

# Glycine Receptor $\alpha 2$ Subunit Activation Promotes Cortical Interneuron Migration

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## SUMMARY

Glycine receptors (GlyRs) are detected in the developing CNS before synaptogenesis, but their function remains elusive. This study demonstrates that functional GlyRs are expressed by embryonic cortical interneurons *in vivo*. Furthermore, genetic disruption of these receptors leads to interneuron migration defects. We discovered that extrasynaptic activation of GlyRs containing the  $\alpha 2$  subunit in cortical interneurons by endogenous glycine activates voltage-gated calcium channels and promotes calcium influx, which further modulates actomyosin contractility to fine-tune nuclear translocation during migration. Taken together, our data highlight the molecular events triggered by GlyR  $\alpha 2$  activation that control cortical tangential migration during embryogenesis.

## INTRODUCTION

The initial phase of corticogenesis is characterized by high rates of cell proliferation and intense cell migration, two critical processes that shape the adult cortex and contribute to functional organization (Bystron et al., 2008). The cerebral cortex develops from distinct progenitor populations that give rise to either excitatory projection neurons or inhibitory interneurons. Interneurons are born in the medial and caudal ganglionic eminences (MGE and CGE, respectively) (Anderson et al., 1997) and reach the cortical wall by navigating in migratory streams, located within the marginal zone (MZ), the subplate (SP), and the subventricular zone (SVZ). These migratory paths are dynamically remodeled during embryogenesis (Wonders and Anderson, 2006; Ayala et al., 2007; Marin and Rubenstein, 2001; Métin et al., 2006).

Interneurons account for approximately 15% of cortical neurons and contribute to local networks where they fine-tune cortical neuron excitability (Seybold et al., 2012).

The unveiling of the molecular and cellular mechanisms that drive interneuron migration has just begun (Ayala et al., 2007; Bellion et al., 2005; Schaar and McConnell, 2005). Tangential migration is controlled by the interplay between extracellular signals and cell-autonomous programs (Caronia-Brown and Grove, 2011; Pla et al., 2006). In the developing forebrain, guidance cues are distributed along the migratory streams and are sensed and integrated by interneurons to ultimately control cytoskeleton remodeling. This ensures dynamic cell-shape changes that are required for neuron migration. Neurotransmitters are extrasynaptically released during corticogenesis and act as signaling molecules in the surrounding of migrating interneurons (Heng et al., 2007; Nguyen et al., 2001; Soria and Valdeolmillos, 2002). Neurotransmission-independent activities of  $\gamma$ -aminobutyric acid (GABA) have been widely characterized in the developing cortex by several groups (Cuzon Carlson and Yeh, 2011; Cuzon et al., 2006; López-Bendito et al., 2003). Activation of type A GABA receptors (GABA<sub>A</sub>Rs) promotes interneuron motility at initial phases of migration but blocks migration once interneurons have reached their final position in the developing cortex. This switch correlates with intracellular chloride gradient reversal in interneurons that have reached the cortical plate (Bortone and Polleux, 2009). While roles of GABA have been investigated in interneuron migration, only limited attention has been given to glycine. Glycine is the smallest amino acid neurotransmitter and activates the glycine receptor's strychnine-sensitive ligand-gated ion channels (LGICs). Activation of glycine receptors (GlyRs) results in chloride ion flux through the cell membrane that regulates neuronal excitability (Lynch, 2009). GlyRs containing the  $\alpha 1$  and  $\alpha 3$  subunits are well known for their functions at spinal cord and brainstem synapses, where they contribute to motor control and signaling pathways linked

to inflammatory pain and rhythmic breathing (Harvey et al., 2004; Manzke et al., 2010). By contrast, GlyRs containing the  $\alpha 2$  subunit are widely distributed in the embryonic brain (Malosio et al., 1991). Due to the inverted chloride gradient in immature neurons, activation of embryonic GlyRs results in membrane depolarization in neuronal progenitors. This has been observed in different systems, including the spinal cord (Scain et al., 2010), the retina (Young-Pearse et al., 2006), the ventral tegmental area (Wang et al., 2005), and the cerebral cortex (Flint et al., 1998; Kilb et al., 2002, 2008).

The present work highlights the functional expression of GlyRs in cortical interneurons and demonstrates their contribution to tangential migration in the developing cortex. We show that endogenous activation of GlyRs promotes neuronal migration by regulating nucleokinesis. This involves a sequence of molecular events: (1) membrane depolarization that triggers voltage-gated calcium channel (VGCC) opening and transient calcium influx; (2) dynamic changes in calcium homeostasis that tune myosin II activity; and (3) actomyosin contractions that support nucleokinesis in migrating interneurons. Thus, our work provides an *in vivo* experimental demonstration of the molecular mechanisms by which GlyRs control interneuron migration in the developing cerebral cortex.

## RESULTS

### GlyRs Containing the $\alpha 2$ Subunit Are Functionally Expressed by Cortical Interneurons

GlyRs containing the  $\alpha 2$  subunit are predominantly expressed in immature neurons from the developing spinal cord (Malosio et al., 1991; Watanabe and Akagi, 1995; Becker et al., 1988) as well as from various embryonic brain regions (Malosio et al., 1991). Brain sections from embryonic day 13.5 (E13.5) Dlx5,6:Cre-IRES-GFP embryos (further named Dlx-GFP in the text) were analyzed to specifically assess GlyR expression in GFP-positive cortical interneurons and their progenitors (Stenman et al., 2003). Immunolabeling demonstrated expression of GlyR  $\alpha 2$  subunits in interneurons (Figures 1A–1D). The subcellular distribution of GlyR  $\alpha 2$  subunits was assessed on cultured MGE that were microdissected from E13.5 Dlx-GFP embryos. Immunolabeling revealed a homogeneous distribution of  $\alpha 2$  subunits over the surface of the soma and growