain has been demonstrated in the CFA and zymosan A models of peripheral inflammation (Harvey et al., 2004). By contrast, GlyR α3 appears to have little or no role in neuropathic pain and the formalin test (Racz et al., 2005; Hösl et al., 2006). In this study, we have investigated the nociceptive phenotypes of GlyR α3 knockout mice in acute models of inflammation and clinically relevant models of persistent inflammatory, neuropathic and visceral pain. Moreover, we investigated the role of GlyR α3 in response to multimodal suprathreshold stimuli-evoking responses, which equate to higher pain scores experienced by human pain patients.

MATERIALS AND METHODS

ANIMALS

Glra3$^{-/-}$ mice on a C57Bl/6 background were produced as described (Harvey et al., 2004). *Glra3*$^{-/-}$ mice and their wild-type littermates were group housed on a standard 12-h light/dark cycle with food and water available *ad libitum* except during behavioural testing.

Experiments were performed on animals of at least 8 weeks of age. For all the experiments detailed in this study, the observer was blinded with respect to mouse genotype. All experiments were carried out in accordance with the UK Home Office Animals Scientific procedures Act (1986).

INDUCTION AND BEHAVIOURAL ANALYSIS OF PAIN STATES

Glra3$^{-/-}$ and wild-type littermates received either unilateral intraplantar injections of 1 mg/ml complete Freund's adjuvant (CFA, 20 µl), 2% (w/v) carrageenan (20 µl) or 50 µg/ml capsaicin (5 µl). Models of rheumatoid arthritis and osteoarthritis were induced by unilateral intra-articular injections of 3% (w/v) kaolin/carrageenan (K/C; 15 µl), or 5 mg/ml monosodium iodoacetate (MIA, 10 µl; Harvey and Dickenson, 2009), respectively. Neuropathic (partial sciatic nerve ligation, PSNL) mice were prepared by unilateral tight ligation of 1/3 to 1/2 of the sciatic nerve using 7-0 silk under isofluorane/O_2 anaesthesia (Malmberg and Basbaum, 1998). Behavioural thermal hypersensitivity (thermal hyperalgesia) was assessed using the Hargreaves' test and the hot plate, and mechanical hypersensitivity (mechanical allodynia) was assessed using von Frey filaments as described (Harvey and Dickenson, 2009). Two baseline measurements were recorded prior to the induction of the pain models. Although both contralateral and ipsilateral limbs were tested, only ipsilateral data are shown for clarity since contralateral data were not significantly different from baseline following the induction of any pain model ($p > 0.05$). Capsaicin-induced acute spontaneous pain behaviours, measured as either biting, flinching, or licking, were measured for 3 min.

SPINAL CORD ELECTROPHYSIOLOGY

Following a laminectomy (L3–L6) *in vivo* electrophysiology was performed in urethane anesthetised (240 mg/kg) *Glra3*$^{-/-}$ and wild-type mice using parylene coated tungsten electrodes (A-M Systems, USA), as previously described (Harvey and Dickenson, 2009). Evoked neuronal responses were recorded from single wide dynamic range dorsal horn neurones receiving inputs from the hindpaw. Transcutaneous electrical stimuli consisted of a train of 16 electrical stimuli (2 ms wide pulses, 0.5 Hz, 3 × C fibre threshold), were delivered by means of pins inserted in to the hindpaw. Post stimulus time histograms were constructed and fibre responses were separated according to the following latencies: A (0–50 ms), C (50–250 ms) and post discharge (250–800 ms). Input (i.e. non-potentiated response) is measured as the number of action potentials in response to the initial stimulus × 16 (total number of stimuli). Wind up values, a measure of the temporal summation of neuronal activity in response to a train of stimuli, was calculated as the total number of action potentials evoked at the end of 16 stimuli minus the input. A wide range of natural stimuli including brush (dynamic mechanical) acetone (innocuous cold), and graded punctate mechanical and thermal stimuli applied using von Frey filaments and a water jet, respectively, were applied to the hindpaw for a period of 10 s. Data were captured and analysed using a CED 1401 interface coupled to a Pentium computer running Spike 2 software (Cambridge Electronic Design).

ACUTE VISCERAL NOCICEPTION

Visceral nociception was assessed using a method adapted from Kamp et al. (2003). Under isofluorane anaesthesia (2.5%, delivered in O_2), an electromyographic (EMG) electrode (insulated copper wire, exposed at the ends and 1 cm centrally) was sewn into the external oblique muscle. A latex balloon (20 mm) attached to a pressure transducer via polyethylene tubing, with the distal 15 mm perforated using a 27-gauge needle to facilitate balloon inflation, was inserted intra-anally (25 mm), and secured by surgical tape to the base of the tail. The anaesthesia was gradually reduced to ~1% isofluorane, and allowed to equilibrate for at least 30 min, such that the animals did not display spontaneous movement but responded to cutaneous hindpaw stimulation. EMG responses were filtered, amplified and recorded in 10 s prior to and during colorectal distensions (10 s) of 10–60 mmHg applied at 4 min intervals. Two consecutive sets of CRD were recorded and averaged. Data are presented relative to the mean wild type maximum response (60 mmHg).

RESULTS

ACUTE NOCICEPTION

Pain thresholds to punctate mechanical stimuli were measured in *Glra3*$^{-/-}$ mice and wild-type littermates using calibrated von Frey hairs, according to the up–down method (Chaplan et al., 1994). Paw withdrawal thresholds to mechanical stimuli were not significantly different between groups ($p > 0.05$; **Figure 1A**). Similarly, thermal thresholds assessed using the Hargreaves' test were indistinguishable

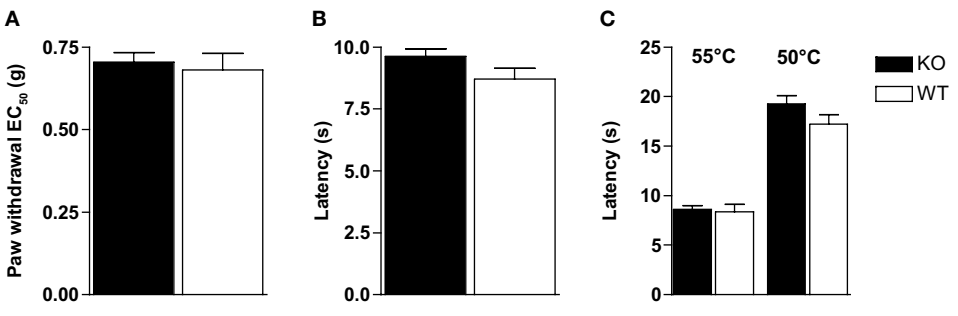

FIGURE 1 | *Glra3*$^{-/-}$ mice show no differences in their acute pain behaviours. (A) Punctate mechanical stimulation using calibrated von Frey (*Glra3*$^{-/-}$, $n = 16$; wild type, $n = 10$). Thermal stimulation using either **(B)** the Hargreaves' apparatus (*Glra3*$^{-/-}$, $n = 30$; wild type, $n = 19$) or **(C)** the hot plate at 50°C or 55°C (*Glra3*$^{-/-}$, $n = 12$; wild type, $n = 12$). Data are presented as mean ± SEM.

between groups ($p > 0.05$; **Figure 1B**). Thermal hypersensitivities were similar across genotypes when measured using the hot plate at either 50°C or 55°C ($p > 0.05$; **Figure 1C**). This absence of an acute phenotype in *Glra3*$^{-/-}$ mice agrees with previous findings (Harvey et al., 2004).

Spinal cord electrophysiology was performed to assess the potential role of GlyR α3 in acute nociception in response to multimodal stimuli. Where behaviour lends information concerning threshold intensities, electrophysiology can be used to inform us about responses to suprathreshold stimuli. Single unit dorsal horn neuronal recordings were performed in *Glra3*$^{-/-}$ ($n = 27$) and wild-type littermates ($n = 22$). The mean depths from the surface of the spinal cord were not significantly different between groups and correspond to the deep laminae of the spinal cord 550 ± 30 μm and 459 ± 38 μm ($p > 0.05$), respectively ensuring that the population of neurons was similar between the two groups, and hence direct comparisons were possible. Responses to electrical, brush, acetone, noxious cold and pinch (**Table 1**); as well as graded mechanical and thermal stimuli (**Figures 2A,B**) were indistinguishable between genotypes ($p > 0.05$).

Table 1 | *Glra3*$^{-/-}$ mice show no alteration in their neuronal responses to multimodal stimuli. Data are presented as mean ± SEM for deep dorsal horn neurones recorded in *Glra3*$^{-/-}$ ($n = 27$) and wild-type mice ($n = 22$). AP represents the mean number of action potentials in response to either a peripheral electrical or 10-s natural stimulus.

	Glra3$^{-/-}$	Wild type
A-fibre threshold (mA)	0.05 ± 0.08	0.07 ± 0.02
C-fibre threshold (mA)	0.94 ± 0.18	0.92 ± 0.16
A-fibre response (AP)	105 ± 10	85 ± 9
C-fibre response (AP)	181 ± 19	143 ± 18
Post discharge (AP)	180 ± 25	155 ± 21
Input (AP)	216 ± 28	199 ± 29
Wind-up (AP)	186 ± 31	118 ± 20
Brush (AP)	67 ± 22	61 ± 14
Pinch (AP)	172 ± 29	154 ± 25
Acetone (AP)	14 ± 5	8 ± 5
1°C	60 ± 11	48 ± 20

INFLAMMATORY PAIN BEHAVIOUR

Consistent with previously reported findings (Harvey et al., 2004) *Glra3*$^{-/-}$ mice displayed showed no difference in the induction of thermal pain sensitisation following CFA (20 μl) injection, but recovered much more quickly compared with wild-type littermate controls (**Figure 3A**). Paw withdrawal latencies to thermal stimulation using the Hargreaves' apparatus were significant versus contralateral responses for wild-type mice days 1–6 and days 1–3 for *Glra3*$^{-/-}$ mice ($p < 0.001$). Consistent with previous findings, *Glra3*$^{-/-}$ mice ($n = 7$) displayed significant differences in thermal hypersensitivity at 4, 5 and 6 days following CFA injection compared with wild-type littermates ($n = 5$, $p < 0.01$).

Acute cutaneous neurogenic inflammation induced by intradermal injection of capsaicin (5 μl; 50 μg/ml) elicited spontaneous pain behaviour in both *Glra3*$^{-/-}$ and wild-type control mice (**Figure 3B**). No significant differences were observed between the type of nociceptive behaviour (i.e. licking, biting and flinching) or the duration of such responses ($p > 0.05$).

Intraplantar injection of carrageenan (20 μl; 2% w/v) elicited significant thermal and mechanical hypersensitivities in both *Glra3*$^{-/-}$ and wild-type control mice at both acute (2–6 h), and sub-acute phases (24 h) ($p < 0.05$–$p < 0.001$; *Glra3*$^{-/-}$, $n = 5$; wild type, $n = 7$; **Figures 3C,D**). Statistical analysis revealed no significant difference in *Glra3*$^{-/-}$ mice in either the magnitude or duration of thermal and mechanical hypersensitivities following carrageenan injection when compared with wild-type control littermates ($p > 0.05$).

Clinically relevant models of inflammation

Intra-articular injection of monosodium iodoacetate (MIA, 7 μl; 5 mg/ml) to induce osteoarthritis (OA) evoked significant behavioural mechanical hypersensitivitiy in both *Glra3*$^{-/-}$ and wild-type control mice when compared with contralateral controls ($n = 6$; $p < 0.001$; **Figure 4A**). Thermal hypersensitivity was not explored since this has been found to be absent in this model (Harvey and Dickenson, 2009). Similarly, a model of rheumatoid arthritis (kaolin/carrageenan; 15 μl, 3% w/v) evoked significant thermal hypersensitivity in both *Glra3*$^{-/-}$ and wild-type control mice when compared with contralateral controls ($n = 6$; $p < 0.05$; **Figure 4B**). No significant differences were observed between genotypes.

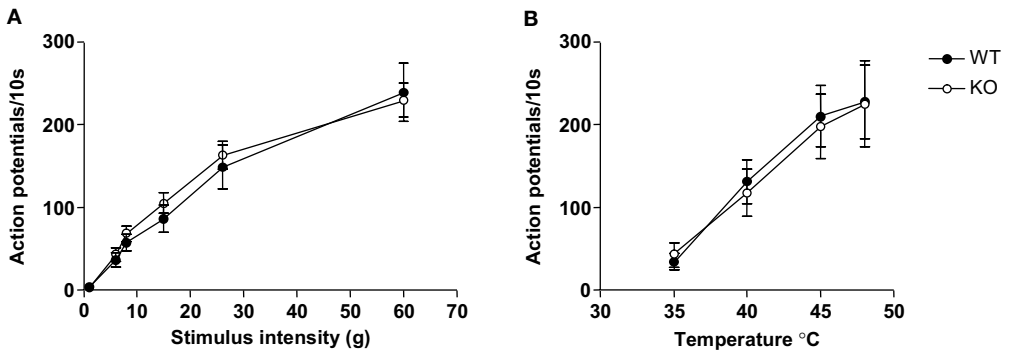

FIGURE 2 | *Glra3*$^{-/-}$ mice show no differences in evoked dorsal horn neuronal responses. (A) Graded mechanical stimuli (von Frey); **(B)** graded thermal stimuli (water jet). Data are presented as mean ± SEM (*Glra3*$^{-/-}$, $n = 27$; wild type, $n = 22$).

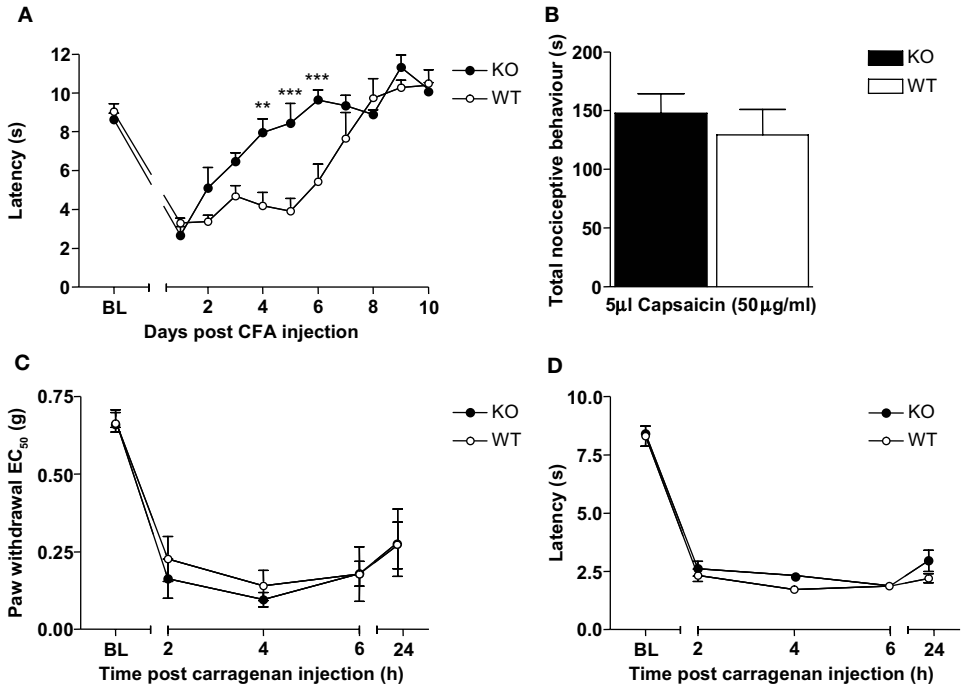

FIGURE 3 | *Glra3*$^{-/-}$ mice display attenuated CFA behaviour, but show no differences in other inflammatory models. (A) Paw withdrawal latencies following unilateral intraplantar CFA (20 µl) were significant versus contralateral responses for wild-type mice days 1–6 and days 1–3 for *Glra3*$^{-/-}$ mice ($p < 0.001$). Paw withdrawal latencies of *Glra3*$^{-/-}$ mice were significantly different from wild-type mice at days 4 ($p < 0.01$, **), 5 and 6 ($p < 0.001$, ***) (*Glra3*$^{-/-}$, $n = 7$; wild type, $n = 5$). **(B)** Total nociceptive behaviour following unilateral intraplantar capsaicin (5 µl, 50 µg/ml) was not significantly different between genotypes (*Glra3*$^{-/-}$, $n = 12$; wild type, $n = 12$). **(C)** Significant mechanical hypersensitivity and **(D)** thermal hypersensitivity was observed at all timepoints following unilateral intraplantar carrageenan (20 µl, 2% w/v) when compared with contralateral controls ($p < 0.05$ –$p < 0.001$) but indistinguishable between genotypes (*Glra3*$^{-/-}$, $n = 5$; wild type, $n = 7$). Data are presented as mean ± SEM and analysed using two-way repeated measures ANOVA, Bonferroni *post hoc* analysis.

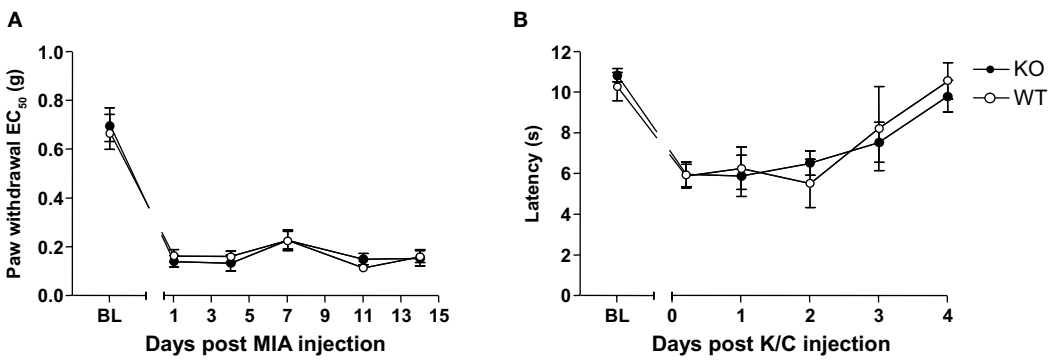

FIGURE 4 | *Glra3*$^{-/-}$ mice show no differences in behavioural hypersensitivity in models of osteoarthritis or rheumatoid arthritis. (A) Significant mechanical hypersensitivity was observed at all timepoints following unilateral intra-articular MIA (10 µl, 5 mg/ml) in both genotypes when compared with contralateral controls ($n = 6$; $p < 0.001$). **(B)** Significant thermal hypersensitivity was observed at days 1 and 2 following unilateral intra-articular K/C injection (15 µl, 3% w/v) in both genotypes when compared with contralateral controls ($n = 6$; $p < 0.05$). No differences were observed between genotypes. Data are presented as mean ± SEM and analysed using two-way repeated measures ANOVA, Bonferroni *post hoc* analysis.

NEUROPATHIC PAIN BEHAVIOUR

Following the induction of peripheral nerve injury using the partial sciatic nerve ligation model, both *Glra3*$^{-/-}$ and wild-type littermates developed robust and persistent mechanical and thermal hypersensitivities from the onset of testing (**Figures 5A,B**). Significant mechanical hypersensitivity was observed in *Glra3*$^{-/-}$ mice at days 3, 5, 7 and 14 ($p < 0.01$–$p < 0.001$) and wild-type mice at days 3–14 ($p < 0.05$–$p < 0.01$) following surgery compared with contralateral controls ($n = 6$). Significant thermal hypersensitivity was observed in *Glra3*$^{-/-}$ mice and wild-type mice at all days following surgery ($p < 0.001$) compared with contralateral controls ($n = 6$). Statistical analysis revealed no significant difference in *Glra3*$^{-/-}$ mice

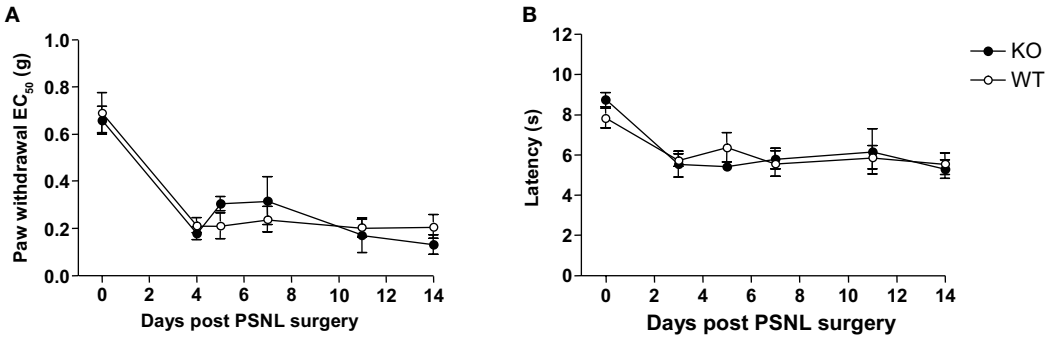

FIGURE 5 | *Glra3*$^{-/-}$ mice show no differences in behavioural hypersensitivitiy following experimentally induced peripheral neuropathy. **(A)** Significant mechanical hypersensitivity was observed in *Glra3*$^{-/-}$ mice at days 3, 5, 7 and 14 ($p < 0.01$–$p < 0.001$) and wild-type mice at days 3–14 ($p < 0.05$–$p < 0.01$) following surgery compared with contralateral controls ($n = 6$). **(B)** Significant thermal hypersensitivity was observed in *Glra3*$^{-/-}$ mice and wild-type mice at all days following surgery ($p < 0.001$) compared with contralateral controls ($n = 6$). No differences were observed between genotypes. Data are presented as mean ± SEM and analysed using two-way repeated measures ANOVA, Bonferroni *post hoc* analysis.

in either the magnitude or duration of thermal and mechanical hypersensitivities following the induction of nerve injury when compared with wild-type control littermates ($p > 0.05$).

VISCERAL PAIN

The visceromotor response (VMR), evoked by the distension of the colon by a balloon inserted intra-colonically, provides a quantifiable measure of visceral pain (visceral hyperalgesia). In rodents, pressures which elicit pain in humans evoke graded abdominal muscle contractions (measured as the VMR), tachycardia and the activation of primary afferent visceral nerves (e.g. pelvic nerve) and subsequent activation of second order projection neurones (Kamp et al., 2003). The response measured at 0 mmHg (with the balloon deflated) was considered as the baseline response; 30 mmHg was considered mildly noxious and 60 mmHg as significantly noxious. Both wild-type and *Glra3*$^{-/-}$ mice displayed coded responses to the graded stimuli (**Figure 6**), however, the responses were not significantly different between the groups ($p > 0.05$).

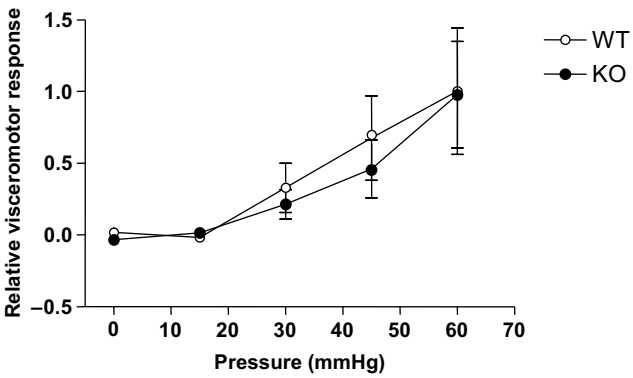

FIGURE 6 | *Glra3*$^{-/-}$ mice show no differences in visceromotor responses to colorectal distension. Isoflurane anesthetised mice display graded visceromotor responses to increasing colorectal pressures. No differences were observed between genotypes (*Glra3*$^{-/-}$, $n = 5$; wild type, $n = 7$). Data are presented as mean (normalised to maximum wild-type control) ± SEM.

DISCUSSION

This study confirms, consistent with previous findings, that GlyR α3 has a significant role in certain inflammatory pain states. In this study, we have used knockout mice to investigate the role of GlyR α3 in a variety of acute, persistent and clinically relevant behavioural pain models as well as neuropathic and visceral pain models. Moreover, *in vivo* electrophysiology has been used to investigate the potential role of these channels in response to suprathreshold stimuli, which equate to intensities that produce high pain scores such as those seen in the clinic.

ACUTE PAIN THRESHOLDS

Consistent with previous findings, acute behavioural thermal and mechanical thresholds in *Glra3*$^{-/-}$ mice were unaltered (Harvey et al., 2004). These behavioural findings are re-enforced by the electrophysiological characterisation of *Glra3*$^{-/-}$ mice to an array of different modalities ranging from threshold to suprathreshold intensities, suggesting that GlyR α3 is not implicated in acute nociception. These characteristics are advantageous when investigating potential therapeutic targets since ideal analgesic targets should be pain-state specific, leaving normal nociceptive processing and thus normal sensation unaltered.

INFLAMMATORY PAIN MODELS

Although the role of GlyR α3 in inflammatory pain has been previously investigated in selected models, this study expands on the current work by investigating its potential role in other inflammatory pain states including capsaicin and carrageenan inflammation and clinically relevant models such as the kaolin/carrageenan (K/C) model of rheumatoid arthritis and the monosodium iodoacetate model of osteoarthritis. CFA-induced behavioural hypersensitivity was reconfirmed in this study, where *Glra3*$^{-/-}$ mice displayed an accelerated rate of recovery, in line with previous findings (Harvey et al., 2004). Since GlyR α3 displayed this inflammatory phenotype, its potential role in other models of inflammation was investigated.

Intraplantar capsaicin elicits immediate spontaneous pain behaviour in rodents through the activation of the vanilloid receptor 1 (VR1/TRPV1). This evoked nocifensive behaviour is thought to arise from the direct activation of C-fibres rather than as a consequence of local inflammation *per se*. Although capsaicin-induced C fibre activation has previously been found to elicit prostaglandin E_2 release in spinal cord slices (Malmberg and Yaksh, 1994), the exact spinal mechanisms have not been fully established. In this study, no behavioural phenotype was observed following intraplantar capsaicin injection in *Glra3*$^{-/-}$ mice.

Intraplantar injection of carrageenan evokes both mechanical and thermal hypersensitivity in rodents. These behaviours can be subdivided into an acute phase (2–6 h) and sub-acute phase (24 h) (Kolhekar et al., 1997). Prostaglandin synthesis occurs both at peripheral and spinal sites since both systemic and intrathecal cyclo-oxygenase (COX) inhibition attenuates nocifensive behaviour. However, intrathecal COX inhibitors were only effective when given prior to carrageenan injection (Dirig et al., 1998). A later study found that spinal CSF levels of PGE_2 peaked in the acute phase but remained significantly elevated up to 24 h (Guay et al., 2004). Taken together, this suggests that there is potentially only a limited time frame where spinal PGE_2 is contributing to the pain behaviours, whereas peripheral or supraspinal sites are involved in both the induction and maintenance of carrageenan-evoked pain behaviours. In this study, no phenotype was observed in either the acute or sub-acute phase of carrageenan behavioural hypersensitivity in *Glra3*$^{-/-}$ mice. Therefore, although spinal prostaglandins are involved in the induction, and to a lesser extent the maintenance of this model, the EP_2 receptor – GlyR α3 signalling pathway is unlikely to represent the downstream substrate.

ARTHRITIC PAIN MODELS

Arthritic pain, as a result of either rheumatoid arthritis or osteoarthritis, represents a major chronic healthcare problem escalating with our aging and obese population (Neugebauer et al., 2007). Although different models exist for their study, the chemical models of MIA, for osteoarthritis, and K/C for rheumatoid arthritis, are popular among pain researchers. In this study, mice displayed significant behavioural hypersensitivity in the paw of the affected limb in both arthritic models, consistent with other literature (Yang et al., 1996; Harvey and Dickenson, 2009). Similar to other peripheral inflammatory models, spinal prostanoids are thought to contribute largely to the observed pain behaviours. Infrapatellar K/C injection leads to a robust and persistent (up to 72 h) increase in spinal PGE_2 levels in rats (Dirig and Yaksh, 1999). In the MIA model of osteoarthritis, NSAIDs are only effective at the early stages of the model, suggesting a switch in the underlying mechanisms at later time points although these have not yet been fully established (Bove et al., 2003; Pomonis et al., 2005; Vonsy et al., 2008). In this study, it would appear that despite good evidence for central prostanoid release, GlyR α3 is not implicated in the underlying pain pathophysiology.

NEUROPATHIC PAIN

The benefits of using NSAIDs to treat neuropathic pain remains controversial, and at best they may only be effective at very early stages following the nerve insult (Ma et al., 2002; Schafers et al., 2004). In this study, analysis of the pain behaviours following the induction of peripheral nerve injury using the partial sciatic nerve injury model revealed that *Glra3*$^{-/-}$ mice developed profound mechanical and thermal hypersensitivities indistinguishable from wild-type littermates. This is in accordance with previous studies investigating the role of GlyR α3 in the chronic constriction injury model of neuropathic pain (Racz et al., 2005; Hösl et al., 2006). It is apparent, therefore, that unlike its clear role in certain types of inflammatory pain, GlyR α3 is not critical for the induction or maintenance of neuropathic pain.

VISCERAL PAIN

Bowel disorders such as irritable bowel syndrome (IBS) are characterised by poorly localised abdominal pain and sufferers display visceral hypersensitivity to experimental colorectal distension compared with healthy volunteers (Verne et al., 2001). To investigate the potential role of GlyR α3 in such bowel complaints we used a method of colorectal distension which produces a quantifiable visceromotor response in lightly anaesthetised mice. In this study, although the magnitude of the VMR was dependent on the applied pressure, no significant differences were observed between genotypes. This contrasts with other studies using the acetic acid writhing test, which reported a significant attenuation in the writhing responses in *Glra3*$^{-/-}$ mice (Racz et al., 2005). This model, however, is often difficult to interpret as little is known about the pathophysiology following acetic acid injection. The acute testing regimen utilised in this study, however, may not be sufficiently long to involve the proposed EP_2 receptor – GlyR α3 signalling pathway. A phenotype using the CRD model might be uncovered by future experiments involving colonic hypersensitivity where VMR are recorded in response to an irritant injected intra-colonically which enhance the VMR to both noxious and non-noxious intensities in rats (Coutinho et al., 1996), although care must be taken to avoid general systemic effects (Deitch et al., 1992).

CONCLUSION

Since the contribution of a particular molecular substrate to the nociceptive phenotype will most likely depend on the origin of the pain (e.g. inflammatory, neuropathic or dysfunctional), the temporal characteristics (i.e. acute or chronic), and the underlying sensitivity (peripheral or central) it is likely that different substrates underlie different pain states.

A selective role of GlyR α3 can be observed based on clear effects in the CFA model, yet there is no clear role in other inflammatory, neuropathic or visceral pain models. This study, therefore, suggests that GlyR α3 may play an important role in mediating PGE_2-induced sensitisation, but only in certain pain states. Previously, GlyR α3 has been found not to contribute to behavioural hypersensitivity following formalin injection (Hösl et al., 2006). Although this model, like most models of peripheral injury, evokes spinal PGE_2 release and is attenuated by COX inhibitors, it most likely reflects a different downstream target since EP2 receptor knockout mice, but not *Glra3*$^{-/-}$ mice, displayed attenuated nocifensive behaviour. This could also hold true for the other inflammatory models investigated in this study and may point to the differential involvement of other prostanoids and EP receptors, located either

centrally or peripherally, that may activate different molecular effectors (e.g. Matsumoto et al., 2005; Hutchinson et al., 2009). What is clear, however, is that different inflammatory pain states are underpinned by different molecular substrates such that their investigation will help to dissect out pain-state specific mechanisms. These observations may be reflected in different clinical phenotypes and, as such, a more mechanistic based approach may be required in the clinic.

ACKNOWLEDGMENTS

Robert J. Harvey and Anthony H. Dickenson conceived the study, Victoria L. Harvey and Anthony H. Dickenson designed and performed the experiments, analysed data and wrote the manuscript with input from all authors. Alex Caley provided mouse genotyping; Robert J. Harvey and Ulrike C. Müller generated and supplied the *Glra3*$^{-/-}$ mice. This work was supported by MRC Project Grant (G0500833) to Robert J. Harvey and Anthony H. Dickenson.

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Defective glycinergic synaptic transmission in zebrafish motility mutants

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Glycine is a major inhibitory neurotransmitter in the spinal cord and brainstem. Recently, *in vivo* analysis of glycinergic synaptic transmission has been pursued in zebrafish using molecular genetics. An ENU mutagenesis screen identified two behavioral mutants that are defective in glycinergic synaptic transmission. Zebrafish *bandoneon* (*beo*) mutants have a defect in *glrbb*, one of the duplicated glycine receptor (GlyR) β subunit genes. These mutants exhibit a loss of glycinergic synaptic transmission due to a lack of synaptic aggregation of GlyRs. Due to the consequent loss of reciprocal inhibition of motor circuits between the two sides of the spinal cord, motor neurons activate simultaneously on both sides resulting in bilateral contraction of axial muscles of *beo* mutants, eliciting the so-called 'accordion' phenotype. Similar defects in GlyR subunit genes have been observed in several mammals and are the basis for human hyperekplexia/startle disease. By contrast, zebrafish *shocked* (*sho*) mutants have a defect in *slc6a9,* encoding GlyT1, a glycine transporter that is expressed by astroglial cells surrounding the glycinergic synapse in the hindbrain and spinal cord. GlyT1 mediates rapid uptake of glycine from the synaptic cleft, terminating synaptic transmission. In zebrafish *sho* mutants, there appears to be elevated extracellular glycine resulting in persistent inhibition of postsynaptic neurons and subsequent reduced motility, causing the 'twitch-once' phenotype. We review current knowledge regarding zebrafish 'accordion' and 'twitch-once' mutants, including *beo* and *sho,* and report the identification of a new α2 subunit that revises the phylogeny of zebrafish GlyRs.

Keywords: glycine, synapse, receptor, transporter, zebrafish, behavior, motility

INTRODUCTION

Glycine receptors (GlyRs) and $GABA_A$ receptors are pentameric ligand-gated chloride channels (reviewed in Moss and Smart, 2001) that mediate inhibitory synaptic transmission. In the vertebrate CNS, GABAergic synaptic transmission is mainly used in the brain, while glycinergic synaptic transmission operates in the brainstem and spinal cord to regulate motor systems. Although GlyRs are found in all vertebrates and perhaps selected invertebrates (Kehoe et al., 2009), mammalian GlyRs have been studied most extensively. Inherited defects in genes encoding the major adult α1β GlyR causes startle disease/hyperekplexia in humans, characterized by noise or touch-induced seizures that result in muscle stiffness and life-threatening neonatal apnea episodes (Sament and Schwartz, 1957; Kirstein and Silfverskiold, 1958; Bakker et al., 2006; Harvey et al., 2008). The biological roles of GlyRs containing the α2, α3 and α4 subunits are less clear, although GlyR α3 is clearly linked to inflammatory pain pathways (Harvey et al., 2004). Glycine also binds to the NR1 subunit of the NMDA receptor, acting as an essential coagonist for excitatory synaptic transmission mediated by glutamate (Johnson and Ascher, 1987; Moriyoshi et al., 1991; Kuryatov et al., 1994). Glycine can also activate the NR3B-containing NMDA receptor in the absence of L-glutamate in heterologous expression systems (Chatterton et al., 2002). However, the *in vivo* physiological significance of glycine-mediated excitatory synaptic transmission is unclear.

Two glycine transporters (GlyTs) also regulate glycinergic synaptic transmission (Eulenburg et al., 2005). GlyTs are thought to take up glycine from the synaptic cleft to terminate glycine-mediated synaptic transmission (GlyT1) and resupply glycine to glycinergic presynaptic terminals (GlyT2). Mouse models of GlyT1 dysfunction exhibit severe motor deficits accompanied by lethargy, hypotonia and hyporesponsivity, and die within 6–14 h after birth as a result of respiratory failure, although wasting and dehydration caused by an inability to suckle may also play a role (Gomeza et al., 2003a; Tsai et al., 2004). Curiously, these symptoms resemble glycine encephalopathy, a disease associated with disruption of the mitochondrial glycine cleavage system, which degrades excess glycine (Applegarth and Toone, 2006). Mutations in the GlyT2 gene cause startle disease/hyperekplexia in humans and congenital muscular dystonia type 2 (CMD2) in cattle (Rees et al., 2006; Charlier et al., 2008; Harvey et al., 2008).

Abbreviations: *acc*, *accordion*; *beo*, *bandoneon*; cDNA, complementary DNA; CMD, congenital muscular dystonia; CNS, central nervous system; DNA, deoxyribonucleic acid; dpf, days post-fertilization; EC, excitation-contraction; ENU, N-ethyl-N-nitrosourea; EST, expressed sequence tag; GABA, γ-aminobutyric acid; GFP, green fluorescent protein; GlyR, glycine receptor; GlyT, glycine transporter; hpf, hours post-fertilization; mRNA, messenger ribonucleic acid; MuSK, muscle-specific kinase; nAChR, nicotinic acetylcholine receptor; NMDA, N-methyl-D-asparatate; NMJ, neuromuscular junction; NR, NMDA receptor; RFP, red fluorescent protein; RNA, ribonucleic acid; RT-PCR, reverse transcription-polymerase chain reaction sequence; TILLING, targeting-induced local lesion in genome; UAS, upstream activating sequence.

Intensive examination of the small freshwater fish, zebrafish, has occurred in the past several decades due to the optical clarity and accessibility of zebrafish embryos and amenability to genetic strategies (Streisinger et al., 1981; Eisen et al., 1986; Driever et al., 1996; Haffter et al., 1996). These studies have enhanced our understanding of the *in vivo* function of genes involved in the regulation of glycinergic synaptic transmission. Two zebrafish mutations, one defective in GlyR function (*bandoneon*) and the other in GlyT1 function (*shocked*), were isolated from behavioral mutagenesis screens (Granato et al., 1996; Cui et al., 2004, 2005; Luna et al., 2004; Hirata et al., 2005; Masino and Fetcho, 2005; Mongeon et al., 2008). Importantly, these zebrafish mutants show physiological and behavioral defects similar to non-human mammalian mutants as well as humans with startle disease and glycine encephalopathy. Thus, zebrafish mutants serve as attractive vertebrate models for childhood neurological disorders. In this review, we discuss the history of the zebrafish mutants *bandoneon* and *shocked* and report the identification of new GlyR cDNA sequences that revise the phylogeny of zebrafish GlyRs.

ADVANTAGES OF ZEBRAFISH AS A MODEL ORGANISM

Zebrafish (*Danio rerio*) have several advantages for the analysis of vertebrate development (Grunwald and Eisen, 2002). First, raising zebrafish is easy. A pair of adult zebrafish can generate 100–200 fertilized eggs in a single spawn. The generation time is 3 months, which is comparable to other vertebrate models such as mice. It is neither expensive nor difficult to maintain thousands of zebrafish in a laboratory. Second, all stages of development occur externally and rapidly, with most organs formed by 5 days of fertilization (Kimmel et al., 1995). The fast pace of development allows one to analyze development in living zebrafish. Third, the embryos are transparent, which makes them amenable for live imaging of individual cells deep within the body such as neurons in the brain. In fact, a number of transgenic zebrafish that express GFP (green fluorescent protein), RFP (red fluorescent protein) or calcium indicators under control of tissue-specific, Gal4-inducible or stress-inducible promoters has been generated to visually monitor the development of tissues and stress response as well as neuronal activity (Amsterdam et al., 1995; Peters et al., 1995; Higashijima et al., 1997, 2003; Long et al., 1997; Scheer and Campos-Ortega, 1999; Halloran et al., 2000). Fourth, the electrophysiological activity of neurons and muscles in zebrafish embryos can be recorded using standard current-clamp and voltage-clamp methods (Prugh et al., 1982; Grunwald et al., 1988; Legendre and Korn, 1995; Drapeau et al., 1999; Fetcho, 2007). Using these methods, the properties of neural circuits that underlie the earliest zebrafish behaviors are beginning to be clarified (Legendre and Korn, 1994; Ribera and Nüsslein-Volhard, 1998; Neuhauss et al., 1999; Ono et al., 2001; Saint-Amant and Drapeau, 2001; Sidi et al., 2003; Kimura et al., 2006; McLean et al., 2007; Tanimoto et al., 2009).

The aforementioned advantages make the zebrafish an excellent system for examining vertebrate development. However, it is the genetic manipulability of zebrafish that has attracted the most attention of biologists (Grunwald and Eisen, 2002; Amsterdam and Hopkins, 2006). Methods for mutagenesis of zebrafish were established in the early 1990's (Mullins et al., 1994) with two large-scale mutant screens completed in Tübingen, Germany and Boston, USA by 1996 (Driever et al., 1996; Haffter et al., 1996). These screens used chemical mutagens and identified more than 4,000 mutants to kick-start large-scale mutagenesis analysis of a vertebrate embryo. The advent of the zebrafish genome sequencing project in 2001[1] has greatly improved the molecular identification of chemically-induced mutations. Retrovirus- and transposon- mediated gene disruption was also developed to generate zebrafish mutants in a systematic manner (Lin et al., 1994; Gaiano et al., 1996a,b; Amsterdam et al., 1999, 2004; Kawakami et al., 2000, 2004; Golling et al., 2002; Sivasubbu et al., 2006; Wang et al., 2007). In addition to these forward genetic approaches, targeting-induced local lesion in genome, combining ENU mutagenesis with large-scale exon sequencing has made it possible to inactivate selected zebrafish genes (Wienholds et al., 2002). More recently, a gene-targeting technique using designed sequence-specific zinc-finger proteins has been demonstrated to be effective in disrupting key genes in zebrafish (Doyon et al., 2008; Meng et al., 2008). Genes can also be knocked down during embryonic stages by the injection of antisense morpholino oligonucleotides into recently fertilized embryos (Nasevicius and Ekker, 2000). These highly stable oligonucleotides can effectively block translation or splicing of a target mRNA to interfere with gene function *in vivo*. Splice-site morpholinos have the major advantage that their efficacy can be monitored by RT-PCR.

Transgenesis is also a powerful tool for analysis of development and gene function in zebrafish. The first zebrafish transgenic lines were generated by injection of DNA into embryos (Stuart et al., 1988, 1990; Culp et al., 1991; Bayer and Campos-Ortega, 1992). Transgenic zebrafish exhibiting cell-specific or inducible gene expression have proven extremely useful for *in vivo* analysis of gene function and developmental processes (Higashijima et al., 1997; Long et al., 1997; Halloran et al., 2000). More recently, virus- and transposon-mediated transgenesis methods have greatly improved the efficiency of generating transgenic zebrafish (Davidson et al., 2003; Kawakami et al., 2004; Ellingsen et al., 2005; Sivasubbu et al., 2006; Kwan et al., 2007; Villefranc et al., 2007). The increased efficiency has significantly enhanced the application of powerful controlled expression methods such as the Gal4/UAS system (Scheer and Campos-Ortega, 1999; Inbal et al., 2006; Scott et al., 2007; Asakawa et al., 2008; Halpern et al., 2008).

Recent technical advances make zebrafish an attractive complement to invertebrate systems such as *C. elegans* and *Drosophila* on the one hand and the transgenic, knockout and knock-in mice on the other. Indeed, zebrafish mutant and transgenic embryos are useful for *in vivo* high-throughput chemical screening, thereby enabling discovery of novel pharmaceutical reagents that may be useful for mitigating human diseases (Peterson et al., 2000, 2004; Stern et al., 2005).

DEVELOPMENT OF LOCOMOTION BEHAVIOR IN ZEBRAFISH

Zebrafish exhibit three distinct behaviors during embryogenesis; spontaneous coiling, touch-evoked escape contractions and swimming. Spontaneous coiling appears after 17 hours postfertilization (hpf) and consists of side-to-side alternating contractions of the axial muscles in the trunk and tail (**Figure 1A**; Saint-Amant and Drapeau, 1998; Downes and Granato, 2006; Pietri et al., 2009).

[1]http://www.sanger.ac.uk/modelorgs/zebrafish.shtml

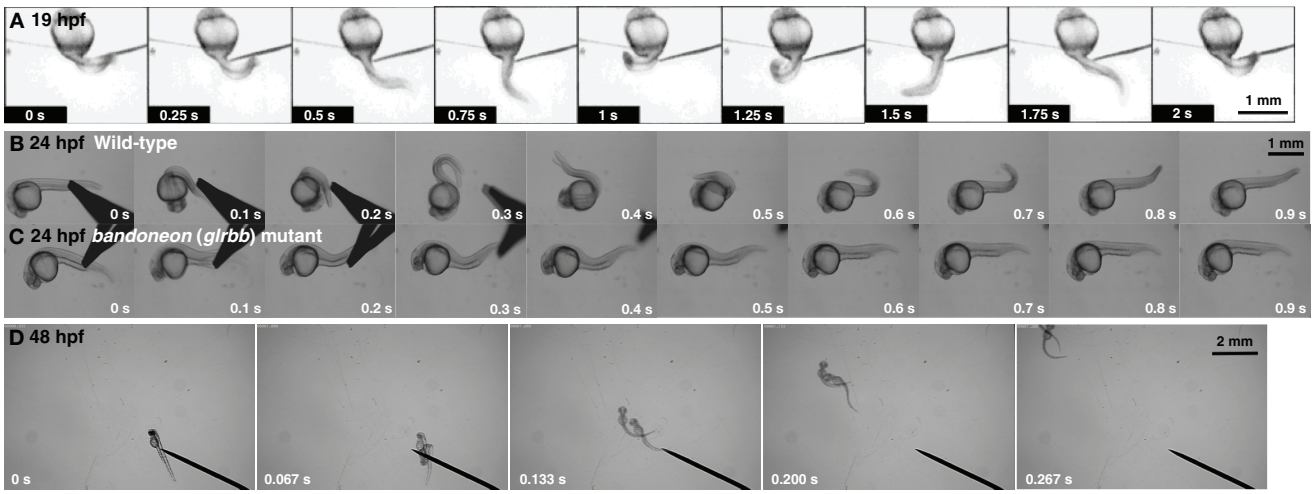

FIGURE 1 | Zebrafish embryos display three early behaviors. **(A)** A 19 hpf wild-type embryo exhibits spontaneous coiling. **(B)** At 24 hpf, a wild-type embryo responds to mechanosensory stimulation with two fast, alternating contractions. **(C)** A *bandoneon (beo)* mutant embryo with a defect in *glrbb* responds to mechanosensory stimulation with bilateral axial muscle contractions that causes the trunk to shorten and bend dorsally. **(D)** At 48 hpf, wild-type embryos swim away following tactile stimulation.

This relatively slow coiling is independent of sensory stimulation, with the frequency of spontaneous coiling peaking at 0.3~1 Hz at 19 hpf and gradually declining to less than 0.1 Hz by 26 hpf. Thus, locomotor circuits are functional as early as 17 hpf. Interestingly, the isolated trunk and tail following transections between somites 5 and 7 also displays spontaneous coiling with a similar time course and frequency compared to intact embryos (Downes and Granato, 2006). However, transections removing the first 10 somites eliminate all spontaneous activity (Pietri et al., 2009). These experiments suggest that the neural network triggering spontaneous coiling is located in the rostral spinal cord between somites 5 and 10.

After 21 hpf, zebrafish embryos respond to touch with escape contractions that typically consists of two to three rapid, alternating contractions of the tail, with muscles contralateral to the side of tactile stimulation contracting first to turn the embryo away from the stimulus (**Figure 1B**; Saint-Amant and Drapeau, 1998; Hirata et al., 2005). Head and yolk stimulation activates trigeminal sensory neurons (Drapeau et al., 2002), whereas trunk and tail stimulation activates Rohon-Beard neurons, which are primary sensory neurons located within the spinal cord and hindbrain of embryonic fish and amphibians. Thus, the neural circuitry responsible for locomotor responses to external stimuli is functional shortly after the appearance of spontaneous coiling. Applying tactile stimuli to spinalized embryos, which were transected rostral to somite 1, evokes the normal touch response (Pietri et al., 2009). By contrast, transections at more caudal locations (somites 1–10) result in progressively weaker responses in progressively caudal locations (Downes and Granato, 2006; Pietri et al., 2009). Thus, the rostral hindbrain is necessary for the full touch-evoked escape response. It has also been reported that the touch response is dependent on AMPA-type glutamate receptor activation (Pietri et al., 2009).

By 28 hpf, tactile stimulation initiates swimming (**Figure 1D**; Saint-Amant and Drapeau, 1998). The frequency of alternating contractions during swimming reaches 30 Hz at 36 hpf, which is comparable with the frequency in adult zebrafish (Buss and Drapeau, 2001). Although spinalized embryos transected between somites 5–7 respond to touch with an initial tail flip, swimming does not follow the initial response in most cases (Downes and Granato, 2006). Thus, it appears that the spinal cord can initiate a touch response, but that supraspinal input is necessary for swimming.

FORWARD GENETICS TO IDENTIFY ZEBRAFISH MUTANTS SHOWING MOTILITY DEFECTS

Since zebrafish embryos display organized behaviors within the first several days of development, behavioral mutagenesis screens are an efficient way to isolate mutants that have defects in the formation and function of neural circuits, including neuronal excitability and synaptic transmission. In the Tübingen screen, Granato and his colleagues reported 166 mutants that showed defective motility at 48–60 hpf (Granato et al., 1996). Mutations that induced obvious developmental defects such as abnormal morphology and increased degeneration were eliminated, since they would also lead to defective responses. Some of the behavioral mutations were linked to muscle defects by simple visual inspection of muscle striation using polarized light. The actin-myosin structure of normal muscle fibers resulted in birefringence (double refraction) when viewed this way, while muscles with defective actin-myosin organization resulted in decreased birefringence (Felsenfeld et al., 1990). Indeed, mutations in dystrophin, laminin, titin, Hsp90 and the cognate cochaperone Unc45b were identified in this manner (Bassett et al., 2003; Etard et al., 2007; Hall et al., 2007; Steffen et al., 2007; Hawkins et al., 2008; Guyon et al., 2009). Several other muscle mutations exhibited defects in excitation-contraction (EC) coupling or muscle structure that resembled human myopathies (Schredelseker et al., 2005, 2009; Zhou et al., 2006; Hirata et al., 2007; Dowling et al., 2009). Since some of these zebrafish mutants exhibit muscle degeneration similar to human diseases, they could be useful for the biological and therapeutic analysis of these diseases (Kunkel et al., 2006; Lieschke and Currie, 2007).

Among the 166 motility mutations isolated from the Tübingen screen, 103 mutants displayed normal birefringence suggesting impairments in the nervous system, neuromuscular junction (NMJ) or functional components of muscle such as EC coupling. These mutants have been further classified into several classes by their responses to touch such as no response, normal but reduced response, vigorous but abnormal response, or simultaneous, bilateral contractions. The latter were named 'accordion' class mutants (**Table 1**), because they respond to tactile stimulation with apparent simultaneous, bilateral contractions of axial

Table 1 | Accordion mutants.

Mutant	Alleles	Mutation	Phenotype and gene defect	References
accordion ***acc***	dta5	Unknown	Embryonic lethal. Touch-induced uncoordinated contraction of trunk muscles resulting in a contracted wavy notochord, 10–20% shorter than wild type. **Mutations I97N, S766F, T848I in the skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase SERCA1 gene *(atp2a1)* on chromosome 3.**	Granato et al. (1996), Odenthal et al. (1996), Hirata et al. (2004), Gleason et al. (2004), Masino and Fetcho (2005)
	mi25i	**I97N**		
	mi289a	**T848I**		
	tc249a	Unknown		
	ti284a†	Unknown		
	tm286	Unknown		
	tn218b	Unknown		
	tp72x	Unknown		
	tq206§	**S766F**		
	ty20	Unknown		
bajan ***baj***	**tf247**	**IVS2-2A > C**	Embryonic lethal. Uncoordinated contraction of trunk muscles, eventually completely immotile. **Intron 2 splice acceptor site mutation in the choline acetyltransferase gene *(chat)* on chromosome 13.**	Granato et al. (1996), Odenthal et al. (1996), Wang et al. (2008)
bandoneon ***beo***	**mi106a**	**R275H**	Embryonic lethal. Touch-induced uncoordinated contraction of trunk muscles resulting in a contracted wavy notochord, slightly bent up, 10–20% shorter than wild type. **Mutations Y79X, L255R, R275H in the GlyR beta subunit gene *(glrbb)* on chromosome 14.**	Granato et al. (1996), Odenthal et al. (1996), Hirata et al. (2005), Masino and Fetcho (2005)
	ta86d	Unknown		
	ta92†	Unknown		
	tf242	Unknown		
	tm115	Unknown		
	tp221	**Y79X**		
	tu230‡	Allele lost		
	tw38f§	**L255R**		
diwanka ***diw***	**ts286**	**Q608X**	Embryonic lethal. Touch-induced uncoordinated contraction of trunk muscles resulting in a contracted wavy notochord, slightly bent up, 10–20% shorter than wild type, small eyes and enlarged hindbrain ventricle. **Intron 4 splice acceptor site and nonsense mutations W447X and Q608X in the procollagen lysine 2-oxoglutarate 5-dioxygenase 3 gene *(plod3)* on chromosome 23.**	Granato et al. (1996), Odenthal et al. (1996), Zeller and Granato (1999), Zeller et al. (2002), Schneider and Granato (2006)
	tv205a	**IVS4-1A > G**		
	tz290	**W447X**		
expander ***exp***	tu12	Unknown	Embryonic lethal. Uncoordinated contraction of trunk muscles resulting in a contracted wavy notochord, 10–20% shorter than wild type. **Unknown gene on chromosome 11.**	Granato et al. (1996), Odenthal et al. (1996), Geisler et al. (2007)
quetschkommode ***que***	ti274	Unknown	Embryonic lethal. Uncoordinated contraction of trunk muscles resulting in a contracted wavy notochord, 10-20% shorter than wild type. **Unknown gene on chromosome 22.**	Granato et al. (1996), Odenthal et al. (1996), Geisler et al. (2007)
ziehharmonika ***zim*** ***ache***	**sb55**	**S226N**	Embryonic lethal. Uncoordinated contraction of trunk muscles resulting in a contracted wavy notochord, 10–20% shorter than wild type. Eventually becoming completely immotile. **Mutations Y139X, G198R and S226N in the acetylcholinesterase gene *(ache)* on chromosome 7.**	Granato et al. (1996), Odenthal et al. (1996), Behra et al. (2002), Downes and Granato (2004)
	tf222a	**G198R**		
	tm205§	**Y139X**		
	tm206‡	Allele lost		
Unresolved	ta222b	Unknown	Unknown.	Granato et al. (1996), Odenthal et al. (1996)

‡Mutant lost, †Viable allele, §Strongest allele. Information compiled from: http://www.eb.tuebingen.mpg.de/core-facilities/zebrafish-stockcenter; http://zfin.org/

muscles, resulting in shortening of the body rather than the normal alternating contractions. Molecular genetic studies have revealed that the 'accordion' phenotype can arise from numerous distinct mechanisms. For example, *accordion* (*acc*) mutants have slow muscle relaxation due to defective clearance of cytosolic Ca^{2+} caused by mutations in *atp2a1*, encoding the sarcoplasmic reticulum Ca^{2+}-ATPase SERCA1, resulting in overlap of contractions by axial muscles on the two sides of mutant fish (Gleason et al., 2004; Hirata et al., 2004). Other 'accordion' class mutants show defects in cholinergic transmission, such as *zeihharmonika* (*zim*) which harbors either missense or nonsense mutations in *ache*, encoding acetylcholinesterase (Behra et al., 2002; Downes and Granato, 2004) and *bajan* (*baj*) which harbors a splice acceptor site mutation in *chat*, encoding choline acetyltransferase (Wang et al., 2008). By contrast, *diwanka* (*diw*) mutants show defective primary motoneuron pathfinding as a result of nonsense mutations in *plod3*, encoding the procollagen lysine 2-oxoglutarate 5-dioygenase 3 (Zeller and Granato, 1999; Zeller et al., 2002; Schneider and Granato, 2006).

Simultaneous contraction of antagonistic muscles was also attributable to bilateral activation of motor neurons caused by impaired reciprocal inhibition in *glrbb*, encoding one of two zebrafish GlyR β subunits (**Figure 1C**; Hirata et al., 2005). Thus, defects in muscle Ca^{2+} storage, cholinergic transmission, motor projection and glycinergic transmission lead to very similar phenotypes in zebrafish embyros. Two additional 'accordion' class mutants, *expander* (*exp*) and *quetschkommode* (*que*) remain to be characterized (**Table 1**; Granato et al., 1996), and it will be intriguing to determine whether the underlying defects fit into one or other of the functional classes above. In order to distinguish between neuronal and muscle defects, one approach is to record the electrophysiological responses of muscles to sensory stimulation (**Figure 2A**). The output of the nervous system and status of the NMJ can be assayed by membrane potential and voltage-clamp recordings following tactile stimulation of zebrafish embryos. For example, recordings from the muscles of *accordion* (*atp2a1*) mutants show normal rhythmic activity corresponding to fictive swimming, indicating that the nervous system is unaffected in *accordion* (**Figures 2B,C**; Hirata et al., 2004). However, highly aberrant, arrhythmic responses can be recorded from the muscles of bandoneon (*glrbb*) larvae, demonstrating that the nervous system output is defective in these mutants (**Figure 2D**; Hirata et al., 2005).

Additional touch-insensitive mutants from the Tübingen screen, such as *alligator* (*ali*), *macho* (*mao*) and *steifftier* (*ste*), have defects in the excitability of sensory Rohon-Beard neurons (Granato et al., 1996; Ribera and Nüsslein-Volhard, 1998; Gnuegge et al., 2001; Pineda et al., 2005). Although the genes responsible for these mutations have not yet been identified, these mutants show reduced Na^+ current amplitudes. By contrast, the zebrafish *twitch twice* (*twt*) mutant showing an aberrant unidirectional startle response was found to harbor nonsense mutations in *robo3*, encoding roundabout 3, a Slit ligand receptor essential for Mauthner cell axon guidance (Burgess et al., 2009). Unsurprisingly, other mutations that affect axon outgrowth also exhibit abnormal behavior (Zeller and Granato, 1999; Zhang and Granato, 2000; Zhang et al., 2004; Schneider and Granato, 2006; Palaisa and Granato, 2007; Tanaka et al., 2007). For example, the mutant *unplugged* (*unp*) is defective in muscle-specific receptor tyrosine kinase (MuSK) and exhibits

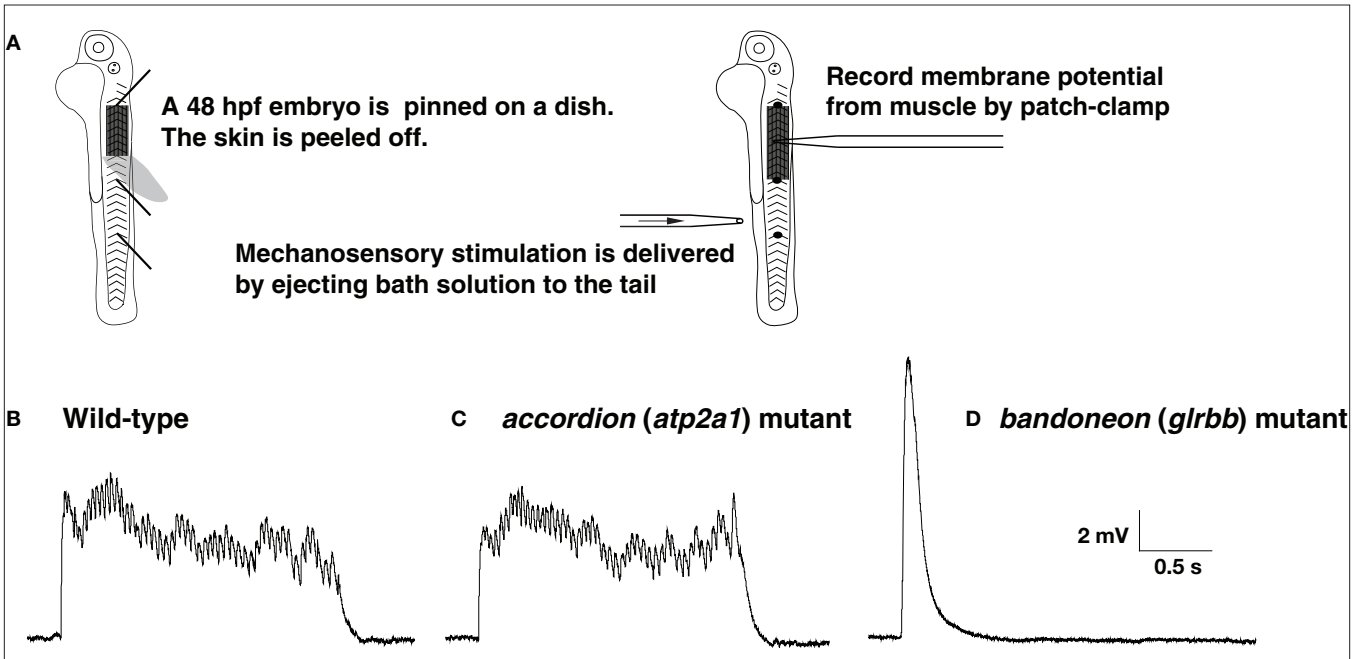

FIGURE 2 | The output of the CNS is normal in *accordion* (*atp2a1*) mutants but abnormal in *bandoneon* (*glrbb*) mutants. (A) Schematic summary of the experimental procedure. **(B)** Wild-type muscle at 48 hpf responds to tactile stimulation with rhythmic depolarizations, representing cyclic muscle contractions during fictive swimming. **(C)** *accordion* (*atp2a1*) mutant muscle respond to tactile stimulation with a similar rhythmic pattern, indicating that the outputs from the CNS are normal. **(D)** *bandoneon* (*glrbb*) mutant muscle responds to tactile stimulation with a short and large voltage response devoid of rhythmicity, suggesting that the CNS outputs are aberrant.

defective initial outgrowth of motor axons (Lefebvre et al., 2007; Jing et al., 2009). Other behavioral mutants exhibiting decreased synaptic transmission and clustering of nAChRs at the NMJ have also been identified including *nicotinic receptor* (*nic*) and *sofa potato* (*sop*) which harbor mutations in the nAChR α and δ subunit genes, respectively. By contrast, mutants *unp* and *twitch-once* (*two*) have mutations in the genes encoding MuSK and the AChR clustering factor rapsyn, respectively (Westerfield et al., 1990; Sepich et al., 1998; Ono et al., 2001, 2002, 2004; Saint-Amant et al., 2008). Curiously, another mutant in the 'twitch-once' class of motility mutants (Granato et al., 1996), *shocked* (*sho*), was recently shown to result from mutations in *slc6a9* encoding GlyT1 (**Table 2**; Cui et al., 2005; Mongeon et al., 2008). Once again, this highlights that central nervous system and muscle defects can result in phenocopying in zebrafish.

ZEBRAFISH GLYCINE RECEPTOR GENES

Inhibitory GlyRs belong to a superfamily of ligand-gated ion channels that includes nAChRs, serotonin ($5HT_3$) receptors and $GABA_A$ receptors (Lynch, 2004). GlyRs are heteromultimers consisting of ligand-binding α and structural β subunits (Grenningloh et al., 1987, 1990a; Langosch et al., 1988), the latter contain a binding site for gephyrin, a multifunctional cytoplasmic linker protein that clusters αβ GlyRs at synapses (Kirsch et al., 1993; Meyer et al., 1995; Feng et al., 1998; Kim et al., 2006). Four α subunit genes (*GLRA1*, *GLRA2*, *GLRA3* and *GLRA4*) and a single β subunit gene (*GLRB*) have been identified in mammals (Grenningloh et al., 1987, 1990a,b; Kuhse et al., 1990, 1991; Akagi et al., 1991). In humans, however, *GLRA4* is a pseudogene. Several variants are also created by alternative splicing and RNA editing (Meier et al., 2005), which may modify functional properties such as agonist specificity, affinity and desensitization kinetics.

In zebrafish, four GlyR α subunits (αZ1, αZ2, αZ3 and αZ4) and two β subunits genes (βa (= βZ) and βb encoded by *glrba* and *glrbb*, respectively; **Table 3**) were initially reported (David-Watine et al., 1999; Imboden et al., 2001a,b,c; Hirata et al., 2005). Phylogenetic analysis suggested that αZ1, αZ3 and αZ4 showed high sequence similarity to the mammalian GlyR α1, α3 and α4 subunits, respectively (Imboden et al., 2001a), and were referred to as zebrafish GlyR α1, GlyR α3, and GlyR α4 (**Figures 3A,C,D**).

Table 2 | Twitch once mutants.

Mutant	Alleles	Mutation	Phenotype and gene defect	References
shocked	ta51e	Unknown	Embryonic lethal. d2, twitch only once, head not straight; d5, head straight, in response to touch just jumps up and falls down, then vibrates with tip of tail. Resting position sideways or upside down. **Mutations G81D and C305Y in the glycine transporter 1 gene (*slc6a9*) on chromosome 2.**	Granato et al. (1996), Odenthal et al. (1996), Luna et al. (2004), Cui et al. (2004, 2005), Mongeon et al. (2008)
sho	**ta229g**[§]	**G81D**		
	te301[†]	**C305Y**		
twitch once	**th26**	**G130E**	Embryonic lethal. d2, twitch only once, head not straight. d5, head straight, Just jumps and falls down, then vibrates with tip of tail. Resting position is sideways or upside down. **Mutation G130E in the muscle rapsyn gene (*rapsn*) on chromosome 18.**	Granato et al. (1996), Odenthal et al. (1996), Ono et al. (2002)
two	tq265b	Unknown		
	tm335	Unknown		

[‡]*Mutant lost,* [†]*Viable allele,* [§]*Strongest allele. Information compiled from: http://www.eb.tuebingen.mpg.de/core-facilities/zebrafish-stockcenter; http://zfin.org/*

Table 3 | Zebrafish glycinergic transmission.

Gene	Location	Protein	Mutant/Knockdown	References
GLYCINE RECEPTORS				
glra1	Chr 14	GlyR α1	*Knockdown*: no phenotype reported	David-Watine et al. (1999), Hirata et al. (2005), McDearmid et al. (2006)
glra2	Chr 9	GlyR α2	Unknown	This review
glra3	Chr 1	GlyR α3	Unknown	Imboden et al. (2001b)
glra4a	Chr 14	GlyR α4a	*Knockdown*: disrupted rhythm-generating networks and reduced the number of spinal interneurons	Imboden et al. (2001a,b), McDearmid et al. (2006)
glra4b	Chr 5	GlyR α4b	Unknown	Imboden et al. (2001b)
glrba	Chr 1	GlyR βa	Unknown	Imboden et al. (2001c), Hirata et al. (2005)
glrbb	Chr 14	GlyR βb	*Bandoneon*: touch-induced bilateral muscle contraction	Hirata et al. (2005)
GLYCINE TRANSPORTERS				
slc6a9	Chr 2	GlyT1	*Shocked*: touch-induced single twitch	Higashijima et al. (2004), Cui et al. (2005), Mongeon et al. (2008)
slc6a5	Chr 7	GlyT2	Unknown	Higashijima et al. (2004)

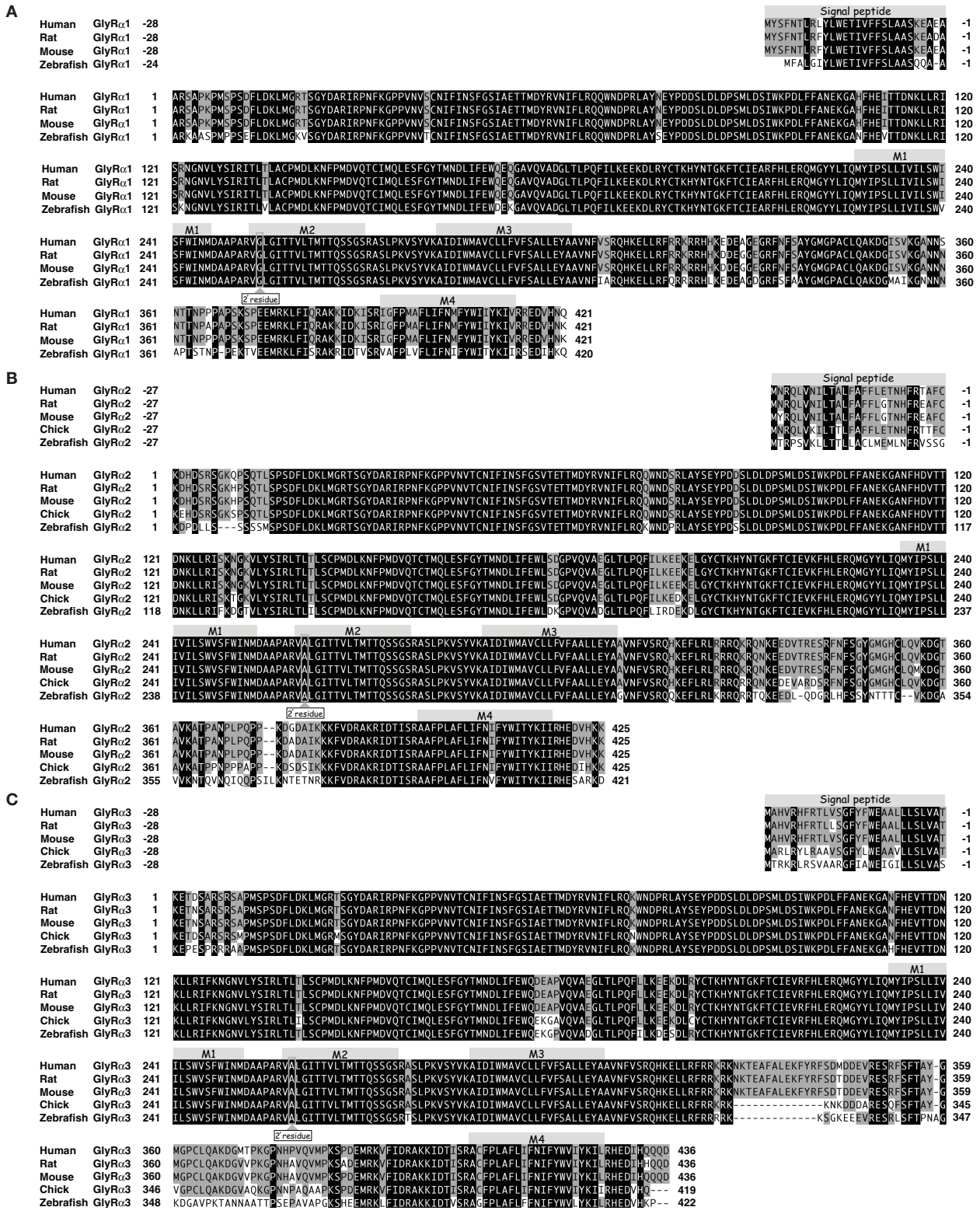

FIGURE 3 | Sequence alignments of zebrafish GlyR subunits with avian and mammalian counterparts. (A) Sequence alignment of human (GenBank accession: NP_000162), rat (NP_037265), mouse (NP_065238) GlyR α1 with zebrafish (NP_571477) GlyR α1. The four membrane-spanning domains are represented as M1-M4. The 2′ residues in M2 are highlighted by grey box. Signal peptides are denoted by negative numbering. **(B)** Protein sequence alignment of human (NP_002054), rat (NP_036700), mouse (NP_906272), chick (XP_001234291) GlyR α2 with zebrafish (GQ406228) GlyR α2. **(C)** Protein sequence alignment of human (NP_006520), rat (NP_446176), mouse (NP_536686), chick (XP_420527) GlyR α3 with zebrafish (NP_694497) GlyR α3.

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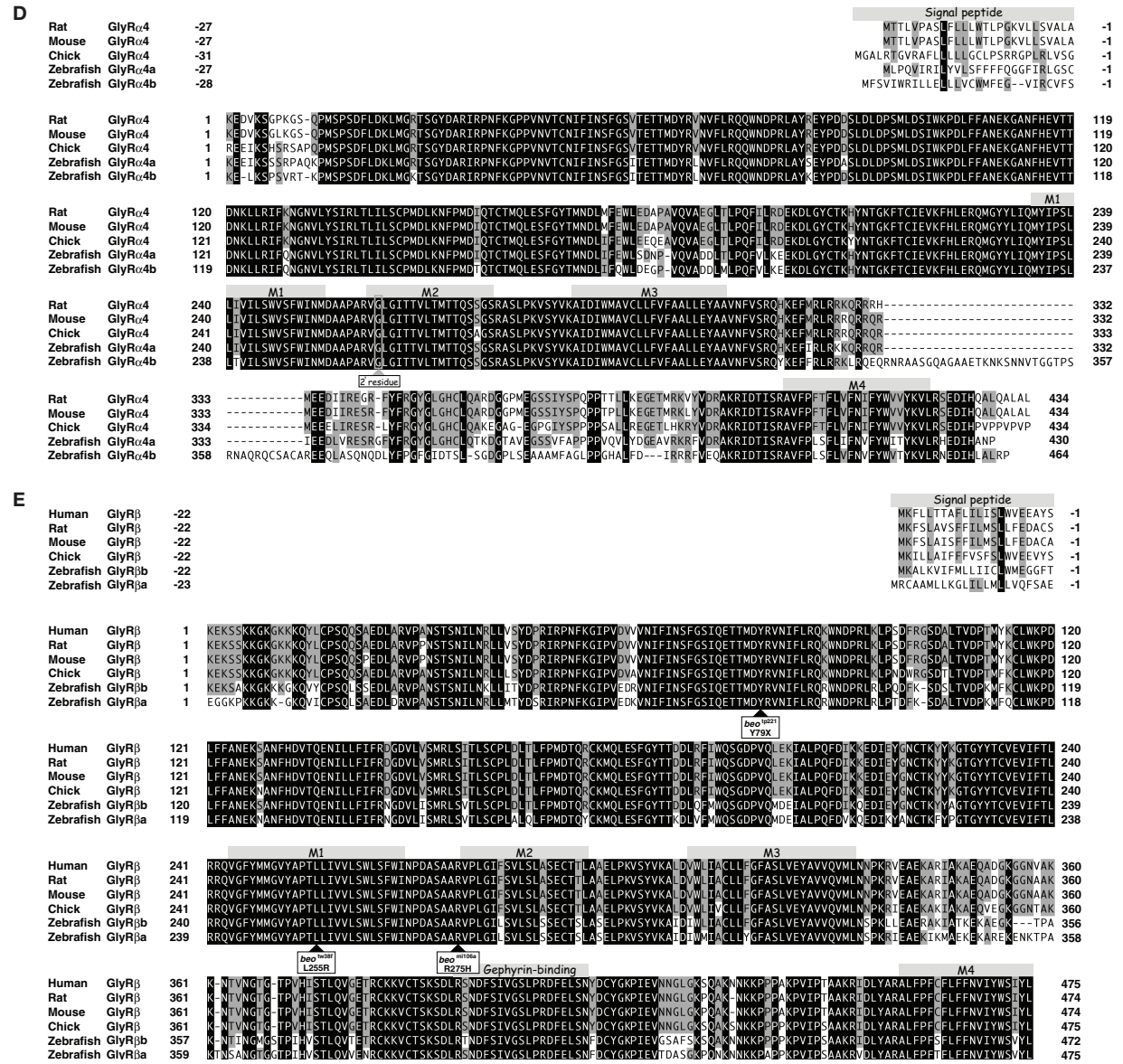

FIGURE 3 | Sequence alignments of zebrafish GlyR subunits with avian and mammalian counterparts. (D) Protein sequence alignment of rat (XP_346351), mouse (NP_034427), chick (XP_001232995) with zebrafish GlyR GlyR α4a (GQ406229) and GlyR α4b (AAH85599). **(E)** Protein sequence alignment of human (NP_000815), rat (NP_445748), mouse (NP_034428) and chick (XP_420379) GlyR β with zebrafish GlyR βb (NP_001003587) and GlyR βa (XP_683646). Position of mutations identified in the three *beo* alleles are represented by arrowheads.

A sequence reported as αZ2 was originally thought to encode a GlyR α2 subunit (Imboden et al., 2001b), but Imboden and colleagues subsequently reclassified this protein as a second α4 subunit based on a more detailed phylogenetic analysis (Imboden et al., 2001a). Thus, αZ2 was renamed α4a, and αZ4 was renamed α4b and the genes were renamed *glra4a* and *glra4b*, respectively. The existence of two distinct orthologs of a mammalian gene is not uncommon in the zebrafish genome, due to the suspected duplication of the whole genome during fish evolution (Amores et al., 1998). Using the most recent zebrafish genome assembly Zv8[2], we identified a novel zebrafish GlyR α subunit gene on chromosome 9 that is likely to encode α2 based on our own phylogenetic and sequence analysis (GenBank GQ406228; **Figure 3B**). We also amplified new zebrafish cDNAs encoding the correct N-terminus of α4a, containing a cleavable signal peptide sequence (GenBank GQ406229), which may explain why the originally isolated α4a (αZ2) required the signal peptide of α1 in functional expression experiments (Imboden et al., 2001b). Therefore, zebrafish have five α subunit (*glra1*, *glra2*, *glra3*, *glra4a* and *glra4b*) and two β subunit (*glrba* and *glrbb*) genes (**Figures 3A–E**). As well as considering overall sequence identity and similarity, we considered other diagnostic criteria in our assignment of orthologs. For example, exon

[2]http://www.ensembl.org/Danio_rerio/Info/Index)

3a and 3b are typically alternatively spliced in the mouse, rat and human GlyR α2 transcripts. But, RT-PCR and genome analysis suggests that zebrafish *glra2*, *glra4a* and *glra4b* do not show alternative splicing of exon 3. However, examining diagnostic residues in the M1-M3 domains proved more useful. The second residue in M2 (also called the 2' residue) is typically glycine in GlyR α1/α4 and alanine in GlyR α2/α3 subunits. This residue has been suggested to influence the conductance of GlyR channels (Bormann et al., 1993) and picrotoxin/picrotin blockade (Wang et al., 2007; Yang et al., 2007). From this point of view, our revised orthology appears to be more accurate, since the zebrafish GlyR α2 subunit has an alanine residue at the 2' position, whereas both zebrafish GlyR α4 subunits harbor a glycine residue at this position.

However, assuming orthology based on sequence identity alone is unwise, and it is also important to consider patterns of gene expression. Hindbrain neurons likely to be reticulospinal interneurons and spinal neurons express both *glra1* and *glra4a* by 24 hpf (Imboden et al., 2001a; Hirata et al., 2005; McDearmid et al., 2006) but *glra4a* was lost by 48 hpf. By contrast, Imboden and colleagues showed robust expression of *glra4a* at 52 hpf in the olfactory pits, mesencephalon, rhombencephalon and somites (Imboden et al., 2001a). However, *glra4b* expression appears to be restricted to the retina in 52 hpf embryos (Imboden et al., 2001a) and the expression patterns of *glra2* and *glra3* remain to be determined. Interestingly, *glrbb* is also expressed by reticulospinal and spinal neurons by 24 hpf, while *glrba* is not expressed in zebrafish until 72 hpf (Hirata et al., 2005). Thus, whilst some data appear contradictory, it is likely that early embryonic GlyRs have the capacity to be heteromeric, with GlyR βa and GlyR βb forming different heteromeric GlyRs based on developmental expression profiles, and the apparent lack of compensation shown in *bandoneon (beo)*.

DEFECTIVE GlyR CLUSTERING IN *BANDONEON*

In the Tübingen screen, seven alleles of the 'accordion' class *beo* mutation (tp221, tw38f, ta86d, ta92, tm115, tf242 and tu230) were isolated (Granato et al., 1996; **Table 1**) and named after the South American accordion-like instrument. From our behavioral screen, we generated another allele (mi106a) and showed that it results from missense and nonsense mutations in *glrbb,* encoding the GlyR βb subunit (Hirata et al., 2005). As mentioned previously, at 24 hpf wild-type embryos responded to touch with multiple coils of the body, which was achieved by alternating trunk contractions (**Figure 1B**). By contrast, *beo* mutants displayed simultaneous contraction of the bilateral axial muscles that resulted in a dorsal flexure and shortening of the body following a tactile stimulation (**Figure 1C**). Later, when wild-type embryos swam in response to touch, *beo* mutants contracted the trunk muscle simultaneously on both sides and failed to swim in response to touch. Although *beo* mutants exhibited an abnormal tactile response, spontaneous coiling was not affected. In addition to behavioral perturbation, *beo* mutants exhibited secondary morphological defects in the notochord and axial muscles that were common to zebrafish behavioral mutants showing excessive contraction of the musculature (Hirata et al., 2004; Lefebvre et al., 2004). In fact, suppression of motor behavior either by a sodium channel inhibitor (tricaine) or a muscle myosin inhibitor (N-benzyl-p-toluene sulfonamide) prevented the morphological perturbations of mutant muscle. The *beo* larvae typically died at 7 days postfertilization (dpf), presumably due to their inability to swim and feed effectively, but cumulative notochord damage may have also contributed to lethality.

Alternation of muscle contractions on the left and the right side of animals requires reciprocal inhibition between left and right sides of the hindbrain and spinal cord (reviewed in Grillner, 2003). Disruption of this inhibition can lead to simultaneous activation of bilateral motor neurons and thus simultaneous muscle contractions on both sides. Normally, tactile stimulation delivered to one side of the body leads to contraction of the contralateral side followed by contraction of the ipsilateral side. To see whether bilateral muscles are simultaneously activated in *beo*, we measured the latency of muscle depolarization following mechanosensory stimulation to the contralateral side and the ipsilateral side. We found that in wild-type muscles, contralateral tactile stimulation results in a muscle response 25 ms faster than ipsilateral stimulation, corresponding to initial touch-induced activation of contralateral muscles followed by ipsilateral muscles (**Figure 4A**). By contrast, the latencies of response to ipsilateral and contralateral stimulation are comparable in *beo*, indicating that tactile stimulation activates both contralateral and ipsilateral muscles simultaneously. Thus, reciprocal inhibition appears to be deficient in *beo*.

Examination of glycinergic synaptic transmission in *beo* by patch-clamp recordings of motor neurons showed that glycinergic, but not glutamatergic synaptic transmission was absent in *beo* (**Figure 4B**). Concordantly, immunolabeling with an anti-GlyR α antibody confirmed that GlyRs were not clustered in *beo* spinal cord as they were in wild-type zebrafish. Interestingly, application of exogenous glycine directly onto motor neurons elicited currents in *beo* motor neurons, suggesting that non-clustered extrasynaptic GlyRs, which may represent homomeric α subunit GlyRs, existed in *beo* mutants (**Figure 4C**). In fact, fetal extrasynaptic GlyRs were thought to be homopentamers of GlyR α2 in rodents (Becker et al., 1988; Malosio et al., 1991; Watanabe and Akagi, 1995). Taken together, these results demonstrated that the GlyR βb subunit was required for synaptic aggregation of GlyRs, corroborating previous findings showing that GlyR β interacted with gephyrin (Meyer et al., 1995; Sola et al., 2004; Kim et al., 2006) a multifunctional cytoplasmic protein that is crucial for the synaptic localization of GlyRs (Kirsch et al., 1993; Feng et al., 1998). The synaptic GlyRs that were eliminated in *beo* could contain either GlyR α1 or α4a, since the corresponding genes appeared to be expressed by hindbrain and spinal neurons during early development. Antisense knockdown of GlyR α4a (but not GlyR α1) reduced glycinergic synaptic transmission and disrupted activity of circuits underlying swimming (McDearmid et al., 2006), suggesting that the early synaptic GlyRs could be α4a/βb heteromers. A complication in this study was that GlyR α4a was referred to as α2 based upon the initial designation of this cDNA as αZ2 (Imboden et al., 2001a,b). The behavioral phenotype associated with α1 and α4a knockdown was not reported, and the translation blocking α4a morpholino used in these studies also caused a reduction in the number of spinal interneurones (McDearmid et al., 2006), a phenotype not examined in *beo* (Hirata et al., 2005). However, the sequences of multiple ESTs and our own αZ4a cDNA cloning suggested that the

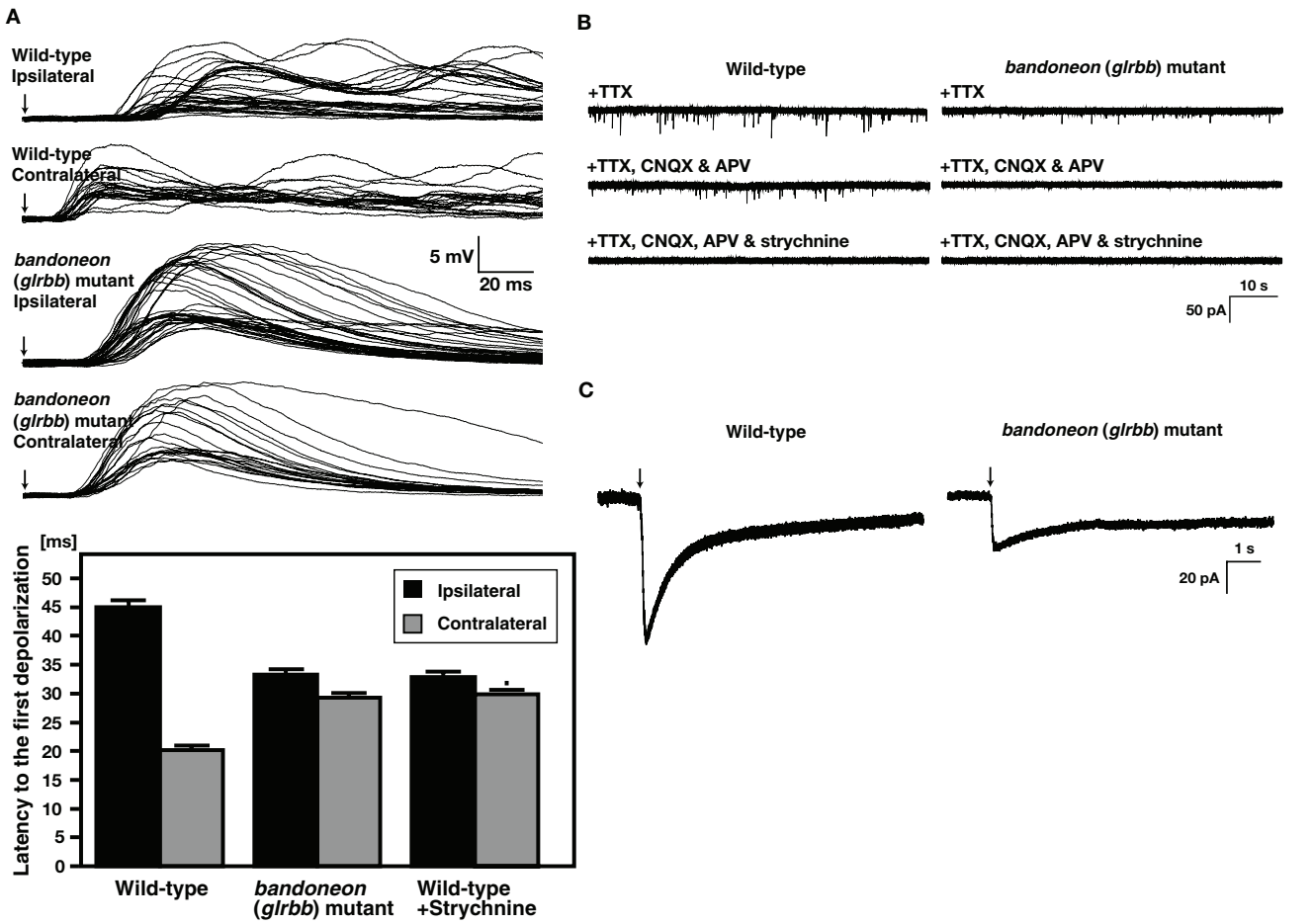

FIGURE 4 | Simultaneously contraction of bilateral axial muscles in *bandoneon* (*glrbb*) mutants due to the loss of glycinergic synaptic transmission. (A) Superimposed voltage responses of muscles evoked by mechanosensory stimulation. Arrows indicate the time of stimulation. The latency of the muscle response to contralateral stimulation was shorter than that to ipsilateral stimulation in wild-type, whereas the latency to ipsilateral and contralateral stimulation was comparable in *beo* mutants. Histograms show that the latency to half-maximal amplitude of the first depolarization was shorter in contralateral stimulation compared to ipsilateral stimulation in wild-type. The latency of the response to tactile stimulation in strychnine-treated wild-type muscles was comparable to latency in *beo* mutants. **(B)** Spontaneous synaptic currents recorded from a wild-type motor neuron in the presence of TTX were decreased in frequency following block of NMDA and AMPA receptors by application of CNQX and APV, respectively. The non-glutamatergic currents in wild-type are eliminated by further application of strychnine, showing that they are glycinergic currents. In *beo*, non-glutamatergic currents in the presence of CNQX and APV are missing, indicating that glycinergic synaptic currents are absent. **(C)** A puff of exogenous glycine induced a current in a wile-type motor neuron and a smaller current in a *beo* mutant motor neuron.

α4a morpholino used by McDearmid et al. (2006) may have been directed against a mis-spliced intronic sequence upstream of *glra4a* exon 2. This does not preclude gene knockdown by interference with *glra4a* splicing, but further studies with other GlyR-directed morpholinos may be warranted to uncover the exact biological roles of the zebrafish GlyR genes.

In humans defects in glycinergic synaptic transmission lead to hyperekplexia. Hyperekplexia is a rare neurological syndrome that is characterized by an exaggerated startle response accompanied by transient muscle rigidity in response to unexpected acoustic or tactile stimuli (Gastaut, 1967; Bakker et al., 2006). More than 20 distinct missense mutations and several nonsense and frameshift mutations have been identified in the GlyR α1 subunit gene (*GLRA1*) to date (Shiang et al., 1993; Bakker, 2006; Harvey et al., 2008). Most missense mutations in the second membrane-spanning domain and its neighboring loops are dominant mutations, whereas most point mutations in the N-terminal extracellular domain and M3-M4 intracellular loop are recessive. Missense and nonsense mutations in *GLRA1* that lead to exaggerated startle reflexes are also found in spontaneous and induced mouse mutants and bovine congenital myoclonus (Gundlach et al., 1988; Buckwalter et al., 1994; Ryan et al., 1994; Pierce et al., 2001; Holland et al., 2006; Traka et al., 2006). To date, no mutations in the other functional GlyR α subunit genes (*GLRA2* and *GLRA3*) have been reported, but in one family compound heterozygous mutations in *GLRB* were associated with hyperekplexia (Rees et al., 2002). Similarly in mice, GlyR β hypomorphs (due to a LINE1 transposable element insertion causing mis-splicing of *Glrb* transcripts) also exhibit abnormal startle responses (Becker et al., 1992; Kingsmore et al., 1994; Mülhardt et al., 1994; Hartenstein et al., 1996).

The mutations underlying three alleles of *beo* have been identified to date (Hirata et al., 2005; **Table 1**). In the tp221 allele, a nonsense mutation (Y79X) is predicted to cause truncation of GlyR βb. By contrast, in tw38f and mi106a, missense mutations L255R and R275H, respectively, were found in the first membrane-spanning domain (M1) and in the intracellular loop between the first (M1) and second (M2) membrane-spanning domains. The R275H mutation in zebrafish GlyR βb affects a highly conserved arginine residue prior to M2 and in fact in human GlyR α1, the corresponding mutation R252H is known to accelerate degradation of GlyR α1 (Rea et al., 2002), suggesting that mi106a mutation is a hypomorph of GlyR βb. Characterization of the remaining *beo* alleles is underway, and may reveal key residues of involved in the function of GlyR βb.

GLYCINE TRANSPORTER 1 (GlyT1) DEFECTS IN *SHOCKED*

Glycine transporters are 12 membrane-spanning domain proteins that belong to Na^+/Cl^--dependent transporter superfamily. In vertebrates, two glycine transporters, GlyT1 and GlyT2, mediate the uptake of glycine from the extracellular space to the cytosol driven by an electrogenic gradient (Eulenburg et al., 2005). GlyT1 is enriched in astrocytes and some excitatory neurons, whereas GlyT2 is enriched in inhibitory glycinergic neurons (Adams et al., 1995; Zafra et al., 1995; Jursky and Nelson, 1996; Cubelos et al., 2005). At glycinergic synapses, astroglial GlyT1 is thought to clear glycine from the synaptic cleft, so terminating neurotransmission. Since glycine serves both as ligand for GlyR activation and as a coagonist and possibly primary ligand for NMDA receptors (Johnson and Ascher, 1987; Kuryatov et al., 1994; Chatterton et al., 2002), GlyT1 regulates both inhibitory and excitatory synaptic transmission. By contrast, GlyT2 is localized to presynaptic terminals of glycinergic neurons and is essential for glycine reuptake, replenishing the pool of releasable transmitter (Zafra et al., 1995; Gomeza et al., 2003b; Mahendrasingam et al., 2003).

Zebrafish *sho* mutants fail to initiate swimming following tactile stimulation at 3 dpf (Granato et al., 1996; Cui et al., 2004; Luna et al., 2004). This phenotype is caused by missense mutations in *slc6a9*, which encodes GlyT1 (Cui et al., 2005; Mongeon et al., 2008). The ta229g allele, which displays the strongest phenotype, results from a G81D missense mutation in the second membrane-spanning domain. Expression of the recombinant zGlyT1 G81D mutant in *Xenopus* oocytes revealed that GlyT1 function is abolished by this mutation (Cui et al., 2005). The milder te301 allele harbors a C305Y missense mutation that is located next to a deduced glycine-binding residue in the pore-forming sixth membrane-spanning domain (Yamashita et al., 2005; Rees et al., 2006; Harvey et al., 2008; Mongeon et al., 2008). Both *sho* mutant alleles display severely compromised tactile-induced locomotion and the frequency of spontaneous coiling is reduced. Tactile stimuli do not evoke escape contractions in *sho* mutants at 24 hpf, unlike wild-type siblings. At later stages, when tactile stimuli induce swimming in wild-type fish, *sho* mutants respond with a few uncoordinated trunk contractions (or not at all) and do not display typical swimming behavior. While sho^{ta229g} mutants normally die within 2 weeks of development, importantly they can be maintained to adulthood by careful feeding. These adult sho^{ta229g} homozygous fish are less active than wild-type zebrafish but fertile, indicating some degree of functional recovery. Interestingly, the weaker sho^{te301} mutants recover by around 4–5 dpf (Mongeon et al., 2008), suggesting that there is a degree of compensation for the loss of functional GlyT1 in zebrafish.

The effects of the loss of GlyT1 function on the nervous system and/or muscles were examined by electrophysiology. Voltage recordings from muscle of wild-type embryos show sustained episodes of rhythmic depolarizations corresponding to swimming following tactile stimulation, whereas *sho* muscle responds with one or two short, arrhythmic depolarizations corresponding to the uncoordinated 'twitch-once' muscle contractions exhibited by *sho* mutants (**Figures 5A,B**; Cui et al., 2004). Similarly, wild-type spinal motor neurons respond to touch with a long burst of action potentials, while *sho* motor neurons responded with only a short burst. Thus, the nervous system output of the CNS is aberrant in *sho*, signifying that the loss of GlyT1 disturbs the CNS function at glycinergic synapses (Cui et al., 2005). Interestingly, the *sho* mutants also exhibit aberrant electrical coupling between axial muscle fibers at 3 dpf (Luna et al., 2004), but this may be a secondary consequence of the CNS defects, since GlyT1 is not expressed in muscle. If the defect in CNS signaling in *sho* is attributable to increased extracellular glycine levels in the synaptic cleft due to the loss of GlyT1 function, one would expect normal responses to be restored in mutants when excess glycine is washed out. To examine this possibility, the hindbrain, which contains many of the neurons mediating responses to tactile stimulation, was exposed to various solutions by perfusion following removal of dorsal roof of the fourth ventricle and responses to tactile stimulation assayed by muscle recordings (Cui et al., 2005). When mutants were perfused with glycine-free solution, they respond with normal swimming behavior (**Figure 5C**) and this functional recovery is inhibited by addition of exogenous glycine (**Figure 5D**). Furthermore, *sho* mutants are more sensitive to the deleterious effects of exogenous glycine on touch-induced swimming compared to wild-type fish (Mongeon et al., 2008). Thus, the putative increase in extracellular glycine due to the loss of GlyT1 is likely to mediate aberrant signaling within the mutant CNS.

One presumption of these perfusion experiments was that extracellular glycine levels were high in the synaptic cleft of *sho* mutants. This excess glycine is predicted to lead to increased inhibition of neurons receiving glycinergic inputs. If this presumption is correct, then blocking glycinergic inhibition should ameliorate the effects of high glycine in *sho* mutants. In fact, application of low concentrations of strychnine to *sho* mutants led to partial recovery of spontaneous coiling in 21 hpf embryos and normal swimming responses in older (40–46 hpf) embryos (Cui et al., 2005). However, the partially recovered response might also be attributed to increased excitatory transmission via NMDA receptors in *sho* mutants. Taken together, it appears that the defective signaling in *sho* is consistent with abnormally high glycine in the synaptic cleft.

GlyT1 knockout mice showed many features that resembled those exhibited by zebrafish *sho* mutants, most notably motor deficits including those involving respiratory neural circuits (Gomeza et al., 2003a; Tsai et al., 2004). Recordings of spontaneous neuronal activity from hypoglossal motor neurons revealed that inspiratory cycling of the respiratory network of the brain stem was nearly eliminated in GlyT1 mutant mice. Much like

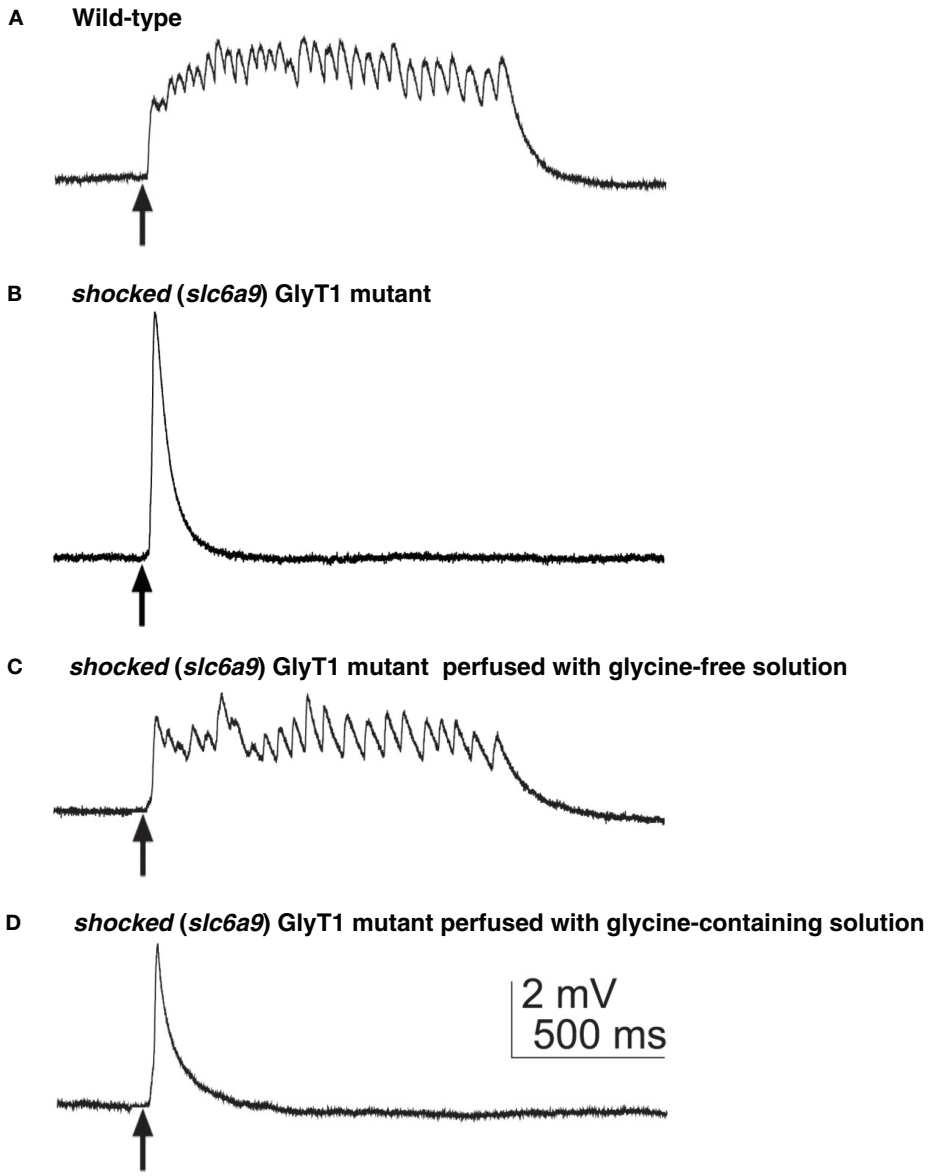

FIGURE 5 | The aberrant motor response of *shocked* (*slc6a5*) mutants defective in GlyT1 is due to high external glycine. (A) Muscle voltage recording from a wild-type embryo showed normal fictive swimming in response to mechanosensory stimulation. Arrows indicate the time of stimulation. **(B)** Muscle recording from a *sho* mutant embryo displaying a large, nonrhythmic depolarization. **(C)** Muscle recording from a *sho* mutant after the hindbrain was exposed and perfused with glycine-free solution demonstrated rhythmic depolarizations similar to fictive swimming. **(D)** Muscle recording from the same *sho* embryo as in **(C)** after switching the perfusion from glycine-free solution to saline containing 0.2 mM glycine again exhibited the aberrant response characteristic of *sho* mutants.

the palliative effect of low concentration strychnine on the swimming circuit of zebrafish *sho* mutants, normal rhythmic activity was restored in hypoglossal motor neurons upon application of low strychnine to brainstem slices from GlyT1 knockout mice. Furthermore, voltage-clamp analyses of hypoglossal neurons were consistent with an increase in extracellular glycine. Thus, the neural defects seen in GlyT1-deficient mice are also likely to be due to increased levels of synaptic glycine, leading to suppression of neural networks. So far, no human GlyT1 defects have been associated with any disease. However, GlyT1 dysfunction has been suggested to play a possible role in glycine encephalopathy (Gomeza et al., 2003a; Harvey et al., 2008), as well as the psychiatric disorder schizophrenia (Freedman, 2003; Tsai et al., 2004), where NMDA receptor hypofunction is suspected. GlyT1 inhibitors elicit activation of NMDA receptors by increasing synaptic glycine levels, thus accelerating the co-agonist action of glycine may be useful pharmacological tools to mitigate some features of schizophrenia (Le Pen et al., 2003). However, whether sequence variations in the GlyT1 gene (*SLC6A9*) are linked to glycine encephalopathy or schizophrenia and whether

GlyT1 inhibitors are useful pharmacotherapies remains to be determined. Interestingly, SNPs in the human GlyT1 gene were recently proposed to be associated with methamphetamine-use disorder (Morita et al., 2008; Bousman et al., 2009). Animal models are clearly required for investigating the biological roles of GlyT1 and for the identification of therapeutic agents for treatment of human disorders related to GlyT1. In this respect, mouse GlyT1 knockouts may be less than ideal, since they die on the day of birth (Gomeza et al., 2003a; Tsai et al., 2004), whereas zebrafish *sho* mutants are accessible and viable.

CONCLUDING REMARKS

Zebrafish *bandoneon* and *shocked* mutants are useful models for understanding of glycinergic synaptic transmission and for clarifying the biological consequences of gene disruption that impinge upon glycinergic signaling *in vivo*. Furthermore, future analysis of other zebrafish mutations may reveal new insights. For example, two 'accordion' class mutants (*que* and *exp*) remain to be analyzed in depth, as well as the crazy fish mutant *techno trousers* (*tnt*). Although these mutants have been suggested to harbor defects in glycinergic transmission because they exhibit exaggerated startle responses in response to touch (Granato et al., 1996), history has taught us that there are several potential phenocopies of glycinergic defects. Equally, it is unclear why defects in *glra1* (encoding GlyR α1) and *slc6a5* (encoding GlyT2) were not uncovered in mutagenesis screens to date, since these are highly mutable genes in other species (Harvey et al., 2008). Importantly, the genetic, developmental, and physiological accessibility of zebrafish make them useful animal models of human syndromes such as hyperekplexia. Small molecule screens using zebrafish mutants have successfully identified several drugs that ameliorate mutant phenotypes (Peterson et al., 2004; Stern et al., 2005). Such screens using zebrafish *sho* mutants have identified several compounds that mitigate the impairment of touch response (Hirata, unpublished). Future comparative and integrative studies using a variety of organisms including zebrafish with defects in glycinergic transmission are a promising strategy for a comprehensive understanding and development of pharmaceutical agents for human diseases defective in glycinergic synaptic transmission.

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Glycine receptors caught between genome and proteome – functional implications of RNA editing and splicing

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Information processing in the brain requires a delicate balance between excitation and inhibition. Glycine receptors (GlyR) are involved in inhibitory mechanisms mainly at a synaptic level, but potential novel roles for these receptors recently emerged due to the discovery of posttranscriptional processing. *GLR* transcripts are edited through enzymatic modification of a single nucleotide leading to amino acid substitution within the neurotransmitter binding domain. RNA editing produces gain-of-function receptors well suited for generation and maintenance of tonic inhibition of neuronal excitability. As neuronal activity deprivation in early stages of development or in epileptic tissue is detrimental to neurons and because RNA editing of GlyR is up-regulated in temporal lobe epilepsy patients with a severe course of disease a pathophysiological role of these receptors emerges. This review contains a state-of-the-art discussion of (patho)physiological implications of GlyR RNA editing.

Keywords: RNA editing, RNA splicing, hippocampus, epilepsy, GABA

GLYCINE RECEPTOR GENES AND SUBUNITS

Glycine receptors (GlyRs) are pentameric chloride (Cl^-) channels. They contribute to inhibition of neuron firing in brain. Expression of GlyR genes (*GLRA1-4*) produces four different α subunits that are capable of forming functional, homomeric channels (Betz and Laube, 2006; Lynch, 2009), while a single gene (*GLRB*) delivers neurons with GlyR β subunit mRNA. Besides modulation of ligand binding (Grudzinska et al., 2005) the GlyR β subunit mediates receptor stabilization at postsynaptic sites (Kirsch et al., 1991; Meyer et al., 1995; Meier et al., 2000, 2001; Meier and Grantyn, 2004). GlyR genes are located on several chromosomes in humans, with *GLRA1* on chromosome 5 (5q32), *GLRA2* on chromosome X (Xp22.1-p21.3) and *GLRA3* as well as *GLRB* on chromosome 4 (4q33-q34 and 4q31.3, respectively). *GLRA4* is a pseudogene (Simon et al., 2004) located on the X-chromosome, position Xq22.2. Expression of all other genes occurs in a wide range of brain regions (Lynch, 2009), and generally it is thought that GlyR α2 expression predominates in the juvenile brain and declines with development (Lynch, 2009). However, at least in retina (Haverkamp et al., 2004) and in the hippocampus (Eichler et al., 2008) GlyR α2 expression persists throughout development.

FOR WHAT PURPOSE DO WE NEED RNA EDITING?

Through RNA editing the genetically encoded information can be modified. Enzymatic deamination of adenosine and cytidine is mediated by adenosine deaminases acting on RNA (ADAR) and apolipoprotein B mRNA editing complex (APOBEC) or activation induced deaminase (AID), respectively (Anant and Davidson, 2001; Seeburg and Hartner, 2003; Honjo et al., 2005). The resulting inosine (equivalent to guanosine) or uracil may then lead to amino acid substitutions in corresponding proteins, provided that the resulting amino acid triplet codons engage different transfer RNAs. These enzyme machineries thus allow for diversification of the proteome or even correction of the genome code. Although the role of diversification by cytidine-to-uracil (C-to-U) RNA editing in generation of immunoglobulin variability is under debate (Honjo et al., 2005) the critical impact of RNA editing on correction of genome codes is well established. For example, the permeability of glutamate receptors for calcium ions increases when adenosine-to-inosine (A-to-I) RNA editing is prohibited, resulting in severe epilepsy (Brusa et al., 1995). Therefore in this case, the A-to-I RNA editing machinery is required for maintenance of physiological brain state through correction of unwanted genome codes (Seeburg and Hartner, 2003). The number of identified RNA-edited transcripts steadily increases, and for example serotonin receptors, potassium channels, GABA and glycine receptors were recently included in this register (Gurevich et al., 2002; Bhalla et al., 2004; Buckingham et al., 2005; Meier et al., 2005).

GlyR C-TO-U RNA EDITING IS NOT SPECIES-SPECIFIC

A cDNA clone corresponding to GlyR $\alpha3^{185L}$ was originally isolated from *Sprague Dawley* rat brain, and molecular analysis revealed the involvement of C-to-U RNA editing in proline-to-leucine substitution at position 185 of the mature GlyR α3 protein (Meier et al., 2005). Molecular analysis of hippocampi from pharmacoresistant temporal lobe epilepsy (TLE) patients further revealed expression of mRNAs coding for the high affinity GlyR $\alpha3^{185L}$ variant (Eichler et al., 2008). In addition, *GLRA2* transcripts of GlyR $\alpha2^{192L}$ were found in these patients. Sequencing of corresponding exons demonstrated lack of genomic $\alpha2^{192L}$ codons, again supporting the involvement of C-to-U RNA editing in proline-to-leucine substitution in

TLE patients (Eichler et al., 2008). However, the amount of RNA-edited *GLR* transcripts was not constant between TLE patients, but increased according to the frequency of secondarily generalized tonic-clonic seizures or the degree of hippocampal sclerosis. That hippocampal sclerosis was associated with increased amounts of RNA-edited *GLRA2/3* transcripts indicates a pathophysiological role of high affinity GlyRs in human hippocampus, which already was suggested by our data on experimentally induced brain lesion (Meier et al., 2005). Consequently, in TLE patients without hippocampal sclerosis the amount of RNA-edited *GLR* transcripts was very low, particularly in case of GlyR α3 (below 1% of *GLRA3* transcripts). This renders quantification of RNA-edited transcripts rather difficult (Nakae et al., 2008). However, our methods for quantification were recently shown to be suitable for detection of RNA-edited messenger fractions below 1% (Eichler et al., 2008).

LEUCINE AT POSITIONS 192 (α2) AND 185 (α1 AND α3) IS RESPONSIBLE FOR HIGH APPARENT GlyR AGONIST AFFINITY

GlyRs are members of the ligand-gated ion channel (LGIC) superfamily, which among others comprises A- and C-type GABA receptors ($GABA_{A/C}R$) and the nicotinic acetylcholine receptor (nAChR). All members share common structural features, particularly in the ligand binding domain (Celie et al., 2005). In fact, all GlyR subunits and $GABA_AR$ β1-3 contain proline at the position corresponding to amino acid 185 of the mature signal-peptide cleaved α3 protein.

However, $GABA_ARs$ that require a high agonist affinity because of their involvement in tonic inhibition of neuronal excitability don't have proline at this position. Instead, $GABA_ARs$ containing α4, α6 or δ subunits are equipped with aliphatic hydrophobic neutral amino acids (such as alanine, leucine or valine) (Meier et al., 2005), and these receptors are well known to respond to low neuronal ambient GABA with tonic chloride currents (Mody, 2001; Wisden et al., 2002; Caraiscos et al., 2004; Mody and Pearce, 2004). Therefore, amino acid substitution at this position of the ligand binding domain should increase the apparent GlyR agonist affinity. Indeed, proline-to-leucine substitution by C-to-U editing of *GLRA3* transcripts was shown to increase the apparent agonist affinity (Meier et al., 2005). Similarly, editing of *GLRA2* transcripts at the corresponding position (192) produces receptors with increased apparent agonist affinity (Eichler et al., 2008).

To further emphasize the critical role of position 185/192 in determining apparent receptor agonist affinity dose-response curves were obtained from HEK293 cells expressing GlyR $\alpha1^{185P}$ or $\alpha1^{185L}$ (**Figure 1**). The short GlyR α1 splice variant lacking insert SPMLNLFQ (Malosio et al., 1991) was used throughout. Again, compared with GlyR $\alpha1^{185P}$, the apparent affinity of $\alpha1^{185L}$ channels was increased several fold [$EC_{50(Glycine)}$ (μM) $\alpha1^{185L}$: 17.7 ± 1.5 vs. $\alpha1^{185P}$: 85.3 ± 4.4, $EC_{50(Taurine)}$ (μM) $\alpha1^{185L}$: 67.0 ± 6. 8 vs. $\alpha1^{185P}$: 378 ± 33, mean ± SD].

It should be noted here that the apparent affinity of GlyRs does not seem to be influenced by RNA splicing because GlyR α1 with insertion ($\alpha1^{ins}$) (Malosio et al., 1991) exhibited similar current profiles (not shown). In addition, compared with α3K, the long splice variant of α3-GlyRs (α3L) (Nikolic et al., 1998) also had increased apparent glycine affinity when RNA-edited (**Figure 2**).

Altogether, proline-to-leucine substitution at positions 192 (α2) and 185 (α1 and α3) confers high apparent agonist affinities to GlyRs. On the contrary, GlyR apparent agonist affinity is not regulated by RNA splicing, except for GlyR α2 where changes in the apparent affinity were observed upon RNA splicing in the region coding for the N-terminal domain (Miller et al., 2004; Eichler et al., 2008).

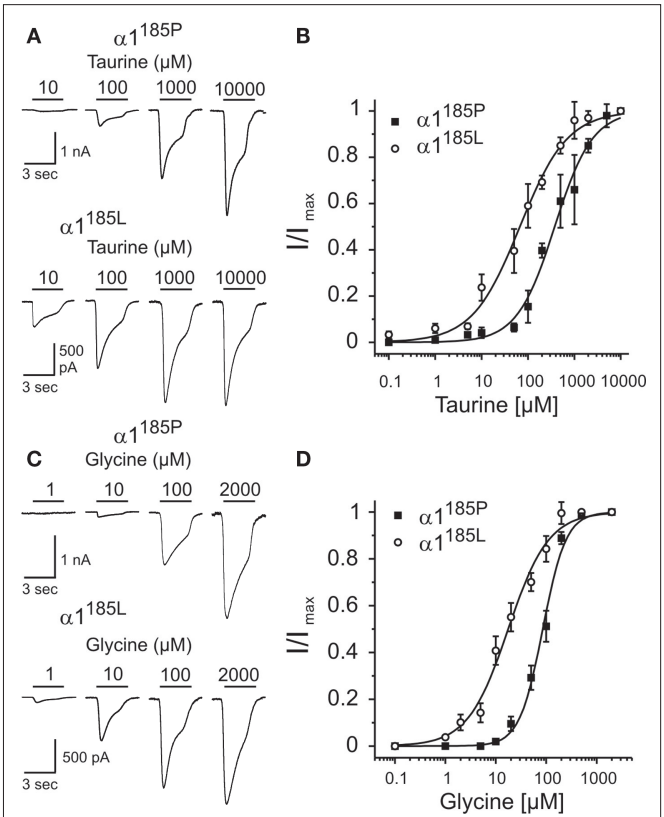

FIGURE 1 | Apparent agonist affinities of GlyR $\alpha1^{185P}$ and $\alpha1^{185L}$. Dose-response curves were obtained from transfected HEK293 cells. **(A,C)** Example traces of Cl^- currents obtained at indicated agonist concentrations. At –70 mV holding potential, maximal current responses ($\alpha1^{185P}$, taurine: 3.3 ± 0.3 nA, glycine: 2.5 ± 0.3 nA; $\alpha1^{185L}$, taurine: 1.7 ± 0.3 nA, glycine: 1.6 ± 0.3 nA) were obtained with 2 mM glycine and 10 mM taurine. Taurine **(B)** and glycine **(D)** dose-response curves of HEK293 cells expressing GlyR $\alpha1^{185P}$ or $\alpha1^{185L}$ are shown. Taurine and glycine current amplitudes were normalized to maximal current amplitudes. Each data point **(B,D)** represents the average current amplitude of 13–21 sampled cells. Hill coefficients: $\alpha1^{185P}$, taurine: 1.06 ± 0.09, glycine: 1.88 ± 0.17; $\alpha1^{185L}$, taurine: 0.84 ± 0.07, glycine: 1.08 ± 0.09. Values represent mean ± SD.

HOW RNA EDITING CAN CHANGE RECEPTOR-CHANNEL KINETICS, A PERSPECTIVE

RNA editing involves a single amino acid substitution in the N-terminal GlyR domain. The main consequence of RNA editing is an increase in the apparent affinities (EC_{50}) of GlyRs for glycine and taurine. RNA editing does not modify GlyR desensitization kinetics (Meier et al., 2005), which is consistent with previous studies showing that receptor desensitization kinetics are mainly affected by change in amino acid sequences in the TM3-4 loop (Legendre, 2001; Breitinger and Becker, 2002; Breitinger et al., 2002). A single amino acid substitution in the N-terminal GlyR domain at positions 192 (α2) or 185 (α1 and α3) could result in a change in the transduction process between agonist binding and channel gating, but it cannot be excluded at this stage that proline-to-leucine

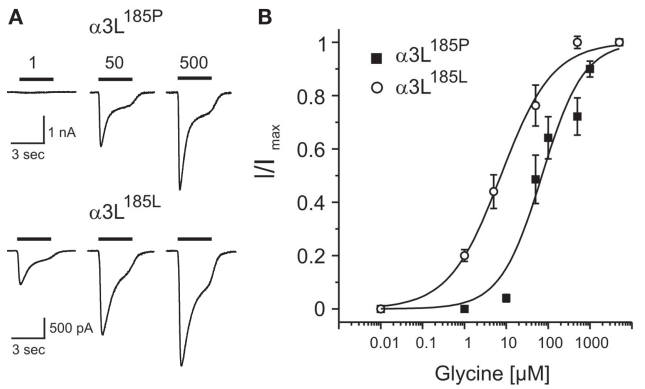

FIGURE 2 | Apparent agonist affinities of GlyR α3L^{185P} and α3L^{185L}. Dose-response curves were obtained from transfected HEK293 cells. **(A)** Example traces of Cl^- currents obtained at indicated agonist concentrations. **(B)** Glycine dose-response curves of HEK293 cells expressing GlyR α3L^{185P} or α3L^{185L} are shown. Glycine current amplitudes were normalized to maximal current amplitudes. Each data point represents the average current amplitude of 13–21 sampled cells. At –70 mV holding potential, the apparent affinities for glycine ($EC_{50(Glycine)}$ [μM]) were 70.9 ± 16.1 (α3L^{185P}) and 7.4 ± 0.9 (α3L^{185L}). Hill coefficients were 0.87 ± 0.17 (α3L^{185P}) and 0.70 ± 0.06 (α3L^{185L}). Thus, the long splice variant of RNA-edited GlyR α3L^{185L} is a high affinity receptor as well. Values represent mean ± SD.

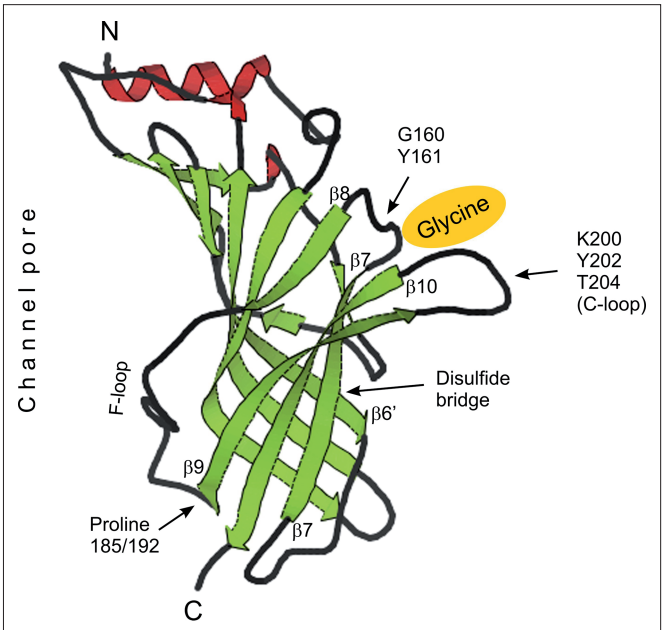

FIGURE 3 | Tertiary structure of AChBP and projection of GlyR α subunit associated structural determinants. Positions of the glycine (yellow) binding pocket, formed by amino acids GY and KYT (C-loop), of the disulfide bridge between β-sheets 6′ and 7 as well as of proline residues at positions 185 (α1 and α3) and 192 (α2) are shown. N- and C-termini and the location of the chloride channel pore are indicated. β-sheets, α-helices and coils are color-coded (green, red and black, respectively). GlyR sequence was projected on the structure view of AChBP downloaded from www.pdb.org. The position of β-sheets served as reference points.

substitution modifies the conformation of the N-terminal domain and hence the access of the ligand to the binding pocket. Changes in EC_{50} values do therefore not necessarily imply changes in the affinity of the receptor for its agonist. Effectively, in the simplest case, the EC_{50} depends both on the agonist dissociation constant (Kd) and on the receptor-channel efficacy (*E*), and EC_{50} = Kd/(1 + E) [for a more detailed explanation see (Colquhoun, 1998; Legendre, 2001)]. Several models have been proposed to describe GlyR activation kinetics, e.g. the "reluctant gating model" (Legendre, 1998) and the "flip model" (Burzomato et al., 2004). The flip model was recently extended to nAChR (Lape et al., 2008), although it has partially been challenged recently (Mukhtasimova et al., 2009). In any case, these studies demonstrate that receptor channels can have intermediate conformational states between binding steps and opening states, which must be taken into account when interpreting changes in EC_{50}. Accordingly, the best way to determine how GlyR RNA editing influences their apparent glycine affinity will be to compare activation kinetics of regular and RNA-edited GlyRs at the single channel level, as was already done in case of the hyperekplexia amino acid substitution α1^{K276E} (Lewis et al., 1998), the hyperekplexia amino acid substitution α1^{Q266H} (Moorhouse et al., 1999) and the spasmodic amino acid substitution α1^{A52S} (Plested et al., 2007). Nonetheless, the net outcome of proline-to-leucine substitution is a gain-of-function GlyR.

CONFORMATIONAL IMPLICATIONS OF GlyR RNA EDITING

The crystal structure of the acetylcholine binding protein (AChBP) was recently determined at high resolution (Celie et al., 2005). Because members of the LGIC superfamily have a common ancestor (Celie et al., 2005) we have used the AChBP structure as a model for the ligand binding domain of GlyR. At least from the point of view of intramolecular location of β-sheets a projection of GlyR sequences on AChBP protein structure can be made (Grenningloh et al., 1987). According to these thoughts proline 185/192 of GlyR α subunits is located directly at the beginning of β-sheet 9 (**Figure 3**, β9), a position that is close to the plasma membrane where these receptors enter the cytosol through transmembrane domain (TM) 1 (**Figure 3**, C-terminus). The glycine binding pocket (**Figure 3**) is formed by two distant amino acid groups (Legendre, 2001), namely GY (160 and 161, respectively) and KYT (200, 202 and 204, respectively). These two domains are separated by β-sheets 7 and 10, and prolines 185/192 are part of the β8/β9-flanked connecting F-loop (**Figure 3**).

Further pursuing these considerations we have arguments that support a conformational role of proline-to-leucine substitution. First, leucine residues have bifurcated non-polar side chains that can mediate interaction with membrane lipids, while proline is frequently found in regions where the protein backbone has to make a sharp turn. In fact, increasing local hydrophobicity at position 250 (located between TM1 and TM2 close to the intracellular side of the plasma membrane) was shown to impact on receptor apparent affinity (Breitinger et al., 2001; Breitinger and Becker, 2002). In this case however, increased hydrophobicity decreased apparent GlyR affinity and favored rapid desensitization. Second, the disulfide bridge that connects β-sheets 6′ and 7 (**Figure 3**) could serve as a hinge (Laube et al., 1993), potentially transmitting conformational rearrangement by proline-to-leucine substitution to the glycine binding pocket. Third, the glycine binding sequence KYT is located within the C-loop (**Figure 3**), which was shown

to undergo conformational change upon AChR agonist binding (Unwin et al., 2002), and fourth, F-loop dynamics are involved in benzodiazepine mediated $GABA_AR$ activation (Padgett and Lummis, 2008). More recently, the F-loop was involved in sensing conformational rearrangements upon ligand docking (Pless and Lynch, 2009). Therefore, we put forward the idea that structural rearrangement by proline-to-leucine substitution will impact on the accessibility of GlyR agonists to the C-loop binding pocket.

GABA ACTIVATES RNA-EDITED GlyRs

Because proline-to-leucine substitution can cause structural rearrangement and therefore impact on accessibility of GlyR agonists to the C-loop binding pocket we have considered possible GABA responsiveness of RNA-edited GlyRs. Non-edited GlyRs can be activated by GABA at concentrations in the millimolar range (De Saint et al., 2001), which apparently precluded any physiological role for GABA on GlyRs. However, an elegant study recently demonstrated the opposite. Lu and colleagues (Lu et al., 2008) show that co-release of GABA and glycine accelerates glycinergic transmission by acting directly on GlyRs. This effect is explained by competition of GABA and glycine with the same binding site. The deactivation phase time constant of a synaptic event reflects the duration of bursts of channel openings (Legendre, 2001). Since burst duration partly depends on the dissociation rate constant of the agonist, a weak partial agonist will evoke bursts of short duration, which will in turn result in a decrease in the deactivation time constant of the synaptic response. It is tempting to speculate that postsynaptic GlyRs facing GABAergic nerve endings as observed in some CNS area such as hippocampus, and brain stem and spinal cord during development, could be activated by synaptic GABA release (Geiman et al., 2002; Levi et al., 2004; Meier and Grantyn, 2004; Muller et al., 2006). However, the concentration of GABA, released into the synaptic clef, must be high enough (>1 mM) to activate GlyRs. This limitation can be overcome as GlyRs acquire a higher agonist affinity if RNA-edited (Meier et al., 2005; Eichler et al., 2008). As GABA is a week competitive partial agonist on GlyRs it is likely that an increase in the apparent affinity for glycine will also result in an increase in the apparent affinity for GABA. This is the case (**Figure 4**).

As shown in **Figure 4** the threshold for GlyR activation by GABA is >0.3 mM in case of GlyR $\alpha3K^{185P}$, whereas it is close to 0.1 mM for RNA-edited GlyR $\alpha3K^{185L}$. Interestingly, application of 10 mM GABA (**Figure 4B**) on outside-out patches containing GlyR $\alpha3K^{185L}$ evoked responses with amplitudes representing ~80% of current amplitudes obtained with saturating glycine concentration (0.1 mM) (Meier et al., 2005). We did not test the effect of GABA concentrations above 10 mM, but according to the amplitude of GABA-evoked responses relative to current amplitudes obtained with 0.1 mM glycine, the apparent affinity of GlyR $\alpha3K^{185L}$ for GABA (EC_{50}) is in the range of 2–3 mM (**Figure 4B).**

At GABAergic synapses the peak concentration of GABA molecules released per vesicle is likely to range from 0.5 to 1 mM (Jones and Westbrook, 1995). Accordingly, GlyR $\alpha3^{185L}$ could be partially activated if located vis-à-vis GABAergic nerve endings. In fact, we have shown recently that GlyR α3K preferentially associates with GABAergic hippocampal nerve endings (Eichler et al., 2009). However, it still remains to be shown that RNA-edited α3K behaves similarly.

A ROLE FOR α3-GlyRs AT GLUTAMATERGIC SYNAPSES?

In addition to these findings, recent analysis of α3-GlyR distribution revealed an unexpected location of the long splice variant of these receptors (α3L) adjacent to glutamatergic nerve endings

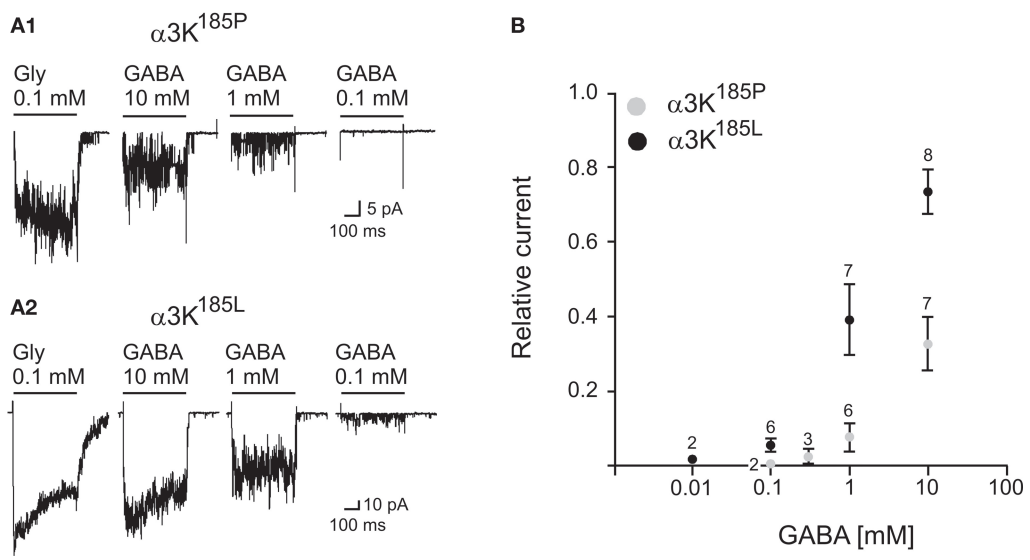

FIGURE 4 | Activation of GlyR $\alpha3K^{185P}$ and GlyR $\alpha3K^{185L}$ by GABA applications. (A1) Example traces of outside-out currents evoked by activation of GlyR $\alpha3K^{185P}$ in response to the application of glycine (0.1 mM) and various concentrations of GABA (Vh = –50 mV; Filter 1 kHz). **(A2)** Example traces of outside-out currents evoked by activation of GlyR $\alpha3K^{185L}$ in response to the application of glycine (0.1 mM) and various concentrations of GABA (Vh = –50 mV; Filter 1 kHz). **(B)** Concentration-response curves for GABA-evoked currents on outside-out patches containing GlyR $\alpha3K^{185P}$ (●) or GlyR $\alpha3K^{185L}$ (●). Measurements were performed on averaged traces. The amplitude of GABA-evoked currents was normalized to the peak amplitude of the current evoked by the application of 0.1 mM glycine (relative current). Numbers above data points indicate the number of measurements per point (mean ± SD).

in hippocampal principle cell layers in adult mice (Eichler et al., 2009). It therefore is conceivable that activation of GlyRs can evoke local chloride currents at glutamatergic synapses, which will reduce the efficacy of these synapses. However, these GlyRs must be activated at the level of glutamatergic postsynaptic domains. Presence of glycine transporter 1 at hippocampal glutamatergic synapses (Cubelos et al., 2005) together with the observation of postsynaptic glutamatergic responses with recurrent small strychnine-sensitive components (Müller et al., 2007) can support this hypothesis, particularly in case of RNA-edited receptors (Meier et al., 2005; Eichler et al., 2008). Again, it remains to be shown whether RNA editing impacts on the subcellular distribution of these receptors.

HIGH AFFINITY GlyR EFFECTIVELY INHIBITS NEURONAL ACTIVITY THROUGH SHUNT INHIBITION

It is now well established that GlyRs are highly mobile entities of the neuronal plasma membrane (Meier et al., 2001; Dahan et al., 2003), which implies that receptors have a certain probability to be encountered at non-synaptic locations.

In the hippocampus, functional glycinergic synapses have not been described, and the majority of α3-GlyR immunoreactivity indeed is found at non-synaptic places (Eichler et al., 2009). Furthermore, it has recently been shown that glycine uptake can regulate hippocampal network activity via GlyR-mediated non-synaptic tonic inhibition (Zhang et al., 2008), and the source of extracellular glycine was suggested to be glial cells (Zhang et al., 2008). A continuous activation of non-synaptic receptors requires a sufficient amount of extracellular glycine or taurine. However, in the central nervous system, the concentration of glycine is tightly regulated by glycine transporters (Gomeza et al., 2003), and the residual glycine concentration in the extracellular space is likely to be close to 0.1 μM in normal conditions (Roux and Supplisson, 2000).

Extracellular glycine can increase temporarily in case of sustained presynaptic activity (Roux and Supplisson, 2000; Supplisson and Roux, 2002), as for example in the epileptic tissue where hypersynchronous high frequency network activity occurs (Fisher et al., 1992; Bragin et al., 2007). Accordingly, this might have important functional consequences, because non-synaptic slightly desensitizing GlyRs could be continuously activated as a result of temporarily altered GlyT activity (e.g. reverse transport) or even due to glycine spillover. Remarkably, the embryonic form of GlyRs, which is composed of five α2 subunits and expressed before synaptogenesis displays functional properties adapted to non-synaptic release of the agonist (Mangin et al., 2003). Therefore, it can sustain a long lasting activation state when the extracellular glycine concentration increases above its activation threshold (Mangin et al., 2003).

Irrespective of the subunit, remarkable features of RNA-edited GlyRs are their high apparent affinities for glycine (Meier et al., 2005; Eichler et al., 2008). Effectively, these receptors can be activated by very low glycine concentrations. In case of GlyR $\alpha3^{185L}$, 300 nM glycine already is sufficient for receptor activation, which in turn results in the occurrence of single channel openings. Independently of the chloride equilibrium potential, the activation of several GlyR $\alpha3^{185L}$ can evoke an inhibition of the neuronal activity. Effectively, the opening of several channels can lead to a decrease in the cell input resistance, which will reduce the propagation of synaptic events and of action potentials, resulting in an inhibitory shunting process (Eichler et al., 2008).

HIGH AFFINITY GlyRs ARE HARMFUL FOR HIPPOCAMPAL NEURONS WITH HIGH CHLORIDE EQUILIBRIUM POTENTIAL

Although GlyRs were reported to have anticonvulsant effects (Kirchner et al., 2003; Song et al., 2006) results obtained more recently (Eichler et al., 2008) point to a pathophysiological role of enhanced GlyR-mediated signaling, particularly in the context of high chloride equilibrium potential as is the case in TLE (Palma et al., 2006). Under these circumstances, neuron silencing triggers up-regulation of the ratio between glutamatergic and GABAergic nerve terminals and is associated with abnormal dendrite morphology (Lohmann et al., 2005; Eichler et al., 2008), both of which phenomena are reminiscent of the TLE histopathology (Loup et al., 2000; Stief et al., 2007). Most importantly, silenced hippocampal neurons were prone to die due to glutamate excitotoxicity, but amongst others could be rescued by expression of the potassium chloride cotransporter type 2 (KCC2) (Eichler et al., 2008). KCC2 expression provides neurons with an efficient chloride extrusion mechanism that decreases their chloride equilibrium potential (Lee et al., 2005). Although the mechanisms underlying this neuroprotective effect of KCC2 remained obscure our results support the previously postulated developmentally dichotomous effects of single cell activity deprivation on neuron integrity within an otherwise active neuronal network (Tao and Poo, 2005).

Furthermore considering altered *GLRA3* splicing in the hippocampus of TLE patients, favouring association of α3-GlyRs with hippocampal GABAergic synapses, the net outcome of anomalous posttranscriptional *GLRA3* processing is predicted to add to the pathophysiology of TLE. In a worst case scenario, provided that the chloride equilibrium potential is high enough (Rivera et al., 2004; Palma et al., 2006; Eichler et al., 2008), up-regulation of RNA-edited α3K-GlyRs (associated preferentially with GABAergic synapses) accompanied by down-regulation of RNA-edited α3L-GlyRs (associated preferentially with glutamatergic synapses) could increase glycinergic signalling at depolarizing GABAergic synapses while reducing inhibitory effects on glutamatergic postsynaptic currents.

EXPERIMENTAL STRATEGIES FOR DETECTION OF RNA-EDITED GlyR PROTEIN

In any case, it is necessary to prove the existence of RNA-edited GlyR protein. However, this figured out to be rather difficult. Because of the position of leucine 185/192 at the interior of the channel pore close to the plasma membrane antibody accessibility is predicted to be low. Indeed, a potentially GlyR $\alpha3^{185L}$-specific antibody obtained by immunization of guinea pigs with peptide EGLTL**L**QFLLK (185L, underlined and bold) and cross-absorption against peptide EGLTL**P**QFLLK (185P, underlined and bold) was not able to visualize RNA-edited GlyR $\alpha3^{185L}$ in transfected HEK293 (not shown). Even variation of fixation protocols and reagents did not ameliorate cellular protein detection. Therefore, it will be necessary to isolate a particular single channel conductance specific to RNA-edited GlyRs or any other differentiating factor in order to validate existence of these proteins

in neuronal tissue. It is conceivable to screen ligand databases for the presence of agonists or antagonists specific to RNA-edited GlyRs. So far, however, poor availability of reliable chloride indicators applicable to living cells was a rather rate limiting step in identification of novel ligands.

CONCLUSION

RNA-edited GlyRs may open avenues to medication of excitability disorders (Eichler and Meier, 2008) because they could provide researchers and clinicians with a battery of new therapeutically relevant drugs, yet these enigmatic receptors still have to be captured at the protein level of investigation. In particular, a possible cell-type specific hippocampal expression has to be determined.

SUPPORTING METHODS

WHOLE-CELL PATCH-CLAMP RECORDINGS

Whole-cell patch-clamp recordings of HEK293 cells were performed 2–3 days after Ca^{2+}-phosphate transfection with α1-GlyR constructs. For electrophysiological recordings, the growth medium was replaced with a bath solution containing (in mM): 145 NaCl, 2.5 KCl, 20 HEPES, 10 glucose, 2 $CaCl_2$, 1 $MgCl_2$, adjusted to pH 7.3. All recordings were carried out at room temperature using patch-clamp amplifier EPC-9 (HEKA Elektronik, Lambrecht, Germany). Patch electrodes were fabricated from borosilicate glass capillaries tubing using a P-97 puller (Sutter Instruments, Novato, CA, USA). The pipette solution contained (in mM): 4 NaCl, 130 KCl, 5 EGTA, 10 HEPES, 10 glucose, 0.5 $CaCl_2$, 4 $MgCl_2$, adjusted to pH 7.3. The pipette-to-bath DC resistance of patch electrodes ranged from 2–4 MOhm. Recordings were performed at a holding potential of −70 mV. Glycine or taurine was locally applied for 3 s via a gravity-driven 5-channel superfusion system. An interval of at least 60 s was left between successive agonist concentrations to allow for recovery from receptor desensitization. Different agonist concentrations were applied randomly. Electrophysiological signals were sampled at a rate of 5 kHz, filtered at 1 kHz and analyzed off-line using software TIDA 5.1 (HEKA Electronics, Lambrecht, Germany). To determine the EC_{50} value for agonists, peak currents obtained with different glycine or taurine concentrations were plotted and fitted to the Hill equation using software Origin 7.0 (OriginLab, Northampton, USA).

OUTSIDE-OUT PATCH-CLAMP RECORDINGS

Outside-out patch-clamp recordings of HEK293 cells were performed 2 days after Ca^{2+}-phosphate transfection with $\alpha 3K^{185P}$ or $\alpha 3K^{185L}$ GlyR constructs. Cells were continuously perfused at room temperature (20–22°C) with bathing solution (2 ml/min) containing (in mM): NaCl 147, KCl 2.4, $CaCl_2$ 2, $MgCl_2$ 2, HEPES 10, glucose 10 (pH 7.2, osmolarity 320 mOsm). Patch-clamp electrodes (5–10 MΩ) were pulled from thick-wall borosilicate glass (Harvard Apparatus, Kent, UK) using a Brown-Flaming puller (Sutter Instrument Co., Navato, USA). They were fire-polished and filled with (in mM): CsCl 130, $MgCl_2$ 4, Na_2ATP 4, EGTA 10, HEPES 10 (pH 7.2, osmolarity 290 mOsm). Currents were recorded using an Axopatch 1D amplifier (Axon Instruments, Foster City, USA). Recordings were filtered at 10 kHz using an eight-pole bessel filter (Frequency Devices, Haverhill, USA), sampled at 50 kHz and stored on a PC computer using pClamp software 10.1. (Axon Instruments, Foster City, USA). The membrane potential was held at −50 mV throughout the experiment. Outside-out single-channel currents were evoked using a fast-flow operating system (Legendre, 1998). Control and drug solution were gravity-fed into two channels of a thin-wall glass theta tube (2-mm outer diameter; Hilgenberg, Malsfeld, Germany) pulled and broken to obtain a 200-µm tip diameter. The outside-out patch was positioned 100 µm away from the glass theta tube, one lumen of the application pipette was connected to reservoirs filled with solutions containing glycine or GABA. The solution exchange was performed by rapidly moving the solution interface across the tip of the patch pipette, using a piezo-electric translator (model P245.30, Physik Instrument, Waldbronn, Germany). Concentration steps of glycine or GABA were applied with an interval of ≥10 s. Exchange time solution (<100 µs) was determined before each set of experiments by monitoring the change in the liquid junction potential evoked by the application of a 10%-diluted control solution to the open tip of the patch pipette.

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Potentiation of glycine-gated NR1/NR3A NMDA receptors relieves Ca^{2+}-dependent outward rectification

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Glycine has diverse functions within the mammalian central nervous system. It inhibits postsynaptic neurons via strychnine-sensitive glycine receptors (GlyRs) and enhances neuronal excitation through co-activation of N-methyl-D-aspartate (NMDA) receptors. Classical Ca^{2+}-permeable NMDA receptors are composed of glycine-binding NR1 and glutamate-binding NR2 subunits, and hence require both glutamate and glycine for efficient activation. In contrast, recombinant receptors composed of NR1 and the glycine binding NR3A and/or NR3B subunits lack glutamate binding sites and can be activated by glycine alone. Therefore these receptors are also named "excitatory glycine receptors". Co-application of antagonists of the NR1 glycine-binding site or of the divalent cation Zn^{2+} markedly enhances the glycine responses of these receptors. To gain further insight into the properties of these glycine-gated NMDA receptors, we investigated their current-voltage (I–V) dependence. Whole-cell current-voltage relations of glycine currents recorded from NR1/NR3B and NR1/NR3A/NR3B expressing oocytes were found to be linear under our recording conditions. In contrast, NR1/NR3A receptors displayed a strong outwardly rectifying I–V relation. Interestingly, the voltage-dependent inward current block was abolished in the presence of NR1 antagonists, Zn^{2+} or a combination of both. Further analysis revealed that Ca^{2+} (1.8 mM) present in our recording solutions was responsible for the voltage-dependent inhibition of ion flux through NR1/NR3A receptors. Since physiological concentrations of the divalent cation Mg^{2+} did not affect the I–V dependence, our data suggest that relief of the voltage-dependent Ca^{2+} block of NR1/NR3A receptors by Zn^{2+} may be important for the regulation of excitatory glycinergic transmission, according to the Mg^{2+}-block of conventional NR1/NR2 NMDA receptors.

Keywords: NMDA receptor, excitatory glycine receptor, voltage block, NR3 subunit, supralinear potentiation, Zn^{2+}, NR1 antagonist, ligand-binding domain

INTRODUCTION

The simplest of all amino acids, glycine, has diverse functions within the mammalian central nervous system (CNS). Glycine mediates synaptic inhibition through hyperpolarizing glycine receptors (GlyRs) and contributes to neuronal excitation by acting as a co-agonist at glutamate-type N-methyl-D-aspartate (NMDA) receptors (see overview in Dingledine et al., 1999; Betz and Laube, 2006). The GlyR is a pentameric protein composed of two types (α and β) of membrane-spanning subunits (Grudzinska et al., 2005). In contrast, the NMDA receptor is a hetero-tetrameric membrane protein (Laube et al., 1998) composed of glycine-binding NR1 and glutamate-binding NR2 subunits (Laube et al., 1997; overview in Cull-Candy et al., 2001) and/or the recently discovered glycine-binding NR3A and NR3B subunits (Sucher et al., 1995; Nishi et al., 2001; Yao and Mayer, 2006). The ligand-binding domains (LBDs) of the NMDA receptor subunits are formed by two extracellular segments S1 and S2 and are thought to be arranged in a dimer-of-heterodimer orientation (Furukawa et al., 2005; Schüler et al., 2008). Binding of the agonist occurs between the two extracellular segments and results in a closure of the LBD and subsequent opening of the ion channel (Armstrong and Gouaux, 2000; overview in Mayer, 2006).

Conventional NMDA receptors composed of two NR1 and two NR2 subunits require the simultaneous binding of glutamate and glycine for efficient channel opening (Johnson and Ascher, 1987; Kuryatov et al., 1994). In contrast, NMDA receptors composed of NR1 and NR3 subunits result in nonselective cation-channels exclusively activated by glycine, termed "excitatory glycine receptors", which are unaffected by glutamate (Chatterton et al., 2002). The physiological role of NR1/NR3 NMDA receptors is however still discussed controversially (Chatterton et al., 2002; Tong et al., 2008). Recombinantly expressed NR1/NR3A and NR1/NR3B receptors generate only small excitatory currents upon activation by glycine (Awobuluyi et al., 2007; Madry et al., 2007a; Smothers and Woodward, 2009). This low functionality cannot be attributed to impaired receptor assembly or decreased surface insertion but derives from opposite roles of glycine which acts agonistically at NR3 subunits and inhibitory through binding to the NR1 subunit (Awobuluyi et al., 2007; Madry et al., 2007a). NR1/NR3A receptor activation by glycine is strongly potentiated in the presence of either the divalent cation Zn^{2+} (ten-fold) or NR1 glycine-site antagonists such as MDL-29951 (MDL; >20-fold). Coapplication of both Zn^{2+} and MDL generates a > 120-fold "supralinear" potentiation of

glycine-induced currents (Madry et al., 2008). In addition, Zn^{2+} alone elicits receptor currents with similar efficacy as glycine at concentrations > 100 μM (Madry et al., 2008).

In the present study we investigated the current-voltage (I–V) dependence and permeation properties of glycine-gated NR1/NR3 NMDA receptors. We found that NR1/NR3A receptors display a strong outwardly rectifying I–V relationship, whereas NR3B subunit-containing receptors (NR1/NR3B and NR1/NR3A/NR3B) do not show a voltage-dependent inward current block at physiological ion concentrations. Further analyses revealed that the voltage-dependent inhibition of ion flux seen with NR1/NR3A receptors is (i) due to Ca^{2+} present in our extracellular recording solution, and is (ii) relieved by potentiating ligands like Zn^{2+} or/and MDL acting at the NR1 subunit. We conclude from these data that voltage-dependent inhibition of NR1/NR3A receptors by physiological concentrations of extracellular Ca^{2+} may be important for regulating excitatory glycinergic transmission, as described for the voltage-dependent Mg^{2+} block of conventional NR1/NR2 NMDA receptors important for glutamatergic signalling.

MATERIALS AND METHODS

MDL-29951 was purchased from Tocris (Biotrend, Cologne, Germany). All other chemicals used were obtained from Sigma (Taufkirchen, Germany). All experimental procedures were done according to German law (Animal licence no. V 54–19c20/15-F126/13; Regierungspräsidium Darmstadt).

cDNA CONSTRUCTS, OOCYTE EXPRESSION AND ELECTROPHYSIOLOGY

The NR1-1a, NR1-1a^{F466A}, NR3A and NR3B expression constructs have been described previously (Kuryatov et al., 1994; Madry et al., 2007a). *In vitro* synthesis of cRNA (mCAP mRNA Capping Kit, Ambion, Austin, TX, USA) was performed as described (Laube et al., 2004; Madry et al., 2007a) in the presence of 115 mM NaCl, 1 mM KCl, 1.8 mM $CaCl_2$ and 10 mM Hepes (pH 7.2). For heterologous expression of NR1/NR3 receptors, 25 ng of cRNA was injected at a NR1:NR3 ratio of 1:3 into *Xenopus laevis* oocytes. Oocytes were isolated and maintained as described previously (Laube et al., 1995). Two-electrode voltage-clamp recording of whole-cell currents was performed according to Madry et al. (2007b). N-methyl-D-Glucamine chloride (NMDG-Cl) was used in external solutions in which NaCl was replaced for analysis of divalent permeability. To monitor the voltage dependence of NR1, NR3A and NR3B receptor combinations, whole-cell current-voltage relationships of saturating glycine-induced currents were recorded in 20 mV-intervals ranging from −90 mV to +30 mV and normalized to the current value obtained at +30 mV. Data points were aligned by using a 3rd-order polynomial fit according to Geiger et al. (1995). To quantify the extent of rectification, the current ratios at 40 mV above and 80 mV below the individual reversal potentials (E_{rev} between −10 and 0 mV) were determined as rectification indices (R_i). The relative divalent to monovalent permeability (P_{div}/P_{mono}) was calculated by the Goldman-Hodgkin-Katz constant field (GHK) voltage equation assuming no anion permeability as described previously (Geiger et al., 1995). The internal concentrations of Na^+ and K^+ used in the calculations were 20 mM and 150 mM, respectively (Katz et al., 2000; Weisstaub et al., 2002). Permeability ratios were calculated for each oocyte and then averaged. In order to avoid the activation of the oocytes' native Ca^{2+}-sensitive chloride currents, all experiments using Ca^{2+} containing extracellular solutions were carried out in oocytes incubated for 30 min at room temperature with the membrane-permeant Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester (BAPTA-AM, 100 μM) prior to electrophysiological recordings (Weisstaub et al., 2002).

STATISTICAL ANALYSES

Values given represent means ± SEM. Statistical significance was determined at the $p < 0.05$ (*), $p < 0.01$(**) and $p < 0.001$ (***) levels using a Student's two-tailed, unpaired t-test.

RESULTS

GLYCINE-GATED NR1/NR3A AND NR1/NR3B RECEPTORS DIFFER IN THEIR CURRENT-VOLTAGE (I–V) RELATIONSHIPS

In a previous study, we described a potentiating effect of the NR1 glycine-binding site antagonist MDL-29951 (MDL) on NR1/NR3A and NR1/NR3B receptors expressed in oocytes (Madry et al., 2007a). When further analyzing the effect of MDL on NR1/NR3A and NR1/NR3B receptor currents elicited by glycine (1 mM), we found that at a negative holding potential (−90 mV) NR1/NR3A receptors were significantly more potentiated by 200 nM MDL than NR1/NR3B receptors, with a potentiation of 8.8 ± 1.2-fold and 2.5 ± 0.1-fold, respectively ($p < 0.001$; **Figures 1A,B**). However, at a positive holding potential (+30 mV), potentiation of the glycine-induced currents by MDL was not different between the two receptor combinations (3.1 ± 0.5-fold and 2.4 ± 0.4-fold, respectively; $p > 0.05$; **Figures 1A,B**). We therefore analyzed whole-cell current-voltage relationships (I–V curves) of glycine-induced currents from NR1/NR3A and NR1/NR3B receptors over a voltage range of −90 mV to +30 mV (**Figures 1C,D**). I–V curves of NR1/NR3B receptors were found to be linear with a reversal potential of around −10 mV (**Figure 1D**), whereas those obtained from NR1/NR3A receptors showed an identical reversal potential but a strong outwardly rectifying behavior, with an inward current block emerging at a holding potential <−30 mV (**Figure 1C**). Interestingly, co-application of 200 nM MDL caused a linearization of the I–V curve for glycine-activated NR1/NR3A receptors (**Figure 1C**), whereas the linear I–V relation of receptors containing the NR3B subunit was not altered in the presence of MDL (**Figure 1D**).

In order to quantify the extent of rectification of NR1/NR3 receptor currents, we determined current ratios at +30 mV and −90 mV and calculated rectification indices (R_i). Based on a reversal potential of −10 mV, linear I–V relationships result in a R_i value of about 0.5 whereas outwardly rectifying I–V curves display R_i values > 1.5. Consistent with the data shown above, MDL potentiation caused a significant change of the R_i for NR1/NR3A receptors (−MDL: 1.65 ± 0.15, +MDL: 0.62 ± 0.08; $p < 0.001$), whereas no difference was seen for NR1/NR3B receptors in the absence and presence of the antagonist (−MDL: 0.43 ± 0.08, +MDL: 0.34 ± 0.02; $p > 0.05$; **Figure 1F**). Finally, we analyzed the I–V curve of triheteromeric receptors composed of NR1, NR3A and NR3B subunits. Maximal inducible currents of these tri-heteromeric NR1/NR3A/NR3B receptors were more than 10-fold larger than those obtained from di-heteromeric NR1/NR3A and NR1/NR3B receptors (data not shown), which is consistent with an efficient tri-heteromeric NR1/NR3A/NR3B receptor assembly (Ulbrich and

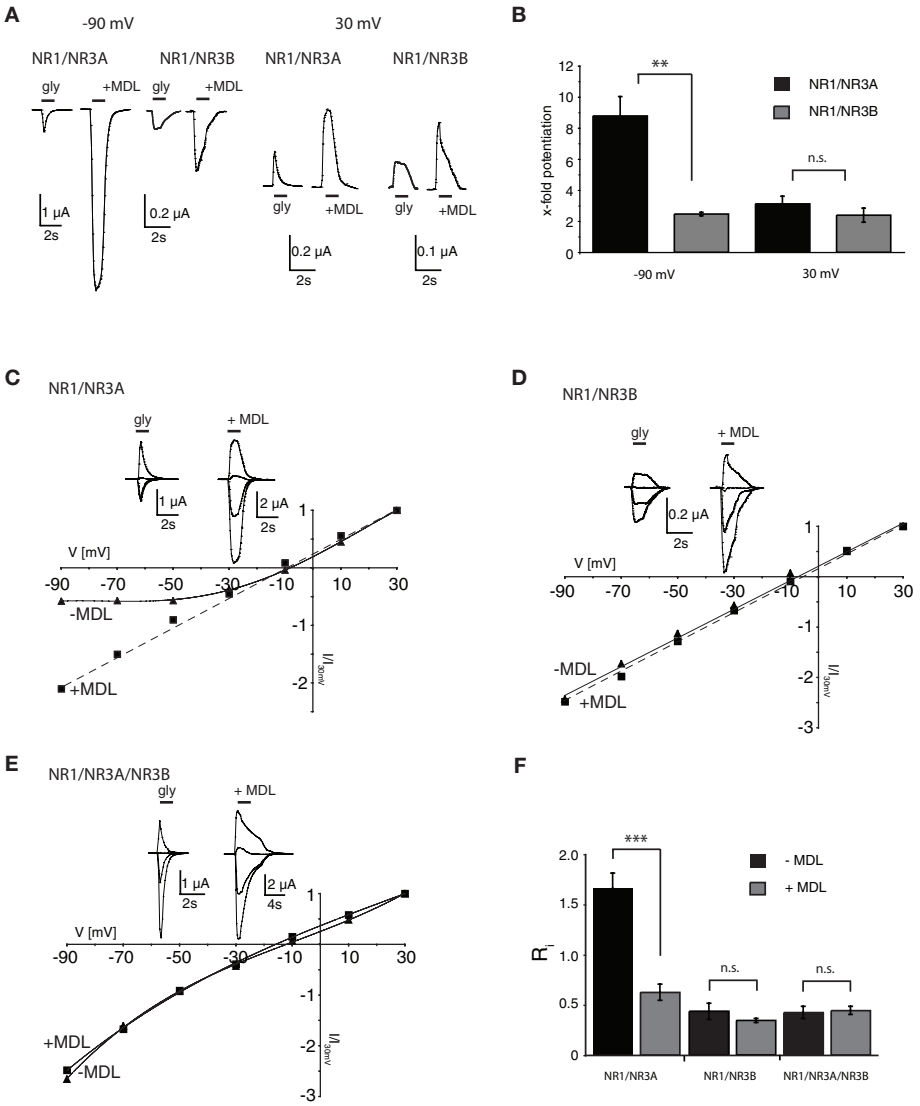

FIGURE 1 | I–V relationships of glycine-gated NR1/NR3 receptors. **(A,B)** Effect of holding-potential on MDL-29951 (MDL) potentiated glycine currents of NR1/NR3A and NR1/NR3B receptors. **(A)** Sample traces at –90mV (left) and +30 mV (right) activated with a saturating glycine concentration (1 mM) in the absence or presence of 200 nM MDL. **(B)** Relative potentiation by MDL of NR1/NR3A (black bars) and NR1/NR3B (gray bars) receptor currents at –90 and +30 mV. Note that MDL-potentiation was at –90 mV about 3-fold larger for NR1/NR3A receptors compared to NR1/NR3B ($p < 0.01$; n = 5). **(C–E)** Normalized I–V plots of NR1/NR3A **(C)**, NR1/NR3B **(D)** and NR1/NR3A/NR3B **(E)** receptor currents recorded from –90 to +30 mV in 20-mV intervals activated by a saturated glycine concentration in the absence (triangle) and presence (square) of 200 nM MDL. Respective sample traces are shown above. Note that NR1/NR3A receptors display an ourwardly rectifying I–V curve in the presence of glycine alone, which becomes linear upon MDL-potentiation. **(F)** Quantification of I–V relationships of NR1/NR3 receptors in the absence (black bars) and presence (gray bars) of 200 nM MDL by determining the rectification index (R_i) of the currents measured at 40 mV above (Δ+40 mV) and 80 mV below (Δ–80 mV) the respective reversal potential.

Isacoff, 2008; Smothers and Woodward, 2009). I–V curves from NR1/NR3A/NR3B expressing oocytes were found to be linear in both, the absence and presence of MDL (**Figure 1E**). Analyses of the R_i revealed values of 0.42 ± 0.06 vs. 0.44 ± 0.04 in the absence and presence of 200 nM MDL for NR1/NR3A/NR3B-receptors, respectively ($p > 0.05$; **Figure 1F**). Thus, MDL caused a linearization of the outwardly rectifying I–V curve of NR1/NR3A receptors by a relief of the voltage-dependent inward current block, whereas NR3B containing combinations showed a linear I–V-relationship irrespective whether MDL was present or not.

DIFFERENTIAL EFFECTS OF Zn^{2+} ON NR1/NR3A RECEPTOR I–V RELATIONS

The divalent cation Zn^{2+} exerts complex and opposing effects at NR1/NR3 receptors. At NR1/NR3A receptors it acts in the lower micromolar concentration range as a positive modulator of glycine-currents and as a full principal agonist at Zn^{2+} concentrations > 100 µM (Madry et al., 2008). In contrast, NR1/NR3B receptors neither become potentiated nor activated by Zn^{2+}. Therefore we wondered whether Zn^{2+}-potentiation of NR1/NR3A receptor currents would display a linear I–V relationship similar to that found for MDL-potentiated receptor

currents. Indeed, co-application of glycine and 50 μM Zn^{2+} fully linearized the outwardly rectifying I–V curve seen in the absence of Zn^{2+} to R_i values resembling those found in the presence of MDL (**Figures 2A,D**). Since maximal potentiation of NR1/NR3A receptors is observed upon co-application of the modulators Zn^{2+} and MDL (Madry et al., 2008), we also analyzed the I–V curves of these supralinearly potentiated NR1/NR3A receptors. In line with a relief of the glycine-mediated inward current block by MDL and Zn^{2+} alone, we found a linear I–V relationship of the glycine-induced currents when both 200 nM MDL and 100 μM Zn^{2+} were co-applied (**Figure 2D**, and data not shown). In conclusion, potentiation of glycine-induced NR1/NR3A receptor currents by Zn^{2+}, NR1 antagonists or a combination of both abrogated the inward current block seen upon application of glycine alone.

Higher concentrations of Zn^{2+} (>100 μM) activate NR1/NR3A receptors with a comparable efficacy as glycine (Madry et al., 2008). Hence, we wanted to know whether both agonists display similar I–V relationships. We found that Zn^{2+}-gated NR1/NR3A receptors displayed a similar inward current block than seen with glycine (**Figure 2B**). Strikingly, MDL potentiation of Zn^{2+}-induced currents was not accompanied by a linearization of the I–V curve, and thus did not cause a decrease of the rectification index (**Figures 2B,D**). Apparently, the voltage-dependent blocks seen with both glycine and Zn^{2+} are differentially affected by MDL. Since both Zn^{2+} and MDL are thought to act via the NR1-LBD, we analyzed the effect of a mutation within the glycine-binding site of the NR1 subunit (phenylalanine 466 to alanine) on glycine-gated I–V relations of NR1/NR3A receptors (**Figure 2C**). Previous mutational analyses have shown that the affinity of both glycine and MDL to the NR1

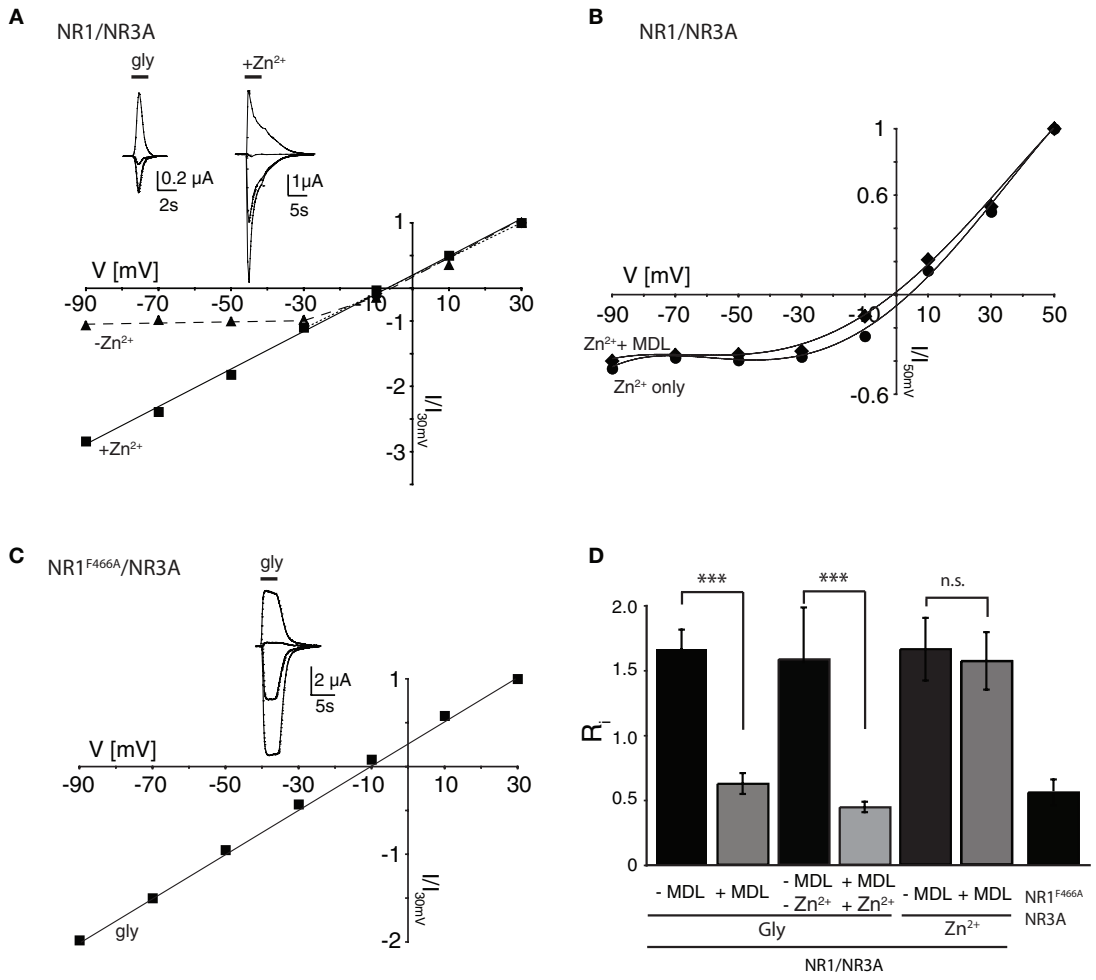

FIGURE 2 | Zn^{2+} mediated effects at NR1/NR3A receptors. (A) Zn^{2+} potentiation of glycine-induced currents at NR1/NR3A receptors. Normalized I–V plot of NR1/NR3A receptor currents activated by a saturating glycine concentration in the absence (triangle) and presence (square) of 50 μM Zn^{2+}. Similar to MDL, also co-application of 50 μM Zn^{2+} with 100 μM glycine causes a linearization of NR1/NR3A receptor I–V relationship. **(B)** MDL potentiation of Zn^{2+}-induced currents at NR1/NR3A receptors. Normalized I–V plot of NR1/NR3A receptor currents activated by a saturating Zn^{2+} concentration in the absence (triangle) and presence (square) of 200 nM MDL. Note that NR1/NR3A receptors display an outwardly rectifying I–V curve upon activation by Zn^{2+} irrespectively of whether MDL is present or not. **(C)** Normalized I–V plot of the LBD-mutant $NR1^{F466A}$/NR3A receptor currents activated by a saturating glycine concentration. Note that $NR1^{F466A}$/NR3A receptors display a linear I–V relation. **(D)** Rectification indices (R_i) of I–V relationships of wt and mutant NR1/NR3 receptors in the absence (black bars) and presence (gray bars) of potentiators.

subunit is reduced by the NR1^{F466A} substitution (Kuryatov et al., 1994; Madry et al., 2007a), and that the potentiating and activating effects of Zn^{2+} at NR1/NR3A receptors are abolished (Madry et al., 2008) by this mutation. Here we found a linear I–V relationship of glycine-induced currents recorded from NR1^{F466A}/NR3A receptors (R_i: 0.49 ± 0.02; **Figures 2C,D**). This indicates that the NR1 LBD is crucial for the rectification behavior of NR1/NR3A receptors.

PHYSIOLOGICAL CONCENTRATIONS OF CA^{2+} CAUSE VOLTAGE-DEPENDENT INHIBITION OF NR1/NR3A RECEPTOR-MEDIATED ION FLUX

Mg^{2+} ions are known to block NR1/NR2 NMDA receptor channels at negative membrane potentials (overview in Cull-Candy et al., 2001). We therefore analyzed whether divalent cations are responsible for the voltage-dependent inward current block seen with NR1/NR3A receptors. First we measured glycine-mediated currents of NR1/NR3A receptors in the absence of divalent cations. I–V relationships obtained under divalent-free conditions were found to be linear, with a rectification index in the range of those seen with glycine-gated NR1/NR3B and NR1/NR3A/NR3B, or potentiated NR1/NR3A receptors (R_i: 0.43 ± 0.04; **Figures 3A,B**). To further test whether the voltage-dependent inhibition of NR1/NR3A receptor-mediated ion flux is due to a specific divalent cation, we analyzed I–V relations of glycine-induced currents in the presence of Ca^{2+}, Ba^{2+} and Mg^{2+} (1.8 mM each). The presence of either 1.8 mM Ba^{2+} or Mg^{2+} resulted only in a minor inhibition of inward current flow with R_i-values of 0.84 ± 0.05 and 0.84 ± 0.03, respectively (**Figure 3B**). In contrast, I–V relations in the presence of 1.8 mM Ca^{2+} revealed a strong inward rectification with a highly significant larger R_{iCa}

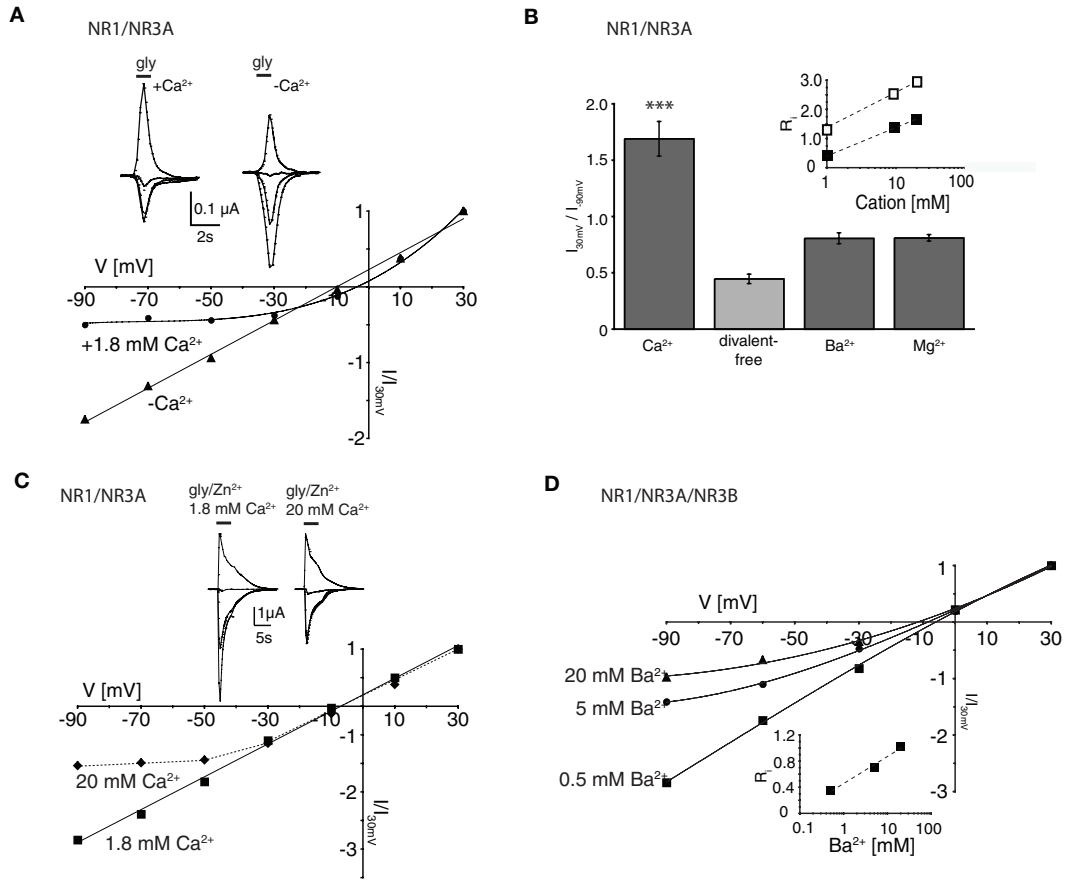

FIGURE 3 | Dependency of I–V relationship on divalent cations for glycine-activated NR1/NR3A receptors. (A) Normalized I–V plot of NR1/NR3A receptor currents activated by a saturating glycine concentration in the absence (triangle) and presence (square) of 1.8 mM Ca^{2+}. Note, that application of a saturating glycine concentration (100 µM) in the absence of any divalent cations results in a linear I–V relationship, whereas 1.8 mM Ca^{2+} causes an inward current block (see also **Figure 1C**). Sample traces are shown above the I–V plot. **(B)** Quantification of divalent-dependent inward current block of NR1/NR3A receptors. Rectification indices (R_i) of I–V relationships of NR1/NR3A receptors in the absence of divalent cations and in the presence of 1.8 mM Ca^{2+}, Ba^{2+} and Mg^{2+} are shown. Inset shows a plot of the three different rectification indices (R_i) fitted against the respective log of (Ca^{2+}) (open symbol) and (Mg^{2+}) (closed symbol). **(C)** Effect of different Ca^{2+} concentrations on Zn^{2+} potentiated glycine-activated currents of NR1/NR3A receptors. Normalized I–V plot of potentiated NR1/NR3A receptor currents activated by a saturating glycine concentration and 50 µM Zn^{2+} in the presence of 1.8 mM (square) and 20 mM (triangle) Ca^{2+}. Note that an increase of the extracellular Ca^{2+} concentration from 1.8 to 20 mM led to an outwardly rectifying I-V-relationship similar to those found under non-potentiated conditions in the presence of low Ca^{2+}. Sample traces are shown above the I–V plot. **(D)** Increasing divalent cation concentrations lead to outwardly rectifying I-V relationships in NR3B containing NR1/NR3 receptors. Normalized I–V plot of NR1/NR3A/NR3B receptor currents activated by a saturating glycine concentration in the presence of 0.5 mM (square), 5 mM (circle) and 20 mM Ba^{2+} (triangle). Inset shows a plot of the three different rectification indices (R_i) fitted against the respective log [Ba^{2+}].

of 1.68 ± 0.09 ($p < 0.001$; **Figure 3B**). To estimate the efficacy of Ca^{2+} and Mg^{2+} to block inward currents, I–V relationships with increasing concentrations (1, 10 and 20 mM) of the two divalent cations were measured. Only higher Mg^{2+} concentrations (>10 mM) resulted in a pronounced inward rectification with R_i-values > 1 similar to those found with low Ca^{2+} concentrations, whereas I–V curves in the presence of 1 mM Mg^{2+} were linear (**Figure 3B,** inset). This is consistent with different affinities of the two cations tested for ion channel block and shows that under physiological divalent cation concentrations Ca^{2+} and not Mg^{2+} determines the I–V relationship of NR1/NR3A receptors.

To test whether potentiated NR1/NR3A glycine currents might be affected at non-physiological elevated Ca^{2+} concentrations, we analyzed the I–V relationship of Zn^{2+}-potentiated (50 μM) glycine-induced currents in the presence of 20 mM Ca^{2+}. This increased Ca^{2+} concentration produced an inward current block at holding potentials <–30 mV (**Figure 3C**) as seen in the absence of Zn^{2+} at low Ca^{2+} (1.8 mM). Based on this result, we re-investigated the divalent cation dependency of the I–V curves of NR1/NR3B and NR1/NR3A/NR3B receptors, which both exhibit linear I–V relationships under physiological salt concentrations. Similarly, increasing the extracellular Ca^{2+} or Ba^{2+} concentration to 20 mM led to the emergence of an outwardly rectifying I–V curve at both NR1/NR3B and NR1/NR3A/NR3B receptors (**Figure 3D**). This implies that in the presence of elevated divalent cation concentrations NR3B subunit containing receptor combinations display an outwardly rectifying I–V relationship as found for NR1/NR3A receptors in the presence of physiological Ca^{2+} concentrations.

In summary, physiological Ca^{2+} conditions are responsible for the outward rectification of glycine-gated NR1/NR3A receptors, whereas potentiated NR1/NR3A and NR3B containing receptors are blocked only at higher Ca^{2+} concentrations. Thus, differences in the affinity of the Ca^{2+} block seem to underlie the differential rectification behavior of NR3A and NR3B subunit containing receptors.

PERMEABILITY FOR DIVALENT CATIONS IS NOT ALTERED IN SUPRALINEARLY POTENTIATED NR1/NR3A RECEPTORS

Removal of a cation-dependent open channel block has been shown in both transient receptor potential (TRP) channels and conventional NMDA receptors by an increase in the passage-rate of the blocking ion through the channel pore (Parnas et al., 2009). Therefore the relief of the Ca^{2+}-mediated block seen with MDL and Zn^{2+}-potentiated NR1/NR3A receptors may derive from an increased Ca^{2+} permeability. To test this hypothesis, we substituted Na^+ with the ion channel pore impermeable compound N-methyl-D-glucamine chloride (NMDG-Cl) and determined I–V curves in the presence of different Ca^{2+} concentrations in order to obtain an approximate estimate of the relative divalent to monovalent cation permeability P_{Ca}/P_{Na}. **Figure 4A** shows that the reversal potentials pooled from three different oocytes become more depolarized as a function of the concentration of Ca^{2+} in the extracellular medium. However, the ratio of P_{Ca}/P_{Na} was <0.25 in all measurements as determined by the Goldman-Hodgkin-Katz voltage equation (see Geiger et al., 1995), confirming a low Ca^{2+} permeability of the NR1/NR3A receptor under non-potentiated conditions (Chatterton et al., 2002). In a next step we compared the

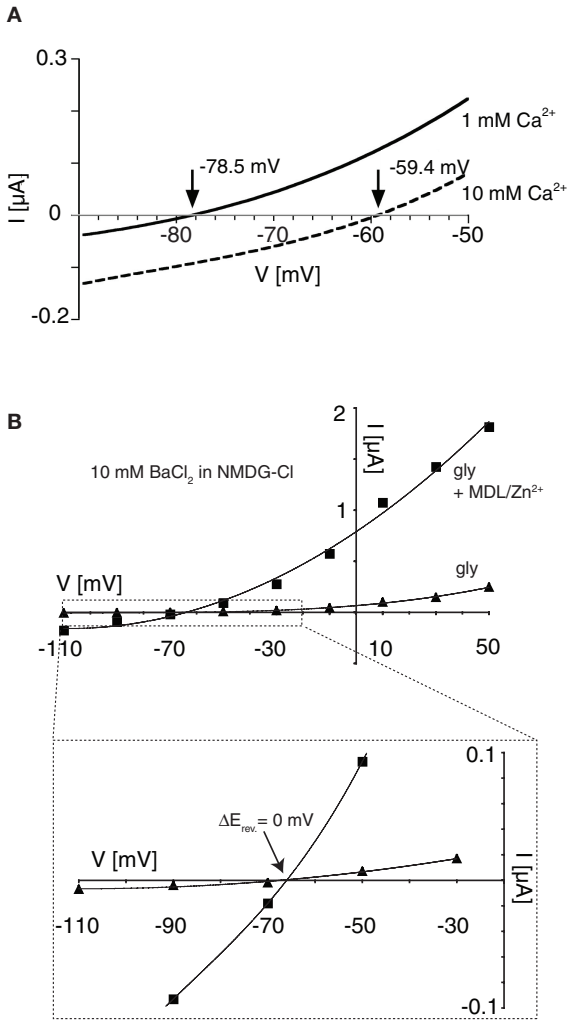

FIGURE 4 | Effect on divalent cation permeability upon supralinear potentiation of glycine-gated NR1/NR3A receptors. (A) Relative divalent to monovalent permeability of NR1/NR3A receptors. Representative I–V recordings around the E_{rev} obtained in Na^+-free ringer containing 1 and 10 mM Ca^{2+}. Arrows indicate the reversal potential (E_{rev}) of each I–V curve. **(B)** I–V recordings of saturated glycine-induced currents (triangles) versus supralinear potentiated currents with 0.2 μM MDL and 50 μM Zn^{2+} (squares) in Na^+-free extracellular solution substituted with 115 mM $NMDG^+$ and 10 mM divalent cation (Ba^{2+}) present. Enlargement illustrates no changes in the respective reversal potentials (–67 mV) for the two conditions.

I–V relationships of glycine-induced currents with that of supralinearly potentiated currents in the presence of divalent cations. Neither the analysis of I–V curves in the presence of 10 mM Ba^{2+} nor using 10 mM Ca^{2+} in the extracellular solution with BAPTA-AM pre-incubated cells revealed any shifts in the reversal potentials (E_{rev} –67 mV; **Figure 4B**). We also tested the accessibility to MK-801, a classical ion channel pore blocker of NR1/NR2 NMDA receptors, which has no effect on glycine-gated NR1/NR3A receptors (Wong et al., 1986; Chatterton et al., 2002). However, even when activated with glycine in the presence of Zn^{2+} and MDL, MK-801 (100 μM) remained ineffective at NR1/NR3A receptors (data not shown). Together these data indicate that the loss of the

voltage-dependent Ca^{2+}-block seen with NR1/NR3A receptors in the presence of MDL and Zn^{2+} is not accompanied by an increased Ca^{2+} permeability of the ion channel.

DISCUSSION

In this article, we show that glycine-gated NR1/NR3A and NR1/NR3B NMDA receptors display a differential sensitivity for Ca^{2+} upon heterologous expression in *Xenopus* oocytes. At negative holding potentials, physiological concentrations of Ca^{2+} (1.8 mM) caused a pronounced inward current block of NR1/NR3A receptors, whereas receptors containing the NR3B subunit were only inhibited at elevated Ca^{2+} concentrations (>10 mM). Interestingly, the voltage-dependent inhibition of NR1/NR3A receptor currents by external Ca^{2+} was abrogated upon co-application of Zn^{2+}, glycine-site antagonist or mutations within the glycine-binding site of the NR1 subunit; all these conditions resulted in a linear I–V relationship. Ion substitution experiments revealed that neither MDL or Zn^{2+} potentiation nor the relief of the Ca^{2+}-block was accompanied by changes in Ca^{2+} permeability. Notably, when NR1/NR3A receptor mediated currents were elicited by Zn^{2+}, MDL co-application did not result in a linear I–V, consistent with different mechanisms underlying glycine and Zn^{2+} agonism.

An interesting finding of this study is that the difference in maximal MDL potentiation of glycine currents seen between NR1/NR3A and NR1/NR3B receptors (Madry et al., 2007a) is due to a pronounced Ca^{2+}-dependent outward rectification of NR1/NR3A receptors. Thus, at physiological Ca^{2+} concentrations NR1/NR3A receptor channels are blocked at negative holding potentials whereas NR1/NR3 receptors containing the NR3B subunit are not affected. Notably, a similar outward rectification of the here described voltage-dependent Ca^{2+} block of the NR1/NR3A receptor exists in conventional NMDA receptors composed of NR1/NR2 subunits. Their voltage-dependent block at resting membrane potentials is mediated by extracellular Mg^{2+} (overview in Cull-Candy et al., 2001). Molecular structures responsible for the Mg^{2+} block have been partially identified and comprise sites in the middle and at the entrance of the channel forming segments of NMDA receptor subunits (overview in Dingledine et al., 1999). For example, asparagine residues of the QRN site in the M2 segment of NR1 and NR2 subunits have been shown to determine the block by Mg^{2+} (Kuner et al., 1996). In addition, a DRPEER motif in NR1 (Watanabe et al., 2002), a tryptophan residue in the M2 regions of NR2 subunits (Williams et al., 1998) and the common SYTANLAAF motif in TM3 (Yuan et al., 2005; Wada et al., 2006) affect the Mg^{2+} block. Comparing the sequences of NR1, NR2 and NR3 subunits reveals a remarkable conservation of these regions, although especially within the QRN site and the SYTANLAAF motif several exchanges between NR1, NR2 and NR3 subunits are found. For example, the corresponding NR3 residue of the QRN site is a glycine. Although all residues mentioned above are highly conserved in NR2 subunits, channels containing NR2A or NR2B subunits are more sensitive to Mg^{2+} block compared with NR2C or NR2D-containing channels, suggesting that additional elements exist that determine subunit specificity to divalent cations. However, the well known physiological function of conventional NMDA receptors in the mammalian brain is to serve as coincidence detectors of presynaptic and postsynaptic activity. This function is achieved through removal of the Mg^{2+} block upon postsynaptic membrane depolarization (Cull-Candy et al., 2001). Likewise, a similar mechanism can be envisaged for NR1/NR3A receptors where release of both, the principal agonist glycine and a second so far unknown ligand may result in a pronounced potentiation of glycine-currents and relief of the voltage-dependent Ca^{2+} block (this study). A previous report has disclosed that the neuromodulator Zn^{2+} (overview in Frederickson et al., 2005) is essential for proper functioning of glycinergic inhibitory neurotransmission (Hirzel et al., 2006). Thus, Zn^{2+} may be similarly essential for efficient activation of NR1/NR3A receptors (Madry et al., 2008).

A second important result of this study is that at least two ligands have to bind simultaneously for abrogating Ca^{2+}-dependent outward rectification of NR1/NR3A receptors. Accordingly, efficient channel gating of NR1/NR3 receptors requires simultaneous occupancy of the NR1 and NR3 LBDs (Awobuluyi et al., 2007; Madry et al., 2007a). Here we show that only ligand-binding to both, the NR3A and NR1 LBD resulted in a linearization of the I–V curve, whereas co-application of the full agonist Zn^{2+} and the NR1 antagonist MDL, both binding within the NR1 LBD, did not abrogate the inward-rectifying Ca^{2+} block. This suggests a remarkable mechanistic similarity in ion channel activation between NR1/NR3A and conventional NR1/NR2 NMDA receptors. Both conventional and glycine-gated NMDA receptors require binding of two ligands within the LBDs of both subunits for efficient channel opening. Thus, only highly cooperative interactions between fully liganded subunits enable the conformational transition of NMDA receptors to the fully open state. Notably, although a remarkable potentiation is found upon binding of both the full agonist Zn^{2+} and MDL, the voltage-dependent Ca^{2+} block is not diminished due to the lack of NR3 LBD occupation. A similar contribution of multiply liganded subunits to efficient channel activation has been proposed for the AMPA receptor, a related member of the iGluR family (Rosenmund et al., 1998). According to this view, occupancy of the NR1 or NR3 binding sites is in principle sufficient to drive channel opening, but additional occupancy of the respective other subunits increases receptor current which might be mediated due to changes in the open probability and/or sub-conductance states of the ion channel. Whatever the precise mechanism(s) of glycine vs. Zn^{2+} action may be, our results show that ion permeability is not changed upon supralinear potentiation of the glycine current. Thus, removal of the Ca^{2+}-dependent channel block is not accompanied by an increase in the passage-rate of Ca^{2+} through the channel pore.

Taken together our data demonstrate fundamental differences in the I–V dependence of di-heteromeric NR1/NR3 receptors that might provide a versatile electrophysiological tool to discriminate NR1/NR3A and NR1/NR3B receptors *in vivo*.

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Glycine and glycine receptor signalling in non-neuronal cells

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Glycine is an inhibitory neurotransmitter acting mainly in the caudal part of the central nervous system. Besides this neurotransmitter function, glycine has cytoprotective and modulatory effects in different non-neuronal cell types. Modulatory effects were mainly described in immune cells, endothelial cells and macroglial cells, where glycine modulates proliferation, differentiation, migration and cytokine production. Activation of glycine receptors (GlyRs) causes membrane potential changes that in turn modulate calcium flux and downstream effects in these cells. Cytoprotective effects were mainly described in renal cells, hepatocytes and endothelial cells, where glycine protects cells from ischemic cell death. In these cell types, glycine has been suggested to stabilize porous defects that develop in the plasma membranes of ischemic cells, leading to leakage of macromolecules and subsequent cell death. Although there is some evidence linking these effects to the activation of GlyRs, they seem to operate in an entirely different mode from classical neuronal subtypes.

Keywords: glycine receptor, cytoprotection, glia, immune cells, renal cells, hepatocytes, endothelial cells

INTRODUCTION

Glycine is one of the main components that mediate fast inhibitory neurotransmission in the central nervous system (CNS). Whilst gamma-amino butyric acid (GABA), interacting with $GABA_A$ receptors ($GABA_A$Rs) acts on the rostral part, glycine-mediated transmission operates in the caudal part of the CNS (brain stem and spinal cord). Activation of glycine-gated ion channels (GlyRs) leads to postsynaptic hyperpolarization induced by chloride influx, or a shunt effect characterized by a drop in membrane resistance consecutive to enhanced chloride conductance. GlyRs are pentameric proteins belonging to the cys-loop family of ligand-gated ion channels. GlyRs are able to form homomeric receptors, composed of α subunits, or heteromeric receptors, composed of α and of β-subunits and in a putative 3β/2α or 2β/3α stoichiometry (Grudzinska et al., 2005). Four different subtypes of α (α1–α4) and one β subunit, encoded by separate genes, have been described to date in most species. GlyRs are typically considered to consist of α1β-heteromers in the mature spinal cord and brainstem, whilst in the foetal/neonatal nervous system homomeric α2 subunit GlyRs are suggested to predominate (for reviews see Betz and Laube, 2006; Kirsch, 2006; Legendre, 2001; Lynch, 2004). However, the GlyR α3 subunit has recently emerged as a key players in inflammatory pain pathways in the spinal cord dorsal horn (Harvey et al., 2004), and it is clear that we do not yet know the full extent of locations and cell types expressing GlyRs. Increasing evidence suggests that inhibitory neurotransmitters, including glycine, exert a more general signalling role than the pure transmission of neuronal inhibition, both within and outside the nervous system. In this respect, it is important to note that GABA and glycine play a key role in developmental processes in neural stem cells and progenitors (reviewed in Nguyen et al., 2001). In this review we focus on non-synaptic roles of glycine and GlyR-mediated signalling in non-neuronal cell types and organs, describing the potential mechanisms of action and biological roles of GlyRs in these cell types.

NEUROGLIAL CELLS

Ontogenetically, neuroglial cells in the CNS can be divided into ectodermal-derived macroglial cells (oligodendrocytes and astrocytes) and mesodermal-derived microglial cells. *Macroglial cells* have the same embryonic origin as neurons. The first reports of neurotransmitter receptors in oligodendrocytes and astrocytes suggested that no functional GlyRs were present on these cell types (Gilbert et al., 1984; Kettenmann et al., 1984a,b). However, ionotropic $GABA_A$ receptors were detected, which mediate depolarizing currents upon ligand binding. This depolarization is explained by GABA-induced chloride efflux, due to the expression of the Na^+–K^+–Cl^- co-transporter (NKCC1) leading to a high intracellular chloride concentration and, hence, to a less negative Nernst potential for chloride (Hoppe and Kettenmann, 1989; Kettenmann et al., 1987). Around ten years later, molecular and functional studies demonstrated that macroglial cells harbour functional α1β GlyRs when studied *ex vivo* in spinal cord slices (Kirchhoff et al., 1996; Pastor et al., 1995). The apparent contradiction with previous *in vitro* studies may be related to cell culture conditions, which might lead to GlyR down regulation. In this context, excess glycine in certain types of cell culture medium has previously been linked to cell death in heterologous GlyR expression experiments (Groot-Kormelink et al., 2002). Nguyen and Belachew further demonstrated, in their oligodendrocyte and neurosphere models, that both GlyRs and glycine transporters (GlyTs) were molecularly and functionally present at different stages of macroglial development (Belachew et al., 1998a,b, 2000; Nguyen et al., 2002). In oligodendrocyte progenitors, glycine was found to depolarize the cell membrane, via the activation of both GlyRs and GlyTs. This depolarization led to the

activation of voltage-gated calcium channels (VGCCs) and, hence, calcium influx. This calcium influx may be one of the crucial signals in the development of oligodendroglial cells. GlyT1 and GlyRs containing the α1 and β subunits were also described in retinal Müller cells. In these cells, glycine was shown to have a depolarizing effect, suggesting an important signalling role in potassium siphoning, or in the regulation of synaptic glycine concentrations (Du et al., 2002; Lee et al., 2005). Our group also found molecular evidence for GlyR expression in different oligodendroglial cell lines (MO3.13, OLN-93, HOG), although the receptors appear to show a cytoplasmic location, which might explain why we could not detect any GlyR-mediated ionic currents (Sahebali et al., 2007). In this respect, Nguyen et al. also found a discrepancy between the number of cells that express GlyRs (80%) and those that exhibit glycine-induced currents (33%; Nguyen et al., 2002). A cytoplasmic location for GlyRs has previously been described in spinal cord neurons (Bechade et al., 1996) and although little is known about GlyR trafficking, it was shown that GlyRs can be ubiquitinated, causing receptor internalization and proteolysis (Buttner et al., 2001). Also chronically blocking GlyR activity with strychnine (1–10 μM) was demonstrated to cause receptor internalization (Levi et al., 1998). Since glycine-gated currents are detected in tissue slices while they are absent in individual cell cultures, one could speculate that GlyRs are present in macroglial cells, but that their plasma membrane location and, hence, their functionality is dependent upon cell culture conditions. It seems indeed likely that some unknown factor(s) in the normal physiological environment of slices might be necessary for plasma membrane expression of GlyRs. Further research is however necessary to identify this (these) factor(s). Downstream events of such factor(s) could at least imply protein kinase activity. In that respect, it has been shown that, in the retina and in auditory nuclei, protein kinases modulate strychnine binding and, hence, GlyR expression (Salceda and Aguirre-Ramirez, 2005; Yan et al., 2007). **Figure 1** summarizes the current findings on glycine and GlyR signalling in macroglial cells. Glycine transporters, astrocytic GlyT1 as well as presynaptic neuronal GlyT2 are of utmost importance for correct glycine recycling both at glutamatergic and at glycinergic synapses, but fall beyond the scope of this review (see Aragon and Lopez-Corcuera, 2003).

Microglial cells, although of mesodermal origin, are considered here because of their intimate spatial relationships to other glial cells and neurons. Over ten years ago, key studies showed that astrocyte-derived glycine and L-serine (between 10 and 100 μM) modulate microglial activity. Micromolar concentrations of glycine induced morphological changes in microglial cells and enhanced lipopolysaccharide (LPS)-induced secretion of nitrogen oxide (NO), superoxide, acid phosphatase as well as metabolic activity. These studies, however, did not focus on the mechanism of this

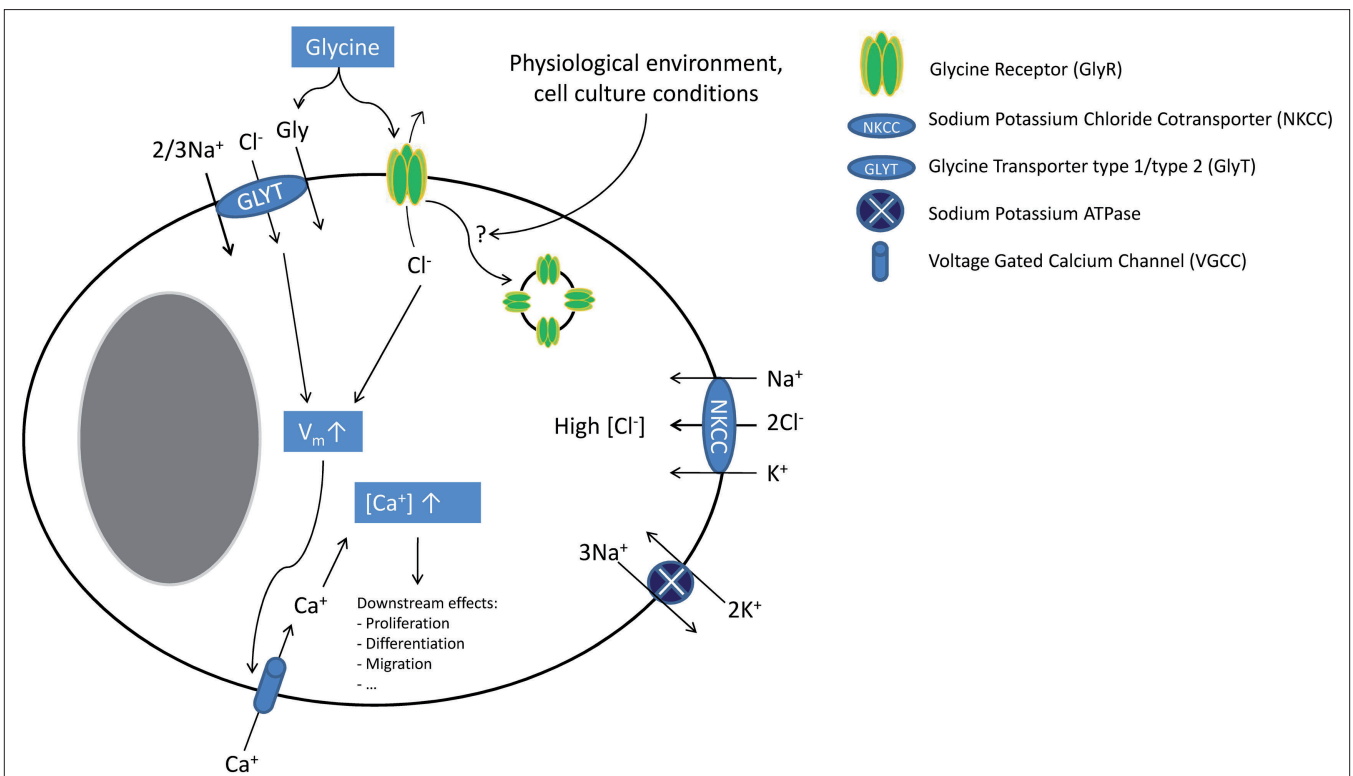

FIGURE 1 | Glycine signalling in macroglial cells. In macroglial cells, NKCC activity leads to intracellular chloride accumulation. Upon ligand binding, GlyR activation causes chloride efflux leading to cellular depolarization (which is further enhanced by electrogenic GlyT activity). This depolarization then activates VGCC. The resulting calcium influx increases the intracellular calcium concentration inducing several downstream effects (e.g. cell proliferation, migration and differentiation). Endocytosis of GlyRs might inactivate these receptors, explaining discrepancies between molecular and functional findings. Although cell culture conditions appear to have an influence on GlyR functionality and possibly endocytosis, the molecular mechanisms regulating of GlyR endocytosis are unknown.

modulatory activity (Tanaka et al., 1998; Yang et al., 1998). Since glycine effects could be mimicked by L-serine, a metabolic precursor of glycine, and since microglial cells were shown to have a low expression of 3-phosphoglycerate dehydrogenase, a key enzyme in L-serine and, hence, glycine biosynthesis, it was suggested that glycine and L-serine could be essential amino acids for microglial cells (Sugishita et al., 2001). This would have implied that the above-mentioned effects could be explained by a metabolic mechanism. This is however contradicted by the high glycine and L-serine synthesis capacities of microglial cells, which seem able to produce glycine and L-serine at concentrations of 88 and 144 μM, respectively (Hayashi et al., 2006). Another study by Schilling and Eder demonstrated that glycine can depolarize BV-2 microglial cells. Glycine-induced currents were insensitive to strychnine (1 μM) or GlyT-antagonists, were chloride-independent and could not be mimicked by the GlyR agonist taurine. Based upon the sodium dependency of glycine currents, their blockade by MeAIB (α-(methylamino)isobutyric acid), the agonist effect of glutamine and their low amplitude, the authors concluded that system A neutral amino acid transporters (SNATs) were responsible for these currents. Moreover, they suggested that these currents, although small, could have a signalling role via membrane depolarization (Schilling and Eder, 2004). Indeed, glycine-induced depolarization was substantial (up to +30 mV) despite the low amplitude of the currents (around 10 pA), and could be explained by the high membrane resistance of microglial cells (Newell and Schlichter, 2005). Although these data suggest that functional GlyRs are not present on microglial cells, our group recently provided molecular evidence for α and β GlyR subunit and gephyrin expression in these cells (J. Van den Eynden, unpublished results). However, the role of these GlyRs remains elusive. Indeed, we also confirmed that the above-mentioned findings in microglial cells were GlyR-independent (Van den Eynden et al., 2008). By analogy with macroglial cells, the discrepancy between molecular and functional data might be explained by cell culture conditions and/or GlyR down-regulation. Future research on brain or spinal cord slices and GlyR trafficking will be necessary to examine this hypothesis.

CONCLUSION

Astrocytes, oligodendrocytes and microglial cells are intimate partners of neurons in the neuronal microenvironment. GlyRs (and GlyTs) have been described on different developmental stages of macroglial cells where they may have a developmental role in the immature CNS, as well as a communication function in the mature CNS. Glycine signalling in microglial cells may be mediated by non-specific amino acid transporters. However, the biological and functional role(s) of microglial GlyRs remain unknown.

IMMUNE CELLS

In recent years, it has become obvious that glycine has immunomodulatory effects on different immune cells. As a common feature, inhibition of immune function was found in most *in vitro* studies, explaining the beneficial effects of glycine that were found in animal studies. For reviews on glycine immunomodulatory effects, the reader can also consult (Wheeler et al., 1999; Zhong et al., 2003). Glycine effects on immune cells have been mainly studied in *macrophages,* in particular using Kupffer cells, the resident macrophages of the liver. When rat were fed with a diet containing 5% glycine for 3 days and then subjected to intravenous LPS injection (an animal model of endotoxic shock), a significant decrease in mortality, liver necrosis, lung damage and serum tumour necrosis factor α (TNFα) levels were found (Ikejima et al., 1996). These beneficial effects were mainly attributed to the inhibitory effects of glycine on Kupffer cells. It was also shown that glycine (0.1–1 mM) blunted LPS-induced calcium fluxes in Kupffer cells, an effect that could be reversed by low concentrations of strychnine (1 μM) and that was not present when a chloride-free buffer was used. Furthermore, glycine was also able to induce uptake of $^{36}Cl^-$ by Kupffer cells (Froh et al., 2002; Ikejima et al., 1996, 1997), an effect that could be mimicked by taurine and β-alanine, two GlyR agonists (Seabra et al., 1998). These findings clearly suggest a role for GlyRs in modulating Kupffer cell functions. Since fluorimetric measurements showed that LPS depolarized Kupffer cells while glycine hyperpolarized them, it was suggested that GlyR-dependent hyperpolarization leads to an inhibition of functional VGCC, which were also found in Kupffer cells (Hijioka et al., 1992). More recently, glycine was shown to inhibit Toll-like receptor 4 upregulation after LPS exposure in Kupffer cells, showing that other signalling pathways may operate in these cells (Xu et al., 2008). Finally, GlyR α1, α4 and β subunit transcripts and proteins were detected in Kupffer cells (Froh et al., 2002). All the above-described glycine effects on Kupffer cells may explain why glycine is beneficial when co-administered in D-galactosamine and ethanol-induced hepatotoxicity models (Bruck et al., 2003; Stachlewitz et al., 1999; Yamashina et al., 2005). It was indeed shown that ethanol increases gut permeability and hence endotoxine uptake, leading to Kupffer cell activation and secretion of cytokines (e.g. TNFα), responsible for liver toxicity (Wheeler, 2003). In alveolar macrophages, glycine was also found to blunt calcium fluxes, to increase $^{36}Cl^-$ uptake and to decrease superoxide and TNFα secretion. The sensitivity of alveolar macrophages to glycine was higher than for Kupffer cells (IC_{50} ≈10 μM, versus ≈300 μM in Kupffer cells). One suggestion for this finding was that, due to the lower alveolar extracellular glycine concentration, there might be less GlyR down-regulation and, hence, a higher GlyR density on alveolar macrophages (Wheeler and Thurman, 1999). This hypothesis was confirmed by animal studies where rats were fed on a glycine-rich diet for 4 weeks. After this period, Kupffer cells had lost their glycine responsiveness, while alveolar macrophages had not, further suggesting GlyR down-regulation after chronic exposure to glycine (Wheeler et al., 2000b). In splenic macrophages similar effects on calcium fluxes and chloride-uptake were observed (Li et al., 2001). Finally, molecular evidence for GlyR α2, α4 and β subunits was found in alveolar and splenic macrophages (Froh et al., 2002). Next to macrophages, also studies concerning glycine effects on other immune cells were conducted. In *T lymphocytes,* there is conflicting evidence regarding the modulatory role of glycine. Glycine was suggested to have anti-apoptotic effects on MOLT4 cells, a human T lymphoblastic leukaemia cell line, at millimolar concentrations (Franek et al., 2002). Stachlewitz found that glycine dose-dependently inhibited proliferation of rat lymphocytes between 0.1 and 1 mM and blunted concanavalin A induced calcium fluxes. This latter effect could be blocked by strychnine (1 μM) or by replacing chloride by gluconate, suggesting the involvement of GlyRs (Stachlewitz et al., 2000).

The authors hypothesized that GlyR activation hyperpolarized the cells, leading to inhibition of VGCCs. However, this is in contradiction with the high chloride concentration (30–60 mM) reported in lymphocytes, which, together with the reported membrane potential of −50 to −70 mV, should lead to depolarization upon GlyR activation (Pilas and Durack, 1997; Tian et al., 1999). The putative implication of VGCCs in lymphocytes is also controversial since calcium-release-activated calcium channels (CRACs) seem to be far more important for lymphocyte activation (for review see Lewis, 2001). Alternatively, we suggest that these inhibiting effects on calcium fluxes could arise from membrane depolarization induced by a GlyR-mediated chloride efflux. This depolarization would cause both a decrease in the calcium electrical gradient and a decrease in CRAC channel conductance, leading to lower CRAC-mediated calcium influx. This mechanism was already suggested to explain $GABA_A$R-mediated inhibitory effects on calcium fluxes in T lymphocytes (Alam et al., 2006; Tian et al., 1999). The inhibitory effects of glycine on rat lymphocyte proliferation (Stachlewitz et al., 2000) are in contradiction with other studies suggesting that (i) glycine does not influence mitogen-induced proliferation of human peripheral blood mononuclear cells (PBMCs) (Sommer et al., 1994) and (ii) that glycine does not modulate *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)- or LPS-induced calcium fluxes in human PBMCs (Alam et al., 2006). A feasible explanation for these apparently conflicting results may be interspecies differences in GlyR expression. In this respect, it is interesting to note that most immune cells studied so far, including T-lymphocytes (J. Van den Eynden, unpublished results) appear to express the GlyR α4 subunit (Alam et al., 2006; Froh et al., 2002). In human, *GLRA4* is a pseudogene due to the presence of a premature stop codon in exon 9, which could explain the absence of functional GlyRs in human studies (Simon et al., 2004). Finally, *neutrophils* also seem to express functional GlyRs. In these cells, glycine inhibits LPS- and fMLP-induced calcium fluxes, increases $^{36}Cl^-$ uptake and decreases superoxide production, all effects that were blocked by 1 μM strychnine (Wheeler et al., 2000a). Rat neutrophils also appear to express GlyR α2, α4 and β subunits (Froh et al., 2002). In human *monocytes*, glycine was found to inhibit LPS-induced TNFα and interleukin (IL)-1 secretion and to enhance IL10 secretion, effects that were strychnine sensitive (1 μM; Spittler et al., 1999). Using flow cytometry, we have also found evidence for GlyR α2 subunit expression in human monocytes and in *natural killer cells*, a cell population where glycine effects were not studied to date (R. J. Harvey and N. J. Horwood, **Figure 2**).

CONCLUSION

Effects of glycine were demonstrated on most immune cells. On monocytes, macrophages and neutrophils, glycine inhibits calcium fluxes leading to a decreased secretion of cytokines (e.g. TNFα). These effects are likely to be mediated by functional GlyRs, although there may be important species differences in subunit composition. Glycine also seems to exert immunomodulatory effects in T lymphocytes, although, in this case, the results are less clear cut.

RENAL CELLS

In the kidney, several *in vitro* studies point to cytoprotective effects of glycine against ischemia. The first evidence of such a protective role of glycine came from a study showing that glycine, produced by the metabolism of glutathione (rather than glutathione itself, as previously thought, Paller, 1986), protects isolated proximal tubules against ischemia in a concentration range of 0.25–2 mM (Weinberg et al., 1987). Since hypoxic conditions lead to a decrease of β-oxidation and, hence, to an increase in acyl-coenzyme A which, in turn, may lead to detergent-like membrane damage, it was initially suggested that glycine effects could be explained by acylglycine formation and, hence, metabolic detoxification. However, it was subsequently shown that this metabolic mechanism plays no major role in glycine-induced cytoprotection (Weinberg et al., 1991a). It

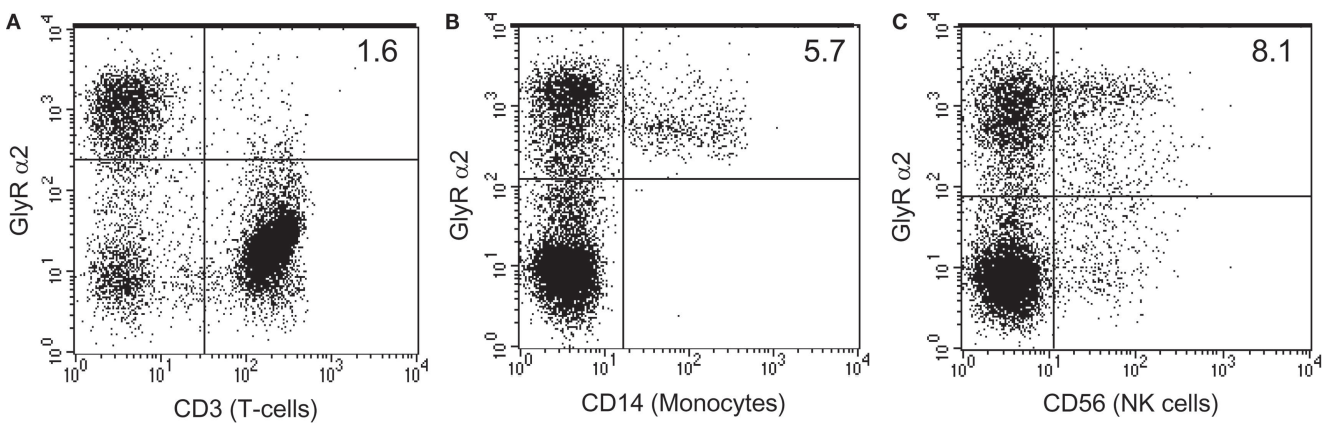

FIGURE 2 | Human monocytes and NK cells express GlyR α2 subunits. Human peripheral blood mononuclear cell subsets were evaluated for the expression of GlyR α2 using a rabbit anti human GlyR α2 antibody (FITC) and either mouse anti-human CD3 (APC), mouse anti-human CD14 (PE) or mouse anti-human CD56 (PE) (BD Pharmingen). Cells were stained according to the manufacturers protocols. GlyR α2 positive cells make up 30% of the total PBMC population. The T cell population (CD3+) accounts for more than 60% of the PBMC population and was mostly negative for GlyR α2 expression **(A)**, whilst all of the monocyte population (CD14+) was positive for GlyR α2 **(B)**. More than half of the natural killer (NK) population (CD56+) was positive for GlyR α2 **(C)**. However, since the T cell population was negative, it is likely that the NKT cells are also negative. Cells that were positive for both GlyR α2 and for the lineage specific cell surface marker appear in the top right hand side quadrant.

was shown that this protection could be mimicked by structurally related amino acids such as L-alanine (Weinberg et al., 1990b), that it was independent of ATP (Weinberg et al., 1997) or calcium (Venkatachalam et al., 1995; Weinberg et al., 1991b) and that it does not protect either against swelling of ischemic cells (Weinberg et al., 1990a) or against plasma membrane breakdown (Venkatachalam et al., 1995). In comparable experimental paradigms, strychnine, in high concentrations (1 mM), was shown to act as an agonist, rather than an antagonist (Aleo and Schnellmann, 1992; Dong et al., 2001). Other GlyR antagonists and chloride channel blockers (e.g. brucine, bicuculline, avermectin B1a, and cyanotriphenylborate) were also demonstrated to exert glycine-like activity (Miller and Schnellmann, 1994; Venkatachalam et al., 1996). Dong and colleagues investigated whether this strychnine protection was due to an (aspecific) intracellular interaction or to an interaction at the cellular membrane, as would be expected for ligand-gated channel-mediated actions. They showed that membrane-impermeable strychnine derivatives maintained their cytoprotective properties in MDCK (Madin–Darby canine kidney) cells, a distal tubular cell line. Moreover, they showed that small modifications to the molecule near the interacting centre for receptor binding abolished the effect (Dong et al., 2001). These results clearly prove specific strychnine interactions with surface-oriented extracellular domains. Although the effects of these compounds are typically receptor dependent, in renal cells these effects do not seem to depend on chloride ion gradients, since replacement of chloride by gluconate could not abolish the effects. In an attempt to explain this apparent contradiction, it has been suggested that under ischemic conditions, dysregulation of GlyRs leads to the development of a pathological pore, allowing the penetration of macromolecules and, hence, cell death (Venkatachalam et al., 1996). This pore formation can be prevented by adding agonists as well as antagonists, which somehow stabilize the GlyR. This hypothesis was supported by Dong and co-authors, who showed that, in ischemic conditions, there is a size-dependent membrane penetration of macromolecular dextrans, with a progressive increase in permeability for larger dextran molecules during the period of ischemia. This permeability defect could be prevented by a cross-linking agent (3,3′ dithiobis(sulfosuccinimidylpropionate)) or by adding millimolar glycine (Dong et al., 1998). Direct evidence for GlyR involvement was recently provided by a study that showed that inhibition of endogenous GlyR expression by RNA interference attenuates cytoprotection in MDCK cells (Pan et al., 2005). They also found that HEK (human embryonic kidney) cells were not protected from ischemic cell death by glycine, unless they were transfected with plasmid constructs expressing the GlyR α1 subunit. GlyR α1 mutants of Y202F or Y202L, which are shown to decrease glycine and strychnine binding, also specifically abolished the effects of glycine and strychnine, respectively, further suggesting the involvement of glycine receptor subunits in cytoprotection At the molecular level, most studies have only found evidence for GlyR β subunit and gephyrin expression at the basolateral membrane of proximal tubule cells (Grenningloh et al., 1987; Miller and Schnellmann, 1994; Sarang et al., 1999). There is also *in vivo* evidence for a protective role of glycine against ischemic conditions in the kidney. For example, when rats were fed with a diet containing 5% glycine, their renal function was improved [higher glomerular filtration rate (GFR) and lower plasma creatinine] after renal ischemia (Yin et al., 2002). These effects might also be relevant under normal physiological conditions since when normal rats are infused with glycine, a decrease in renal vascular resistance is observed, leading to an increase in renal plasma flow and, hence, in GFR. In addition, glycine also decreases proximal tubular reabsorption (Thomsen et al., 2002).

CONCLUSION

Numerous *in vitro* and *in vivo* studies provide evidence for a cytoprotective role of glycine against kidney ischemia. This effect seems to be mediated by GlyRs with unusual properties: (i) there is little evidence that GlyR α subunits are expressed in kidney cell types, (ii) glycine effects can be mimicked by both GlyR agonists and antagonists and (iii) these effects do not depend upon the chloride gradient. Currently, porous defects are thought to develop in plasma membranes of ischemic cells as a consequence of a molecular perturbation of GlyR channels, that lead to leakage of macromolecules and, hence, to cell death. Glycine and GlyR antagonists might stabilize this unusual GlyR of unknown subunit composition accounting for cytoprotective effects.

HEPATOCYTES

In the liver, ischemia-reperfusion injury is a serious problem after liver transplantation that often leads to primary graft dysfunction. Thus, glycine is a potential cytoprotective agent of particular interest for the liver. The specific role of glycine in hepatic ischemia-reperfusion injury was recently reviewed (Habib et al., 2006). A few years after the first report of a cytoprotective role of glycine in renal ischemia, Marsh and colleagues showed that isolated hepatocytes exposed to cold ischemia in the presence of glycine (3 mM) experienced a significant improvement of cellular injury compared to controls (Marsh et al., 1991). This effect was shown to be independent of glutathione synthesis (Marsh et al., 1991; Ozaki et al., 1994). Comparable effects were observed with high concentrations of alanine (10 mM) and strychnine (1 mM) as well as under conditions of metabolic inhibition (chemical hypoxia; Dickson et al., 1992; Marsh et al., 1993; Nagatomi et al., 1997; Sakaida et al., 1996). Also for hepatocytes, the strychnine effects were shown to be specific (Dong et al., 2001). Glycine hepatoprotective effects were independent of ATP levels or intracellular acidosis, and glycine could not protect against injury caused by calcium ionophores or by oxidative stress (Marsh et al., 1993; Sakaida et al., 1996). It was also shown that glycine could inhibit calcium-dependent non-lysosomal protease activity during ischemia (Ferguson et al., 1993; Nichols et al., 1994). High concentrations of glycine (2–10 mM), but also L-alanine (10 mM) or strychnine (1 mM), were shown to be protective and to non-specifically block influx of different ions (e.g. sodium, cobalt or nickel; Carini et al., 1997; Frank et al., 2000). Although it was shown that blocking sodium influx by itself was cytoprotective and was dependent upon chloride influx, glycine was still protective in a sodium- or chloride-free buffer. Glycine was hypothesized to block non-specific 'leak' channels, which might increase in number and permeability with time (Frank et al., 2000). Glycine protects hepatocytes form ischemic cell death, but it does not prevent the mitochondrial permeability transition (MPT), contrary to cellular acidosis or sodium-free culture conditions, both preventing the MPT (Qian et al., 1997). In a review on the role of the MPT in ischemic cell death

of hepatocytes, it was argued that glycine acted downstream of the MPT, at a time point where cells are completely deprived of ATP and destined for necrotic cell death (Kim et al., 2003). Glycine was also shown to protect against hepatotoxicity induced by different agents (e.g. valproate, paracetamol, cadmium chloride or copper chloride), an effect that can only partly be explained by metabolic detoxification of acyl-coenzyme A by means of acylglycine formation (Deters et al., 1998; Vance et al., 1994). To date, only one study has described clear GlyR-related modulatory effects in hepatocytes. Qu and colleagues demonstrated that glycine blocks prostaglandin E_2- and epinephrine-induced calcium fluxes in hepatocytes, an effect that is chloride-dependent and antagonized by low concentrations of strychnine (10 μM). However, no direct link between calcium modulation and cytoprotective effects could be made (Qu et al., 2002). Molecular evidence for GlyRs in hepatocytes is scarce. A GlyR β subunit splice variant was described in liver homogenates (Oertel et al., 2007), but its cellular origin is unclear (hepatocytes, Kupffer cells or endothelial cells). To date, no GlyR α subunit gene expression has been described (Froh et al., 2002; Grenningloh et al., 1987). *In vivo* evidence also supports a protective role of glycine against liver ischemia and other pathologies. Several animal studies have shown that glycine has beneficial effects during liver transplantation. When given intravenously to donors or when given to acceptors (den Butter et al., 1993; Ito et al., 2008; Schemmer et al., 1999), glycine improved survival and decreased liver enzyme concentrations. It is however noteworthy that besides a direct cytoprotective effect on hepatocytes, there is also an important immunomodulatory contribution via the suppression of Kupffer cell activity *in vivo*. Indeed, a positive correlation was found between the protective effects of glycine and the decrease in Kupffer cell-derived TNFα and proteases (Duenschede et al., 2006; Schemmer et al., 1999; Yamanouchi et al., 2007). Further evidence for Kupffer cell involvement in beneficial glycine effects came from an animal study that showed that glycine, when given to donors, decreases levels of TNFα and liver enzymes, an effect that can be blocked by adding low levels of strychnine (5 μM; Zhang et al., 2005). As strychnine antagonism is only established for immunomodulatory effects on Kupffer cells, but not for cytoprotective effects on hepatocytes, these findings strongly support a crucial role for Kupffer cells. As mentioned earlier, beneficial effects of glycine in alcoholic liver disease were mainly due to Kupffer cells as well. Also in animal models of endotoxine and hemorrhagic shock, glycine modulation of Kupffer cells and inhibition of TNFα-secretion seem to be crucial for its beneficial effects (Mauriz et al., 2001; Neyrinck et al., 2005; Wang et al., 2004; Yang et al., 2001; Zhong et al., 1999). On the other hand, it was shown that destruction of Kupffer cells by gadolinium chloride (GdCl) was far less potent than glycine in animal liver transplantation studies, suggesting that not all *in vivo* findings can be explained by modulation of Kupffer cells (Rentsch et al., 2002, 2005). Also, in animal models of cholestasis, where bile cannot flow from the liver to the duodenum it was shown that beneficial glycine effects (lowering of TNFα levels, liver necrosis and liver enzymes) remained when Kupffer cells were destructed by GdCl (Fang et al., 2003; Froh et al., 2008). Finally, some of the cytoprotective effects of glycine could also be attributed to glycine interacting with (sinusoidal) endothelial cells (see "Endothelial cells"). The above-mentioned cytoprotective effects of glycine against liver ischemia were considered solid enough to trigger clinical trials. Preliminary studies with few patients were undertaken showing, in one first study, that 2 mM glycine added to the perfusion solution during transplantation induced a significant decrease in liver enzymes and evoked less complications (Arora et al., 1999). A second study with seven patients confirmed that a one-week long daily infusion of glycine post-operatively (corresponding to ~2 mM plasma concentration) significantly decreased plasma liver enzyme levels (Schemmer et al., 2001, 2002). These preliminary results led to a multicenter, randomized, placebo-controlled double blind clinical trial that is currently under investigation (HEGPOL, "Hepatoprotective Effects of Glycine in the Postoperative Phase"; Luntz et al., 2005).

CONCLUSION

In the liver, glycine protects against ischemic injury what may have clinical applications to reduce complications in liver transplantation with preliminary clinical trials showing promising results. However, the molecular mechanism of the glycine effect remains elusive and nothing is known about the precise role or composition of hepatic GlyRs. As for the kidney, one hypothesis is that glycine could block non-specific pore formation, preventing plasma membrane leakage and, eventually, cell death. Besides direct cytoprotective effects on hepatocytes, glycine modulatory effects on Kupffer and endothelial cells have to be taken into account especially in *in vivo* studies.

ENDOTHELIAL CELLS

By analogy to the situation in the liver and in the kidney, glycine was also suggested to have cytoprotective effects in endothelial cells subjected to hypoxia or challenged with ionomycine, cyanide, hydrogen peroxide or maitotoxin (Estacion et al., 2003; Nishimura and Lemasters, 2001; Nishimura et al., 1998; Weinberg et al., 1992). These effects were independent of pH, ATP levels or calcium and could be mimicked by L-alanine. Estacion and co-workers found that glycine protective effects on cell death were taking place at the late phase of maitotoxin cytotoxicity and could prevent pore-formation in the cytoplasmic membrane, which would normally lead to cell death. Nishimura and co-workers also hypothesized that glycine could block the opening of a "death channel" during hypoxia. They found that sinusoidal endothelial cells were going through different stages during ischemia. During early hypoxia, a cation influx appears as a consequence of a decrease in Na^+/K^+ ATPase activity. However, this is not accompanied by an anion influx and causes only moderate cell swelling. In a next stage, called the metastable state (characterized by the MPT), anions start flowing inside cells through a "death channel" leading to an increase in colloid osmotic pressure, to bleb formation and, eventually, to the rupture of plasma membranes and to cell death. Based on the finding that glycine slows anionic calcein entrance, decreases bleb formation and prevents entrance of propidium iodide and macromolecules into cells, it was suggested that glycine could somehow inhibit this "death channel" (Nishimura and Lemasters, 2001). Besides protecting against ischemic necrosis, glycine also seems to prevent apoptosis of sinusoidal endothelial cells induced by vascular endothelial growth factor (VEGF) deprivation. This protective effect is blocked by strychnine (1 μM) and is possibly mediated by enhanced bcl-2 expression (Zhang et al., 2000). In this context, VEGF-induced cellular calcium influxes can be completely

prevented by addition of 1 mM of glycine in CPA cells, a bovine endothelial cell line. This effect is clearly GlyR-dependent since it can be blocked by micromolar concentrations of strychnine and is eliminated in chloride-free buffer conditions. Furthermore, GlyR β subunit expression was found at the mRNA and protein level and glycine could decrease the proliferation and the migration of endothelial cells (Yamashina et al., 2001). This inhibiting effect of glycine on endothelial cells may explain why glycine was found to be tumour suppressive in melanoma and liver tumours. In this respect, it is of interest to mention that glycine inhibited tumour growth in the advanced stages only, which is when neo-angiogenesis has an important role in tumour progression because of oxygen need (Rose et al., 1999a,b). Later, it was demonstrated that glycine could indeed inhibit angiogenesis, leading to a decreased wound healing and tumour growth (Amin et al., 2003), suggesting that glycine effects on endothelial cells may be of future interest for research into atherosclerosis (McCarty et al., 2009). Glycine-mediated vascular effects were also attributed to NMDA receptors (NMDARs) in some studies. For example, Mishra and colleagues showed that glycine lowers the blood pressure in normotensive rats, while it increases it in hypertensive and L-NAME (NO synthase inhibitor)-treated rats. These effects were attributed to NMDAR activation on both endothelial and vascular smooth muscle cells, with the former being NO-dependent and prevailing under normotensive conditions and the latter prevailing under hypertensive conditions (Mishra et al., 2008). NMDAR-mediated vascular effects were also suggested to mediate glycine vasodilatory effects and, hence, to increase GFR in the kidney (Deng et al., 2002; Slomowitz et al., 2004).

CONCLUSION

In endothelial cells, some modulating effects of glycine are present that appear to be mediated by GlyRs. Potential effects of glycine on migration and proliferation may explain beneficial glycine effects on tumour growth and wound healing. As in the liver and in the kidney, some of the cytoprotective effects of glycine could be mediated by a non-specific pore formed under ischemic conditions.

OTHER CELL TYPES

In *cardiomyocytes,* a few recent studies showed beneficial glycine effects under ischemia-reperfusion conditions. For example, 3 mM glycine increases the cell viability of isolated cardiomyocytes and of isolated rat hearts after ischemia/reperfusion (Ruiz-Meana et al., 2004). Based on the findings that glycine prevented mitochondrial swelling and calcein release, it was suggested that glycine somehow inhibits the MPT. However, this contrasts with other findings in the kidney and in the liver where the general assumption is that glycine acts downstream of the MPT (Kim et al., 2003). In another study, glycine infusion into animal donor hearts was beneficial for right ventricular function after transplantation (Warnecke et al., 2006). Lastly, a recent study showed that 2 mM glycine could protect isolated rat heart function and viability in an experimental LPS-induced decreased cardiac function model. This protective effect of glycine was linked to an attenuation of LPS- and hypoxia-induced calcium influx. Interestingly, the authors again detected the presence of GlyR β subunits, but not GlyR α subunits (Qi et al., 2007). GlyRs are also well described in *sperm* in humans, pigs, mice and golden hamsters where they are involved in the zona pellucida-initiated acrosome reaction, an indispensable step in the fertilization process. Glycine induces the acrosome reaction at micromolar concentrations and this effect can be blocked by very low concentrations of strychnine (50 nM). In the *spasmodic* and *spastic* mouse models (characterized by GlyR α1 and β subunit mutations, respectively), deficiencies in the acrosome reaction are found. Molecular studies have detected GlyR α1, α3 and β subunits in the periacrosomal plasma membrane (Bray et al., 2002; Kumar and Meizel, 2008; Llanos et al., 2001, 2003; Meizel, 1997; Melendrez and Meizel, 1995, 1996; Sato et al., 2000a,b, 2002). Interestingly, GlyR α4 subunit transcripts were also found in the male genital ridge in chicken (Harvey et al., 2000). Finally, cytoprotective effects of glycine in ischemia-reperfusion injury have also been described in *other organs,* such as in the gastro-intestinal system, in skeletal muscle and the lungs (Ascher et al., 2001; Gohrbandt et al., 2006; Iijima et al., 1997; Lee et al., 2001, 2002; Mangino et al., 1996; Tariq and Al Moutaery, 1997). However, in those models, no clear cellular targets for glycine have been identified, so indirect effects via immune cells or even the nervous system have not been eliminated.

CONCLUSION

The effects of glycine, mediated by classical or unconventional GlyRs, have been described in many different non-neuronal cell types and both inside and outside the nervous system. Broadly, glycine effects can be classified into two main types: cytoprotective and modulatory, an overview of which can be found in **Table 1**.

Modulatory effects of glycine were mainly found in immune cells such as macrophages and are associated with clear molecular and pharmacological evidence of functional GlyRs. For example, glycine in macrophages induces uptake of $^{36}Cl^-$ and inhibits calcium flux in a dose-dependent manner, in a concentration range of 0.1 and 1 mM. Pharmacological findings also support the involvement of GlyRs in these effects: blockage is observed by low concentrations of strychnine (1 μM), similar effects are elicited by taurine and β-alanine, but not by L-alanine, and these effects are eliminated by replacement of chloride in buffers. At the molecular level, both GlyR α and β subunits have been described on these cells, although there may be important species differences. Besides immune cells, comparable modulatory effects were observed in glial cells, endothelial cells and spermatozoa. Although the GlyRs on these cells are clearly identical in subunit composition to selected neuronal GlyRs, it is remarkable that, to our knowledge, no direct electrophysiological evidence for functional GlyRs exists in immune cells. Although, technically, there is no problem performing patch-clamp experiments on these cells, the only non-neuronal cells where GlyR-mediated ionic currents have been directly measured are glial cells, and even in this case currents could only be recorded from spinal cord slices, as opposed to cultured cells. This suggests a complex regulation of GlyR expression in non-neuronal cells and further research is required to confirm the existence of functional GlyRs on these cell types. In this regard, an emphasis on correct cell culture conditions may represent the best way forward. *Cytoprotective effects* were mainly studied in renal cells, hepatocytes and endothelial cells, and have obvious clinical and therapeutic importance in the protection that is provided under ischemic conditions. There is some evidence linking these effects to GlyRs, although these receptors seem to function in an entirely different mode as described for the classic neuronal GlyRs.

Table 1 | Most often evoked mechanisms responsible for glycine effects in non-neuronal cells.

	Cytoprotective effect	Modulatory effect
Cells most studied	Renal cells	Immune cells
	Hepatocytes	Macroglial cells
	Endothelial cells	Endothelial cells
Main effect	Protection against ischemic necrosis	Modulation of proliferation, migration, differentiation, apoptosis,…
Active concentrations	Up to 10 mM	0.1-1 mM
Calcium flux modulation	No	Yes
Uptake of 36-chloride	No	Yes
Chloride dependency	No	Yes
Pharmacological findings	No strychnine block described	Effects blocked by low concentrations of strychnine (1 μM)
	Effects mimicked by structurally related amino acids (e.g. L-alanine, L-serine)	Effects mimicked by taurine and β-alanine
	Effects mimicked by high concentrations (1 mM) of strychnine and other chloride channel blockers	
	No mimicking by taurine	
Molecular findings	Only β-subunits	Both α- and β-subunits
Electrophysiological findings	None	Only in slice preparations of macroglial cells
Suggested mechanism	Glycine sensitive death pathway	GlyR dependent modulation of calcium signalling
	Unknown role of GlyR (subunit)s	

Glycine effects also seem to be more non-specific in the sense that the observed effects can be mimicked by other structurally related amino acids (e.g. L-alanine) and are caused by higher concentrations of glycine (up to 10 mM). Other findings inconsistent with GlyR-mediated effects are the 'agonist-like' effects of strychnine in high concentrations (1 mM) and of non-specific chloride channel blockers, as well as the chloride-independent effects. At the molecular level, mainly GlyR β subunits have been detected, which are not reported to form functional homomeric GlyRs in recombinant systems. It is however noteworthy that the GlyR β subunit distribution in the CNS is far more extended compared to GlyR α subunits, suggesting that β subunits may have some as yet unknown function (Malosio et al., 1991). The current hypothesis concerning the cytoprotective effects of glycine against ischemia is tenuous at best, since it requires the existence of a glycine-sensitive pore that is activated in later stages of ischemic cell death, leading to membrane leakage of macromolecules. Moreover, this pore is stabilized and/or blocked by glycine, GlyR antagonists and some chloride blockers. Molecularly, this pore seems to be some multimeric protein, somehow associated with GlyR subunits which can stabilize the pore upon ligand binding. More research is certainly needed to further explore this intriguing hypothesis. Finally, when considering glycine-related effects in non-neuronal cells, it is important to realize that other mechanisms do exist, some of which maybe also important for the reported modulatory and cytoprotective effects, but fall beyond the scope of this review. These include the NMDAR-mediated glycine effect in the vascular system, transporter-mediated effects in microglial cells or possible metabolic effects in microglia or the liver.

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Glycinergic transmission in the mammalian retina

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Glycine and γ-aminobutyric acid (GABA) are the major inhibitory neurotransmitters in the retina. Approximately half of the amacrine cells release glycine at their synapses with bipolar, other amacrine, and ganglion cells. Glycinergic amacrine cells are small-field amacrine cells with vertically oriented dendrites and comprise more than 10 different morphological types. The retinal distributions of glycine receptor (GlyR) α1, α2, α3 and α4 subtypes have been mapped with subunit-specific antibodies. GlyRs were clustered at postsynaptic hot spots which showed selective distributions for the different subunits. As a rule, only one α subunit was expressed at a given postsynaptic site. The kinetic properties of GlyRs were measured by recording spontaneous inhibitory postsynaptic currents (sIPSCs) from identified retinal neurons in wild-type, *Glra1*$^{spd\text{-}ot}$, *Glra2* and *Glra3* knockout mice. From observed differences of sIPSCs in wild-type and mutant mice, the cell-type specific subunit composition of GlyRs could be defined. OFF-cone bipolar cells and A-type ganglion cells receive prominent glycinergic input with fast kinetics that is mainly mediated by α1β GlyRs (decay time constant τ ~ 5 ms). By contrast, All amacrine cells express α3β GlyRs with medium fast kinetics (τ ~ 11 ms). Narrow-field (NF) and wide-field amacrine cells contain predominantly α2β GlyRs with slow kinetics (τ ~ 27 ms). Lastly, ON-starburst, narrow-field and wide-field amacrine cells in *Glra2* knockout mice express α4β GlyRs with very slow kinetics (τ ~ 70 ms).

Keywords: glycine receptors, sIPSCs, retina, synapses, *Glra1*$^{spd\text{-}ot}$ mice, *Glra2*$^{-/-}$ mice, *Glra3*$^{-/-}$ mice

INTRODUCTION

Glycine and GABA, the major inhibitory transmitters of the mammalian retina, are preferentially localized in different types of amacrine cells which fulfill specific roles in the processing of visual signals (Pourcho, 1996). GABA-ergic amacrine cells are wide-field amacrine cells providing lateral interactions across the inner plexiform layer (IPL; Lin and Masland, 2006). They are involved in the generation of receptive field surrounds (Flores-Herr et al., 2001) and in the computation of direction selective light responses (Taylor and Vaney, 2003). Glycinergic amacrine cells are small-field cells, whose dendrites are primarily involved in local interactions between different sublaminas of the IPL, such as the OFF- and ON-sublamina (Hsueh et al., 2008).

Glycine has been localized in 40–50% of all retinal amacrine cells (Marc, 1989; Pourcho, 1996; Pow and Hendrickson, 1999), which exist in more than 10 distinct morphological types (Badea and Nathans, 2004; MacNeil and Masland, 1998; Menger et al., 1998; Vaney, 1990). They receive synaptic input from bipolar cells at ribbon synapses and from other amacrine cells – both GABAergic and glycinergic – at conventional chemical synapses. Their output synapses contact bipolar cells, other amacrine cells and ganglion cells (Jusuf et al., 2005; Pourcho and Owczarzak, 1991a,b; Sassoè-Pognetto et al., 1994). Some glycinergic amacrine cells bear interplexiform processes ascending towards the outer plexiform layer (OPL; Kolb and West, 1977).

The diversity of types of glycinergic amacrine cells is paralleled by the striking heterogeneity of glycine receptors (GlyRs). All four α subunits of the GlyR have been localized to specific synapses within the mammalian retina (Haverkamp et al., 2003, 2004; Heinze et al., 2007; Sassoè-Pognetto et al., 1994). To date, selective agonists or antagonists that distinguish different isoforms of synaptic GlyRs have not been identified (Betz and Laube, 2006; Harvey and Betz, 2000; Legendre, 2001; Lynch, 2004). However, mutant mice are available that have dysfunction of specific GlyR subunits and thus it became possible to study details of the glycinergic synaptic transmission in the mammalian retina (Ivanova et al., 2006; Majumdar et al., 2007; Weiss et al., 2008).

It has to be emphasized that the morphological types of glycinergic amacrine cells, their circuits, and the distribution of GlyRs are closely similar when different mammalian retinas are compared (Masland, 2001). In this review we concentrate on the mouse retina because mutants are available that express green fluorescent protein (GFP) in specific types of amacrine cells (Haverkamp et al., 2009; Heinze et al., 2007), and also mutants which lack specific GlyR subunits.

There are more than 10 different types of bipolar cells, at least 30 types of amacrine cells and approximately 15 types of ganglion

cells in any mammalian retina (Masland, 2001). They are involved with different retinal circuits and fulfill specific roles in visual processing. In this review it will be shown that they express different sets of synaptic GlyRs.

RESULTS

LOCALIZATION OF GLYCINE IN AMACRINE CELLS

The first anatomical demonstration of glycine as neurotransmitter in the mammalian retina was through uptake of tritiated glycine followed by autoradiography (Ehinger and Falck, 1971). Amacrine cells were prominently demarcated, whilst some bipolar cells were weakly labelled. More recent demonstrations of glycine in the retina applied immunolabelling with antibodies against glycine or against the glycine transporter GlyT1 (Menger et al., 1998; Pow, 1998; Pow and Hendrickson, 2000). **Figure 1** shows a vertical section through a mouse retina that was double immunolabelled for glycine and for GlyT1 (Haverkamp and Wässle, 2000). Strong glycine immunoreactivity can be observed in amacrine cell bodies and their dendrites descending into the IPL (**Figure 1A**). Weak glycine expression is also found in putative ON-cone bipolar cells in the centre of the inner nuclear layer (INL). The section was also immunolabelled for GlyT1 (**Figure 1B**) which labels all glycinergic amacrine cells but not bipolar cells (**Figure 1C**).

Bipolar cells do not express GlyT1 but they receive glycine by diffusion through electrical synapses (gap junctions) from glycinergic amacrine cells (Vaney et al., 1998). However, there is no evidence that bipolar cells release glycine in addition to L-glutamate, the well established transmitter released by bipolar cells. In other parts of the CNS, GlyT1 has been localized to glial cells, whilst GlyT2 is now known to represent the presynaptic neuronal glycine transporter (Zafra et al., 1995). Surprisingly, GlyT2 does not appear to be expressed in the mammalian retina (Zafra et al., 1995). Thus, uptake of tritiated glycine, glycine immunolabelling and GlyT1 expression all indicate that half of the amacrine cells of the mammalian retina are glycinergic (Marc and Liu, 1985; Pow and Hendrickson, 1999; Wässle et al., 1986) and that this is the main source of releasable glycine in the retina.

MORPHOLOGICAL TYPES OF GLYCINERGIC AMACRINE CELLS

The most prominent and also most numerous glycinergic amacrine cell is the AII amacrine cell which transfers the light signal from rod bipolar cells into the cone pathway (Kolb and Famiglietti, 1974). **Figure 2A** shows the typical bistratified morphology of a neurobiotin injected AII amacrine cell from the mouse retina and **Figure 2B** shows the dense array of AII cells immunostained for parvalbumin in the rat retina. In the outer IPL, AII cell lobular dendrites provide glycinergic, chemical output synapses onto OFF-cone bipolar cell axon terminals. In the inner IPL, AII cells receive input from rod bipolar cells and are engaged via electrical synapses (gap junctions) with ON-cone bipolar cell axon terminals. Further glycinergic, small-field amacrine cells were identified in the cat retina by combined Golgi-staining and glycine uptake (Pourcho, 1980; Pourcho and Goebel, 1985). In a systematic survey, Menger et al. (1998) identified at least eight different glycinergic amacrine cells in the rat retina. More recent studies on glycinergic amacrine cells in the retina have utilised a transgenic mouse (GFP-O) which expresses green fluorescent protein (GFP) under the control of the thy-1 promoter (Feng et al., 2000; Heinze et al., 2007). Five

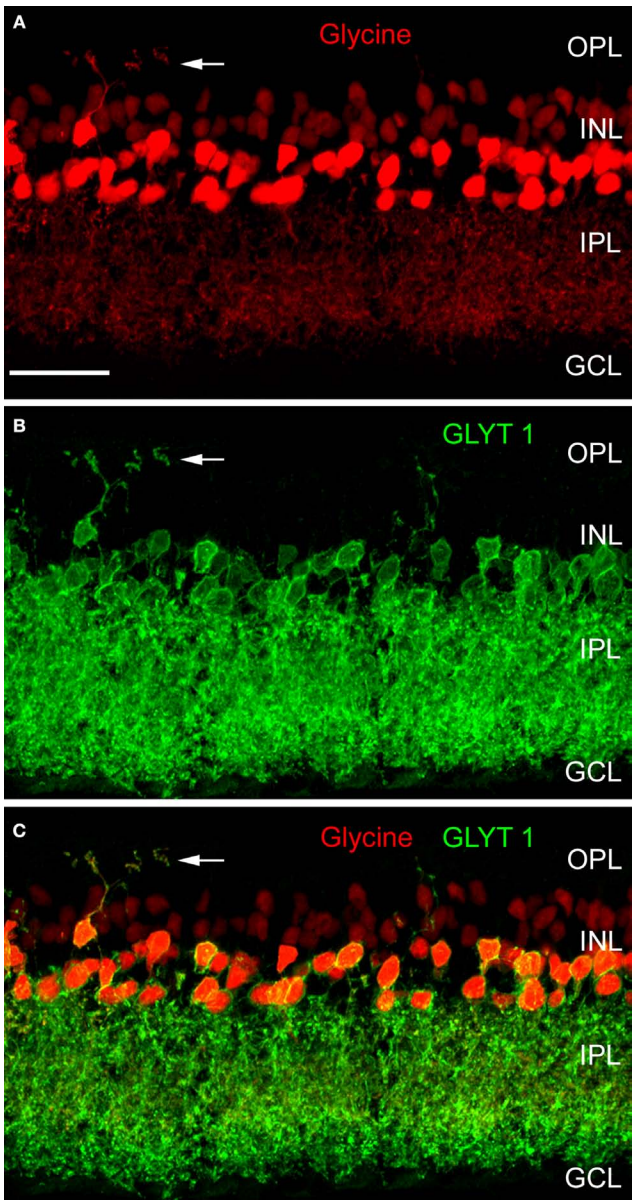

FIGURE 1 | Glycinergic amacrine cells of the mouse retina (modified from Haverkamp and Wässle, 2000). A vertical section was double immunolabelled for glycine (red) and the glycine transporter GlyT1 (green). **(A)** Strong glycine immunofluorescence is found in amacrine cells at the INL/IPL border. Weak glycine immunofluorescence is also present in the cell bodies of ON-cone bipolar cells in the center of the INL. **(B)** GlyT1 immunofluorescence is found in amacrine cell bodies and their dendrites descending into the IPL. **(C)** The superposition of **(A)** and **(B)** shows that only the amacrine cells but not the bipolar cells express GlyT1. The arrows indicate an interplexiform process ascending to the OPL (OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL; ganglion cell layer; Scale bar: 25 μm).

such cells are illustrated in **Figures 2C–G**, with double labelling for calretinin in order to reveal the different sublaminas of the IPL. Although they are all small-field amacrine cells, the level of stratification of their dendrites within the IPL is clearly different. The cells in **Figures 2C–E** have small, diffuse dendritic trees confined to the outer, middle and inner IPL, respectively. By contrast,

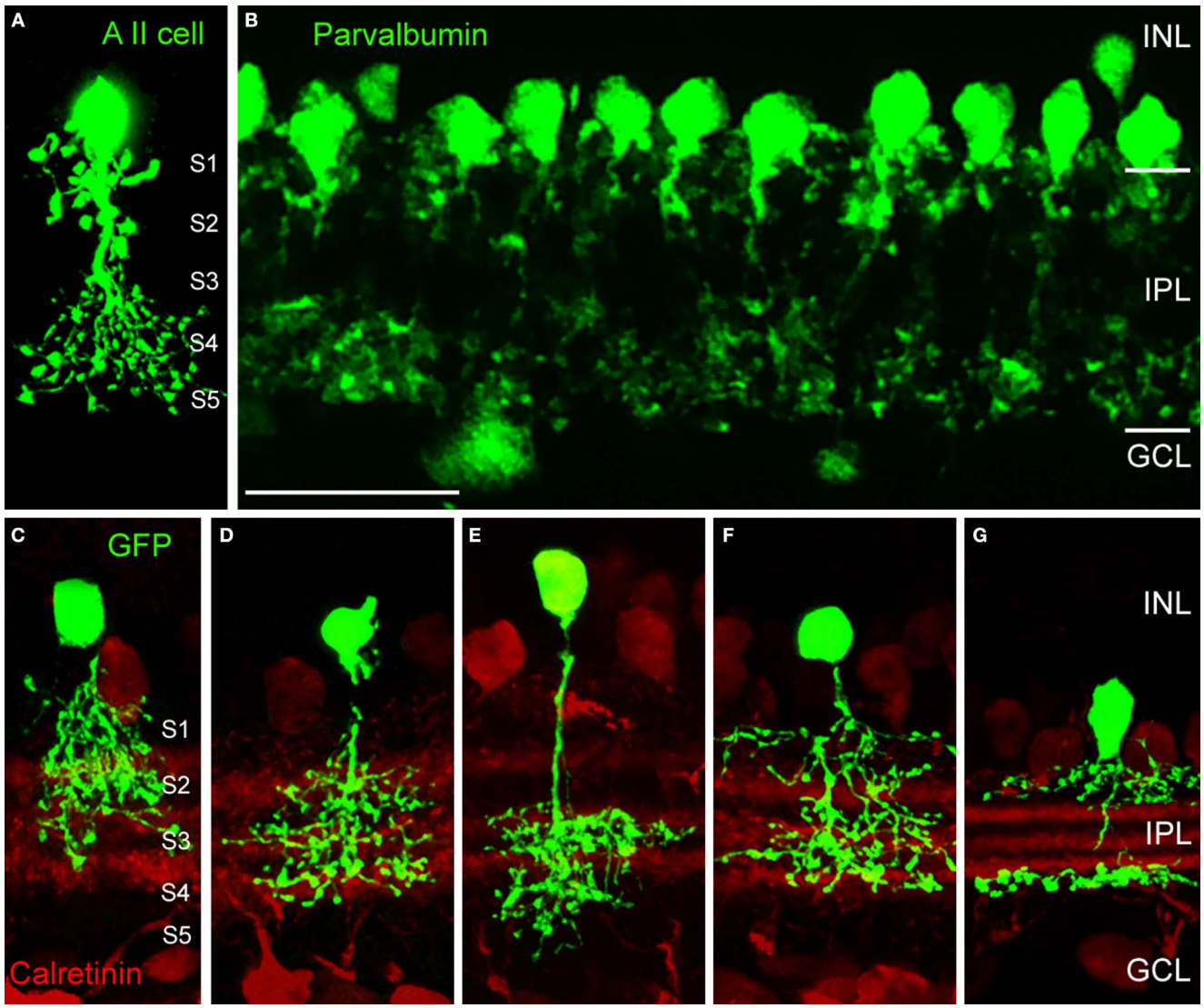

FIGURE 2 | Morphological types of glycinergic amacrine cells (modified from Heinze et al., 2007; Weiss et al., 2008). (A) Vertical section of an AII amacrine cell of the mouse retina that was filled with neurobiotin during patch-clamp recordings. The IPL is subdivided into 5 sublaminas of equal thickness. **(B)** The array of AII amacrine cells of the rat retina immunostained for parvalbumin (Scale bar: 25 μm). **(C–G)** Glycinergic amacrine cells expressing GFP (green fluorescent protein) in the thy1-GFP-O mouse (Feng et al., 2000). **(C)** Type 2 cell. **(D)** Type 3 cell. **(E)** Type 4 cell. **(F)** Type 7 cell (all according to the scheme of Menger et al., 1998). **(G)** A8 cell (according to Kolb et al., 1981).

the cells in **Figures 2F,G** have a bistratified appearance. More than 10 distinct types of glycinergic amacrine cells have been identified from such morphological criteria (MacNeil and Masland, 1998) and more may be found in future studies.

SYNAPTIC LOCALIZATION OF GLYCINE RECEPTORS

The postsynaptic glycine receptor (GlyR) is a ligand-gated chloride channel composed of ligand-binding α and β subunits. The β subunits bind to the receptor clustering protein gephyrin (reviewed by Harvey and Betz, 2000; Legendre, 2001; Lynch, 2004; Vannier and Triller, 1997). Molecular cloning has revealed four genes encoding the α subunits (α1, α2, α3, α4) and only one gene encoding the β subunit (Harvey et al., 2000). In the adult two copies of the α subunit and three copies of the β subunit form the pentameric receptor protein (Grudzinska et al., 2005). Subunit selective antibodies have recently become available that recognize the four GlyR α subtypes (Harvey et al., 2004; Haverkamp et al., 2003, 2004; Heinze et al., 2007). When these antibodies were applied to lightly fixed sections of the mammalian retina, they each produce a distinct punctate immunofluorescence pattern (**Figure 3**). Electron microscopy has suggested that these puncta represent clusters of GlyRs at postsynaptic sites (**Figure 3B**; Sassoè-Pognetto et al., 1994). The GlyR α1 subunit is expressed in a sparse population of puncta in the OPL, which represent synapses between glycinergic interplexiform processes and bipolar cell dendrites (**Figures 3A,C**). In the outer IPL (stratum S1 and S2) GlyR α1 immunoreactivity is found in large puncta, which

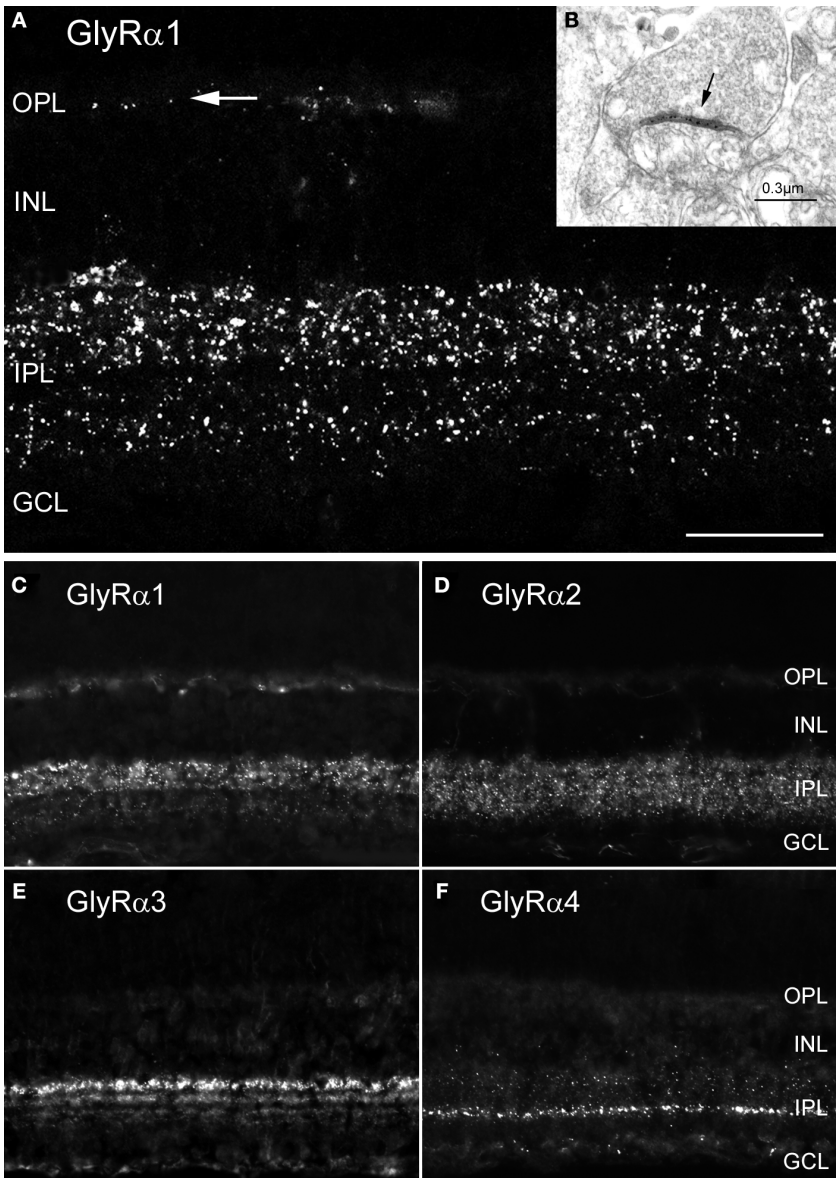

FIGURE 3 | Synaptic localization of GlyR subtypes in the rodent retina (modified from Heinze et al., 2007; Sassoè-Pognetto et al., 1994). **(A)** Fluorescence micrograph of a vertical section through a rat retina that was immunostained for the GlyR α1 subunit. The arrow points to a few synaptic clusters in the OPL. **(B)** Electron micrograph showing a synapse (arrow) expressing the GlyR α1 subunit. The antibody (mAb2b) recognises an extracellular epitope and, therefore, the synaptic cleft is immunolabelled. **(C–F)** Vertical sections through the mouse retina. **(C)** GlyR α1 immunoreactive puncta are most prominent in the outer IPL. **(D)** GlyR α2 immunofluorescence is more evenly distributed across the IPL. **(E)** GlyR α3 expression is found in four bands. **(F)** GlyR α4 immunoreactivity is most prominent in a small band at the sublamina 3/4 border [Scale bar: 25 µm in **(A)**; 65 µm in **(C–F)**].

occur at high density. They represent synapses between AII amacrine cells and OFF-cone bipolar cells (Sassoè-Pognetto et al., 1994). In the inner IPL (stratum S3–S5) smaller GlyR α1 immunoreactive puncta can be observed representing synapses onto ganglion cell dendrites and rod bipolar cell axons (Ivanova et al., 2006; Majumdar et al., 2007). The GlyR α2 subunit is more uniformly distributed across stratum 1–4 (**Figure 3D**), and GlyR α2 immunoreactive puncta occur at the highest density amongst the four α subunits (Haverkamp et al., 2004). The GlyR α3 subunit (**Figure 3E**) shows four bands of higher density of puncta (Haverkamp et al., 2003). Lastly, the GlyR α4 subunit (**Figure 3F**) shows a band of high density of puncta at the border between stratum 3 and 4 (Heinze et al., 2007). This characteristic distribution of subunits across the IPL suggests that the GlyR subtypes are expressed at different synapses and are involved with different neuronal circuits.

CO-LOCALIZATION OF GLYR SUBUNITS AT POSTSYNAPTIC SITES

Since synaptic GlyRs are composed of 2α and 3β subunits (Grudzinska et al., 2005) there is the possibility of two different α subunits co-existing in a single heteromeric GlyR. In addition, it

is possible that two different GlyR subtypes, such as α2β and α3β GlyRs, co-distribute at the same postsynaptic sites. In both cases, the immunoreactive hot spots should coincide. However, when retinal sections were double labelled for the GlyR α1 subunit and the other three GlyR α subunits, no statistically significant coincidence rate of immunoreactive puncta was observed (**Figures 4A–C**). When retinal sections were double labelled for the GlyR α2 and α3 subunits a coincidence rate of 26.7% was found (Haverkamp et al., 2004). In retinal sections double labelled for the GlyR α3 and α4 subunits no significant coincidence rate was found (Heinze et al., 2007). In sections double labelled for the GlyR α4 and α2 subunits, 31.5% of the α4 immunoreactive clusters also contained the α2 subunit (**Figures 4D–F**). The results indicate that – as a rule – postsynaptic GlyR clusters contain only one type of α subunit. The exception is approximately one-third of synapses immunoreactive for GlyR α2 that can also contain the α3 or the α4 subunits.

EXPRESSION OF GLyRs BY IDENTIFIED NEURONS

In order to reveal the involvement of selected GlyR subtypes with different retinal circuits, identified neurons were immunostained for the different GlyR α subunits. **Figure 5A** shows an EGFP labelled A-type ganglion cell in a whole mount of the thy-1 GFP-O mouse retina (Majumdar et al., 2007). The retina was also immunolabelled for the GlyR α1 subunit (**Figure 5B**) demonstrating that many GlyR α1 immunoreactive puncta decorate the dendrites of this A-type ganglion cell. This suggests the cell receives glycinergic input through synapses that contain the GlyR α1 subunit. A-type ganglion cells were also double labelled for the other α subunits (data not shown), and a small number of puncta coincided with the A-type dendrites. Quantification of these results showed that the predominant input is through GlyR α1 containing synapses. However, there is also a small but significant input through synapses expressing the other GlyR α subunits (Majumdar et al., 2007).

Double labelling approaches also enable the identification of the presynaptic partner of synaptic GlyRs in the retina. **Figure 5C** shows a vertical view of a Type 3, glycinergic amacrine cell in the thy-1 GFP-O mouse retina. A single optical section through this EGFP-expressing cell together with GlyR α2 immunostaining is shown in **Figure 5D**. Many GlyR α2 immunoreactive puncta coincide with dendritic varicosities of the Type 3 cell. Since this cell is a glycinergic amacrine cell, the puncta may represent input synapses the cell receives from other, glycinergic amacrine cells or output

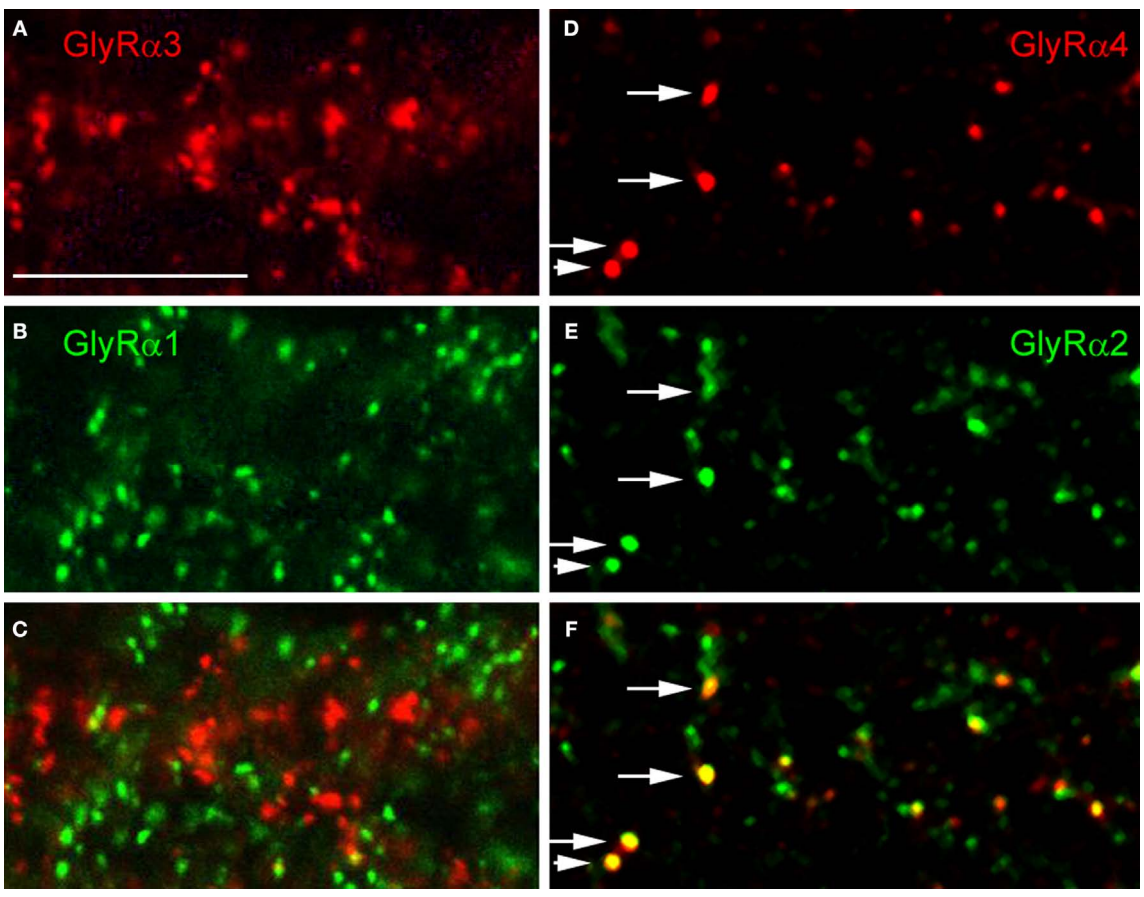

FIGURE 4 | Colocalization of GlyR subunits at postsynaptic sites (modified from Haverkamp et al., 2003; Heinze et al., 2007). (A) GlyR α3 immunoreactive puncta. **(B)** Same section as in **(A)**, immunostained for GlyR α1. **(C)** Superposition of **(A)** and **(B)** shows that GlyR α3 and GlyR α1 immunoreactive puncta are not colocalized. **(D)** GlyR α4 immunoreactive puncta. **(E)** Same section as in **(D)**, immunostained for GlyR α2. **(F)** superposition of **(D)** and **(E)** shows that GlyR α2 and GlyR α4 immunoreactive puncta sometimes colocalize (arrows). (Scale bar: 10 μm).

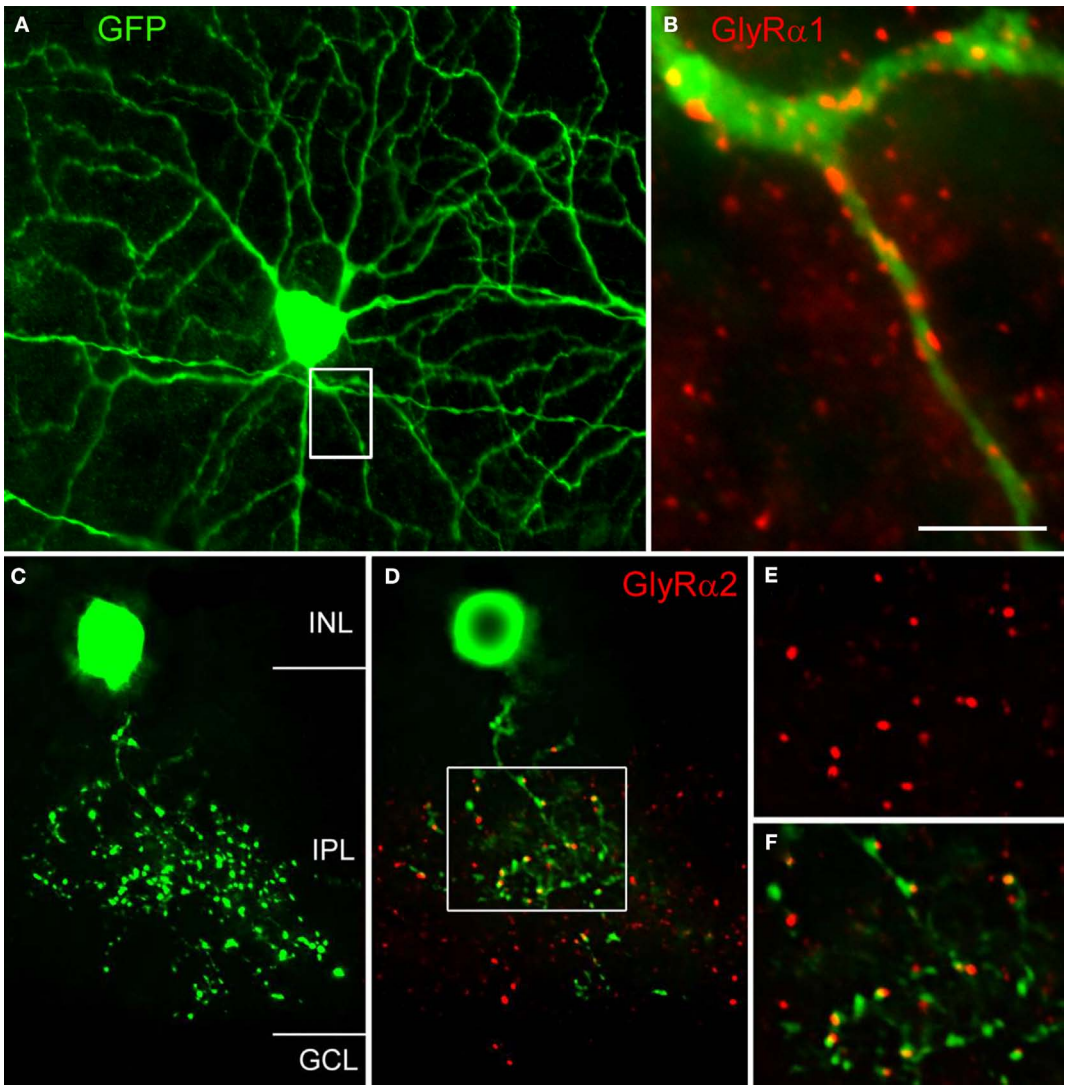

FIGURE 5 | Expression of GlyRs by identified neurons (modified from Majumdar et al., 2007). **(A)** This A-type ganglion cell expressed EGFP in a whole mount of the thy1 GFP-O mouse (Feng et al., 2000). The whole mount was also immunostained for GlyR α1. **(B)** The boxed area from **(A)** is shown at higher magnification and the dendrite of the A-type ganglion cell is decorated by GlyR α1 immunoreactive puncta. **(C)** Vertical view of a Type 3 amacrine cell from the thy1 GFP-O mouse retina (collapsed stack of confocal sections). **(D)** Single confocal section of the cell in **(C)** also immunostained for the GlyR α2 subunit. The boxed area is shown at higher magnification in **(E)** and **(F)**. **(E)** GlyR α2 immunoreactive puncta. **(F)** Dendritic varicosities and GlyR α2 immunoreactive puncta superimposed [Scale bar: 60 µm in **(A)**, 10 µm in **(B)**, 17 µm in **(C)** and **(D)**, 10 µm in **(E)** and **(F)**].

synapses the cell makes onto other, non-labelled neurons. As can be seen from the magnified micrographs in **Figures 5E,F**, the red GlyR α2 immunoreactive puncta are always slightly displaced from the green varicosities. We interpret this result as indicating synaptic GlyR α2 clusters which are expressed by unknown neurons that are postsynaptic to this Type 3 cell.

The two examples of cells presented in **Figure 5** suggest a correlation between the morphological type of a given neuron and the molecular signature of the glycinergic synapse it receives or makes. In this context one interesting question is whether the presynaptic neuron instructs the postsynaptic cell to express certain GlyR subunits or whether a given postsynaptic neuron expresses an exclusive GlyR subtype. We addressed this question by carrying out a detailed physiological characterisation of selected synaptic GlyRs.

GLYCINE RECEPTORS EXPRESSED BY BIPOLAR CELLS

Bipolar cells receive glutamatergic, synaptic input from photoreceptors in the OPL and provide synaptic output onto ganglion and amacrine cells in the IPL. Bipolar cell axons in the IPL receive synaptic input from both GABAergic and glycinergic amacrine cells. There are about 10 different types of cone bipolar (CB) and one rod bipolar (RB) cell in the mammalian retina (reviewed by Wässle et al., 2009). The major subdivision is into OFF- and ON-CB cells, which are hyperpolarized and depolarized, respectively, by a light stimulus. RB cells are ON bipolar cells. Axons of OFF-CB

cells terminate in the outer half of the IPL, those of ON-CB and RB cells in the inner half (Wässle, 2004).

Patch-clamp recordings performed from bipolar cells in slices of the mouse retina (Ivanova et al., 2006) enabled the study of GlyRs by the application of exogenous glycine, and by recording and analyzing glycinergic spontaneous inhibitory postsynaptic currents (IPSCs) in the presence and absence of selected antagonists. The patch electrode was filled with a solution containing neurobiotin and Alexa 488 which diffused into the bipolar cells during the recordings. The slices were immunostained after the experiments and thus the bipolar cell type could be identified unequivocally. The glycinergic IPSCs measured in wild-type mouse retinas were also compared with those from two mutant mouse retinas, one deficient in the GlyR α1 subunit (*Glra1*$^{spd\text{-}ot}$, "*oscillator*", Buckwalter et al., 1994; Kling et al., 1997) and one deficient in the GlyR α3 subunit (*Glra3*$^{-/-}$, Harvey et al., 2004; Haverkamp et al., 2003). Mice deficient in the GlyR α3 subunit did not show an apparent phenotype (Harvey et al., 2004; Haverkamp et al., 2003). Homozygous *Glra1*$^{spd\text{-}ot}$ mice die at about 3 weeks of age (Buckwalter et al., 1994) and juvenile *Glra1*$^{spd\text{-}ot}$ mice, of the age of 16–18 days, were used for the experiments. The results were compared to measurements in wildtype mice of the same age.

The exogenous application of glycine elicited large-amplitude glycinergic currents in all OFF-CB and RB cells, whilst ON-CB cells exhibited only very small, if any, glycinergic currents (Eggers and Lukasiewicz, 2006; Ivanova et al., 2006). Co-application of the selective GlyR antagonist strychnine (3 μM) blocked these glycine-induced currents as expected. Wang and Slaughter (2005) have also shown that the $GABA_A$ receptor antagonists bicuculline and gabazine are also competitive antagonists of homomeric α1 and α2 subunit GlyRs expressed in HEK293 cells or on retinal neurons at high micromolar IC_{50}s. However, glycine-induced currents recorded from bipolar cells were not affected by either bicuculline (100 μM) or gabazine (3 μM) (Ivanova et al., 2006). It has also been reported (Han et al., 2003) that DCKA (5,7 dichlorokynurenic acid), an antagonist of the glycine-binding site of NMDA receptors, blocks the slowly desensitizing glycine-induced current in the tiger salamander retina. Picrotoxinin is also a specific blocker of GlyRs in recombinant expression systems (Pribilla et al., 1992). Application of picrotoxinin (50 μM) reduced the peak currents in bipolar cells to 93% but application of DCKA (500 μM) did not inhibit glycine-induced currents on bipolar cells. These results suggest that bicuculline, gabazine, picrotoxinin and DCKA are not useful pharmacological tools for differentiating the types of GlyRs expressed by bipolar cells.

Studies using knockout mice were more revealing. While there was no significant difference between glycine-induced currents from bipolar cells in wild-type and *Glra3*$^{-/-}$ mice, glycine-induced currents could not be elicited in any bipolar cell in *oscillator* mice (*Glra1*$^{spd\text{-}ot}$) even when glycine was applied at a concentration as high as 10 mM. A more detailed analysis of bipolar cell GlyRs involved the study of sIPSCs. **Figure 6A** shows a recording of spontaneous IPSCs (sIPSCs) from an OFF CB cell at low temporal resolution. When $GABA_AR$ (3 μM gabazine) and the $GABA_CR$ (100 μM TPMPA) antagonists were applied (**Figure 6A**, 2) sIPSCs remained and the frequency was slightly increased. However, when strychnine was co-applied with both GABAR antagonists (**Figure 6A**, 3) the

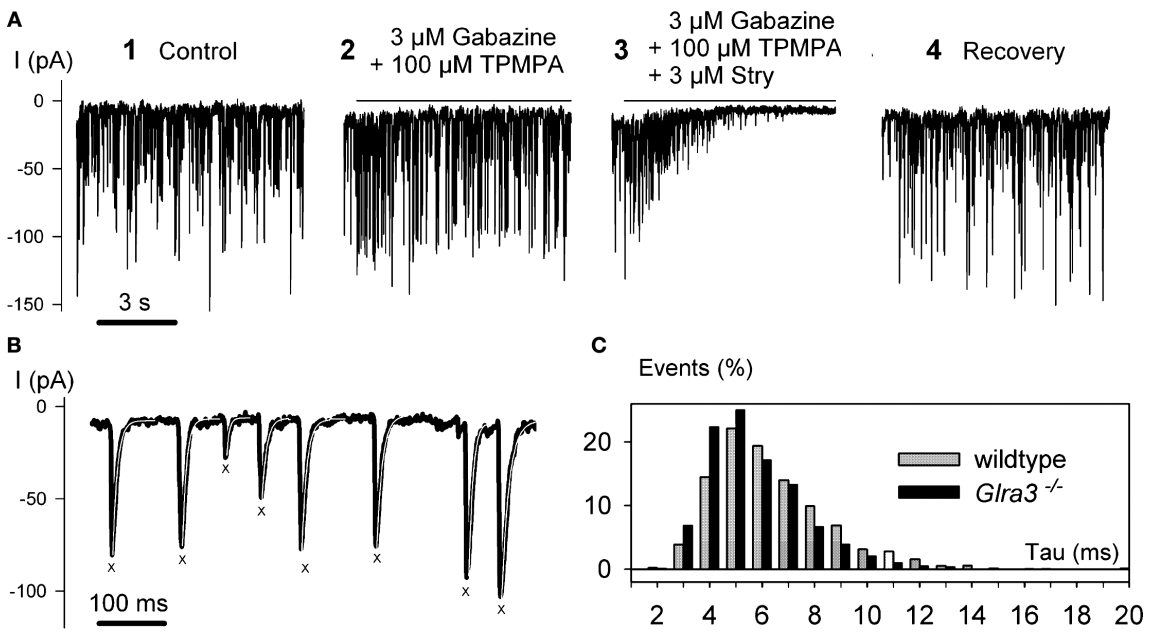

FIGURE 6 | Recordings of spontaneous IPSCs from OFF-CB cells of the mouse retina (modified from Ivanova et al., 2006). (A) At a holding potential of V_H = –50 mV, large sIPSCs were recorded (1). Co-application of the GABAR antagonists gabazine and TPMPA (1,2,5,6 tetrahydrpyridine-4-yl-methylphosphonic acid) did not block the IPSCs (2). However, co-application of GABAR antagonists and strychnine (Stry) abolished the IPSCs (3). **(B)** IPSCs from trace 2 in **(A)** are shown at higher temporal resolution. IPSCs with monotonic rising and decay phase are marked by X and their decay phase was approximated by a single exponential function with the time constant τ (white lines superimposed on black traces). **(C)** The histogram represents the relative frequencies calculated from 17 cells recorded in wild-type mice (light bars), and 15 cells recorded from *Glra3*$^{-/-}$ mice (dark bars).

sIPSCs completely disappeared, demonstrating that they represent glycinergic sIPSCs. The trace in **Figure 6B** shows the IPSCs at higher magnification, enabling the kinetics of single sIPSCs to be analysed. Superimposed events were excluded from the analysis, and only those with monotonic rising phase, lacking inflections and returning to the baseline without contamination from subsequent events were selected (marked by X in **Figure 6B**). We found that the decay phase could be described by a single exponential function with a decay time constant τ. In a total of 17 OFF CB cells studied in retinal slices from wild-type mice, the sIPSCs had a mean amplitude of –70 ± 38 pA, a mean rise time of 1.1 ± 0.3 ms and a mean decay time constant τ = 5.9 ± 1.4 ms (total number of OFF CB cells $n = 17$, total number of IPSCs analysed: $N = 2296$). Glycinergic sIPSCs were also recorded from OFF-CB cells in retinal slices from *Glra3*$^{-/-}$ mice. The IPSCs had a mean amplitude of –81.9 ± 34 pA, a mean rise time of 1.1 ± 0.2 ms and a mean decay time constant τ = 5.1 ± 1.0 ms ($n = 15, N = 3273$). The histogram in **Figure 6C** compares the decay time constants of glycinergic IPSCs recorded from wild-type and *Glra3*$^{-/-}$ mice. No significant difference between the two distributions was found (Kolmogorov-Smirnov test, $p > 0.05$). The result suggests that the GlyR α3 subunit is not an essential component of GlyRs on OFF-CB cells. We also recorded 17 OFF-CB cells from the retinas from *Glra1*$^{spd\text{-}ot}$ mice, and did not observe glycine-induced currents or glycinergic sIPSCs. By contrast, amacrine and ganglion cells in *Glra1*$^{spd\text{-}ot}$ mice showed both glycine responses and glycinergic sIPSCs (Majumdar et al., 2007; Weiss et al., 2008). The total lack of glycine responses in *Glra1*$^{spd\text{-}ot}$ mice suggests that the GlyR α1 subunit is an essential component of GlyRs on OFF CB cells.

GLYCINE RECEPTORS ON AII AND NARROW-FIELD AMACRINE CELLS

Glycinergic amacrine cells comprise at least 10 different morphological types (**Figure 2**). The GlyRs expressed by AII amacrine cells and by the narrow-field (NF) amacrine cells Types 5/6 and Type 7 (Menger et al., 1998) were studied by patch-clamp recordings in mouse retinal slices. During the recordings, the cells were filled with a fluorescent marker (**Figures 7A**) to aid morphological identification. Application of exogenous glycine induced Cl^--currents both in AII and NF amacrine cells that were blocked by the co-application of strychnine (3 μM) and reduced by the co-application

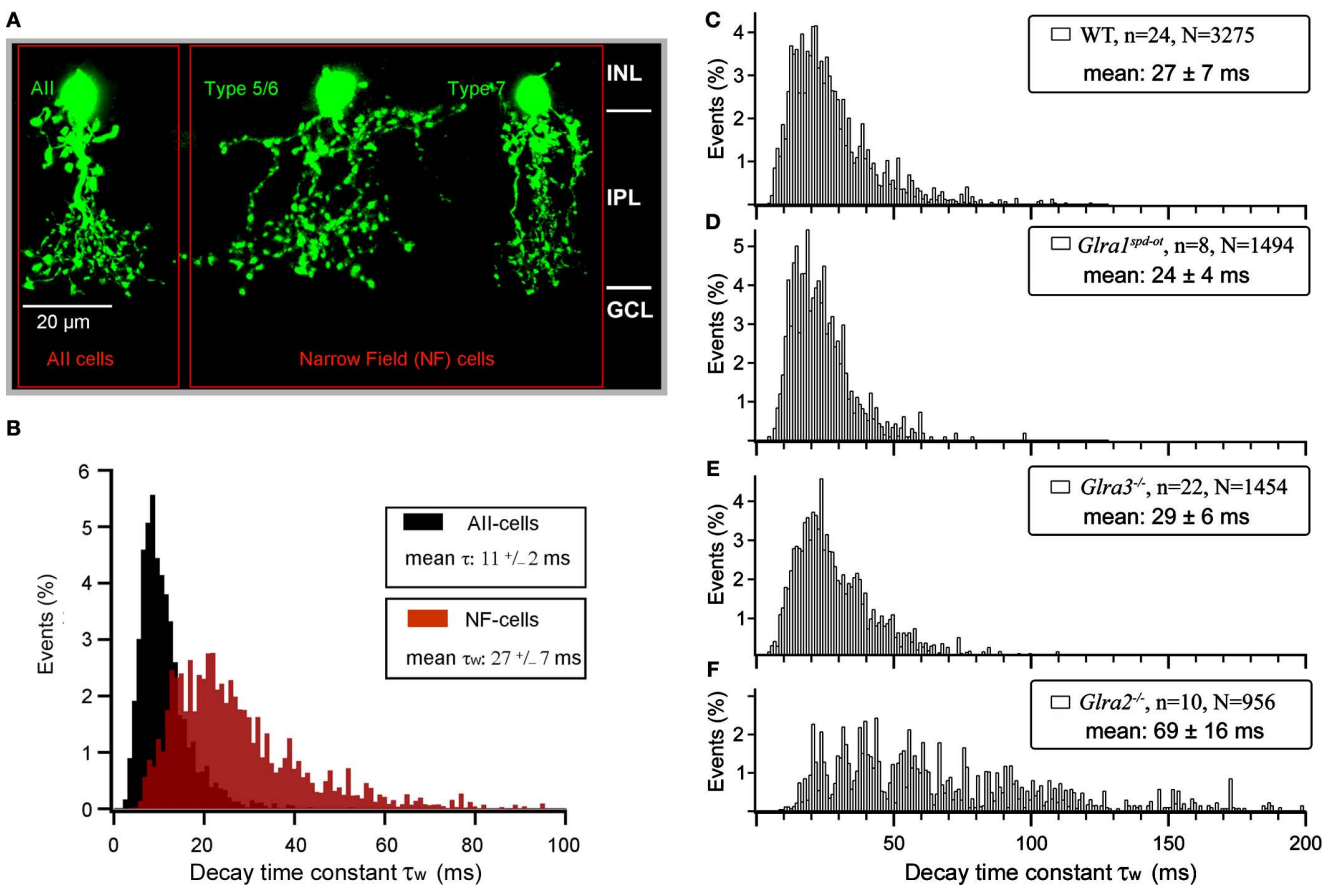

FIGURE 7 | Glycinergic sIPSCs of small-field amacrine cells of the mouse retina (modified from Weiss et al., 2008). (A) Collapsed stack of confocal sections through an AII amacrine cell (left), a Type 5/6 cell (center) and a Type 7 cell (right). They are referred to as narrow-field (NF) amacrine cells. **(B)** Frequency histogram of decay time constants of glycinergic sIPSCs recorded from AII cells in wild-type mice (black histogram). The brown histogram shows the sIPSCs recorded from NF cells in wild-type mice. **(C)** Decay time constants τ_ws of glycinergic sIPSCs recorded from NF amacrine cells of wild-type mice. **(D)** τ_ws recorded from *Glra*$^{spd\text{-}ot}$ mice. **(E)** τ_ws recorded from *Glra3*$^{-/-}$ mice. **(F)** τ_ws recorded from *Glra2*$^{-/-}$ mice.

of picrotoxinin to 73.6 ± 1.8% in AII cells and to 69.9 ± 1.8% in NF cells. Glycine-induced currents were also measured in *Glra1*$^{spd\text{-}ot}$, *Glra2*$^{-/-}$ (Weiss et al., 2008) and *Glra3*$^{-/-}$ mouse retina. Comparable to *Glra3*$^{-/-}$ mice, no obvious phenotype was observed in *Glra2*$^{-/-}$ mice (Weiss et al., 2008; Young-Pearse et al., 2006). In the case of NF amacrine cells the currents elicited in the three mutant mouse lines were not different from those measured in wild-type mice. In AII amacrine cells the currents were significantly reduced in *Glra3*$^{-/-}$ mice but not in the other two mutants.

The analysis of sIPSCs revealed significant differences between AII and NF amacrine cells (**Figure 7B**). Glycinergic sIPSCs recorded from AII amacrine cells could be approximated by a single decay time constant. The histogram of decay time constant τ from AII amacrine cells showed a peak at ~10 ms and a mean of 11.2 ± 2.0 ms ($n = 33$; $N = 2407$). Glycinergic sIPSCs recorded from NF amacrine cells could be approximated in 80% of the cells by a single exponential function, in 20% of the cells a bi-exponential fit had to be applied from which a single weighted decay time constant was calculated. The histogram of decay time constants τ_w of NF amacrine cells in **Figure 7C** shows a peak at ~20 ms and a mean of 27 ± 7 ms ($n = 24$; $N = 3275$). The difference of the decay time constants between AII cells and NF cells was statistically significant (Weiss et al., 2008).

In order to reveal the GlyR subunits that are responsible for these differences, sIPSCs were also recorded in *Glra1*$^{spd\text{-}ot}$, *Glra2*$^{-/-}$ and *Glra3*$^{-/-}$ mutant mice. In the case of AII cells, the decay time constants measured in wild-type, *Glra1*$^{spd\text{-}ot}$ and *Glra2*$^{-/-}$ mice were not significantly different. We also attempted to record glycinergic sIPSCs from AII cells in *Glra3*$^{-/-}$ mice, however, in a total of $n = 50$ cells recorded, no glycinergic sIPSCs could be measured. This suggests that the GlyR α3 subunit is a necessary constituent of synaptic GlyRs on AII cells. In the case of NF cells, sIPSCs were measured in all three mutant mice (**Figures 7C–F**). No significant differences in the distribution of decay time constant τ_w were observed when results from wild-type (**Figure 7C**), *Glra1*$^{spd\text{-}ot}$ (**Figure 7D**) and *Glra3*$^{-/-}$ (**Figure 7E**) mice were compared. By contrast, decay time constants measured from *Glra2*$^{-/-}$ mice (**Figure 7F**) were significantly longer. This difference was highly significant (Kolmogorov-Smirnov $p < 0.01$) and suggests that the α2 subunit shapes the kinetics of GlyRs in NF amacrine cells.

GLYCINE RECEPTORS EXPRESSED BY OTHER RETINAL NEURONS

Glycine induced currents and sIPSCs were also recorded from displaced wide-field, putative GABAergic amacrine cells (Majumdar et al., 2009). These GlyRs had slow kinetics (mean $\tau > 25$ ms; Majumdar et al., 2009; Veruki et al., 2007) comparable to NF amacrine cells. ON-starburst (cholinergic amacrine cells) had sIPSCs with extremely long decay time constants (mean $\tau \sim 70$ ms), which did not differ between wild-type and the three mutant mice (Majumdar et al., 2009). Since GlyR α4 immunoreactive puncta (**Figure 3F**) occur at higher density along the dendrites of ON-starburst amacrine cells, it is possible that GlyRs of ON-starburst cells are dominated by the α4 subunit. This would in turn suggest that GlyRs containing the α4 subunit have slow kinetics.

There are approximately 15 different types of ganglion cells in any mammalian retina. The GlyRs expressed by A-type ganglion cells of the mouse retina were also investigated both in wild-type and mutant mice (Majumdar et al., 2007). In the wild-type retina, glycinergic sIPSCs of A-type ganglion cells have fast kinetics (mean $\tau = 3.9 \pm 2.5$ ms). Glycinergic sIPSCs recorded from *Glra2*$^{-/-}$ and *Glra3*$^{-/-}$ mice did not differ from those of wild-type mice. However, the number of glycinergic sIPSCs was significantly reduced in *Glra1*$^{spd\text{-}ot}$ mice and the remaining sIPSCs had slower kinetics. These results show that A-type ganglion cells receive preferentially kinetically fast glycinergic inputs, mediated by GlyRs containing α1 subunits.

DISCUSSION

All four GlyR α subunits are clustered in synaptic hot spots (**Figure 3**) that show characteristic distributions across the IPL of the mouse retina (Heinze et al., 2007). Gephyrin is responsible for clustering GlyRs to postsynaptic sites by linking the GlyR β subunit to the cytoskeleton (Kirsch et al., 1993; Vannier and Triller, 1997). In gephyrin deficient mouse retinas no GlyR clusters could be detected (Fischer et al., 2000) which suggests that synaptic GlyRs in the retina are always heteromeric, i.e. composed of α and β subunits. In the adult, two copies of the α subunit and three copies of the β subunit form the pentameric receptor protein (Grudzinska et al., 2005). Thus, it is theoretically possible that two different α subunits are present in a heteromeric GlyR. From double-labelling experiments using subunit-specific antibodies we found that – as a rule – only one type of α subunit is present in a given receptor. However, colocalization of two GlyR α subunits within the same synaptic cluster has also been observed. In the case of the GlyR α2 and GlyR α3 subunits we found a coincidence rate of 26.7% (Haverkamp et al., 2004), whilst in the case of GlyR α2 and GlyR α4 subunits 31.5% of the GlyR α4 clusters also contained GlyR α2 (Heinze et al., 2007).

To date, selective agonists or antagonists that distinguish different isoforms of synaptic GlyRs have not been identified (Betz and Laube, 2006; Harvey and Betz, 2000; Legendre, 2001; Lynch, 2004). However, mutant mice with specific knockouts or spontaneous mutations of GlyR α1, α2 or α3 subunit genes have proven to be useful tools for the analysis of GlyRs expressed by different retinal neurons. If a given retinal cell type expresses exclusively α1β, α2β or α3β synaptic receptors, one would imagine that both glycine-induced currents and glycinergic sIPSCs should be abolished in the corresponding mutant which is the case for bipolar cells of *Glra1*$^{spd\text{-}ot}$ mice. However, the situation is usually more complex, because given retinal neurons express more than one type of synaptic GlyR (Majumdar et al., 2007, 2009; Weiss et al., 2008). These synaptic receptors might be localised at the same postsynaptic site and small glycine-containing vesicles released from the presynaptic terminal could activate both receptors simultaneously. Hence the sIPSC will be composed of the pooled responses of both receptors. However, it is also possible that GlyRs are composed of α2α3β or α2α4β subunits and such receptors may have unusual and characteristic kinetics. Moreover, there may be a compensatory up-regulation of expression of other GlyR subunit genes, ameliorating the loss of the subunit affected by the knockout/mutation (Heinze et al., 2007). Keeping these provisos in mind, GlyRs of the mammalian retina have the following properties:

The GlyR α1 subunit is clustered in large synaptic hot spots in the OFF-sublamina (**Figure 3A**), which represent synapses from AII amacrine cells onto OFF-bipolar cell axon terminals (Sassoè-Pognetto et al., 1994). GlyR α1 immunoreactive puncta in the inner IPL (**Figures 3A and 5B**) are located on ganglion cell dendrites

(Majumdar et al., 2007). Rod bipolar cells receive input along their axons descending through the IPL, while their axon terminals do not coincide with GlyR α1 hot spots (Ivanova et al., 2006). Studies with recombinant GlyRs have shown that the expression of the GlyR α1 subunit results in channels with fast kinetics (Betz and Laube, 2006; Harvey et al., 2000; Legendre, 2001; Lynch, 2004). This is consistent with GlyR-mediated synaptic currents (sIPSCs) recorded from the adult brain stem, where GlyR α1-containing synapses show a fast decay time constant ($\tau \sim 6$ ms; Singer et al., 1998). OFF-cone bipolar, rod bipolar and A-type ganglion cells of the retina have GlyR α1-containing synapses which also have fast decay time constants (τ bip ~ 5.9 ms, τ gang ~ 3.9 ms; Ivanova et al., 2006; Majumdar et al., 2007).

GlyR α2 immunoreactive synapses (**Figure 3D**) are distributed more evenly across the IPL and are the most frequent type of glycinergic synapses in this region (Haverkamp et al., 2004). GlyR α2 is not expressed by bipolar cells (Ivanova et al., 2006), but is confined to amacrine and ganglion cells. Since in the neonatal brain stem and spinal cord GlyR α2 is the predominant subunit (Becker et al., 1988; Malosio et al., 1991; Singer et al., 1998; Smith et al., 2000; Takahashi et al., 1992) it was possible to study the kinetics of synapses containing α2 GlyRs. Spontaneous IPSCs recorded from neonatal GlyRs had slow decay time constants ($\tau \sim 14$ ms; Singer et al., 1998). In the retina, narrow-field (NF) amacrine cells receive their predominant glycinergic input through synapses containing GlyR α2 (**Figure 7**) and spontaneous IPSCs recorded from these synapses also have slow decay time constants ($\tau \sim 27$ ms). A-type ganglion cells (Majumdar et al., 2007) also received a small portion of their glycinergic input through synapses expressing GlyR α2. This was demonstrated both by physiological recordings and immunostaining. In other ganglion cell classes this input through GlyR α2 expressing synapses was more prominent (Majumdar, unpublished results).

GlyR α3 expressing synapses have been described in the dorsal horn of the spinal cord (Harvey et al., 2004), but their kinetic parameters are not yet known in detail (Heindl et al., 2007). Synapses containing GlyR α3 in the mouse retina are aggregated in four sublayers of the IPL (**Figure 3E**) and their density is reduced along the two sublayers where the dendrites of starburst amacrine cells are found. It was found that dendrites of AII amacrine cells in sublamina 1/2 express GlyR α3 immunoreactive puncta (Haverkamp et al., 2003). Haverkamp et al. (2003) observed also GlyR α3 immunoreactive puncta on the axon terminals of OFF-cone bipolar cells. The physiological results, however, did not confirm a glycinergic input of cone bipolar cells that would be mediated by the GlyR α3 subunit (Ivanova et al., 2006). It is, therefore, possible that the GlyR α3 labeled puncta were not localized on the bipolar cell axon terminals but on nearby amacrine cells processes. A-type ganglion cells too expressed GlyR α3 immunoreactive puncta along their dendrites (Majumdar et al., 2007). Physiological recordings have shown that AII amacrine cells have glycinergic synapses containing the GlyR α3 subunit (Weiss et al., 2008). Spontaneous IPSCs recorded from AII cells had decay time constants ($\tau \sim 11$ ms) which were slower than those of synapses containing GlyR α1 but faster than those harbouring GlyR α2. In the rat retina sIPSCs of AII amacrine cells also exhibited fast kinetics (Gill et al., 2006).

To date, synapses containing GlyR α4 have only been described in the mammalian retina (Heinze et al., 2007). They are sparsely distributed across the IPL with a significantly higher density within the sublamina where the processes of ON-starburst amacrine cells ramify. Double labeling experiments with antibodies against choline acetyltransferase and GlyR α4 suggested that processes of ON-starburst cells express GlyR α4 in synaptic hot spots (Heinze et al., 2007). The kinetics of sIPSCs of synapses containing GlyR α4 can only be estimated from circumstantial evidence. As mentioned above, sIPSCs of NF amacrine cells receive their predominant glycinergic input through synapses harbouring GlyR α2. However, in the *Glra2*$^{-/-}$ mouse NF amacrine cells still received a small number of slow glycinergic sIPSCs, which are most likely to represent synapses containing GlyR α4 (**Figure 7F**). The decay time constants of these slow sIPSCs ($\tau \sim 70$ ms) were much slower than those recorded from synapses containing GlyR α2 (Weiss et al., 2008). Recent recordings of glycinergic sIPSCs from ON-starburst cells which are decorated by GlyR α4 immunoreactive puncta have shown that GlyRs of ON-starburst cells in *Glra2*$^{-/-}$ mice also have slow kinetics ($\tau \sim 70$ ms). Although they did not differ between wild-type, GlyR α1, and GlyR α3 subunit mutant mice, the final proof that such slow GlyRs contain the α4 subunit will require the generation of *Glra4*$^{-/-}$ mice.

In conclusion, more insights into the functional role of GlyRs in retinal processing can only come from measurements of light responses. Early experiments in the intact cat eye, involving extracellular recordings from ganglion cells and iontophoretic application of glycine and its antagonist strychnine, showed that the light responses of all ganglion cells become more sustained upon the application of strychnine (Bolz et al., 1985). With the same approach it was shown that in the dark adapted retina light responses of OFF-ganglion cells were completely blocked by the application of strychnine (Müller et al., 1988). This confirms that the signal transfer from AII amacrine cells to OFF-cone bipolar cells involves a glycinergic synapse. More recently light responses were studied in retinal slices and retinal whole mounts. Recordings from mouse rod bipolar cells showed that they receive three light driven inhibitory inputs: a fast input mediated by $GABA_A$ receptors, an intermediate input through GlyRs and a slow input through $GABA_C$ receptors (Eggers and Lukasiewicz, 2006). Modulating the relative proportions of these inhibitory inputs will change the characteristics of rod bipolar cell output. Recordings from A-type ganglion cells of the mouse retina (Manookin et al., 2008; Murphy and Rieke, 2008; van Wyk et al., 2009) demonstrated that the light driven responses of OFF-A type ganglion cells were mediated by direct glycinergic inhibitory inputs. The light responses of local edge detector (LED) ganglion cells of the rabbit retina are dominated by inhibitory inputs mediated by glycinergic amacrine cells (van Wyk et al., 2006). These examples show that glycinergic inhibition not only modulates the light responses of retinal neurons but it is also instrumental for creating specificity, preferentially by crossover inhibition between the ON- and the OFF-channels.

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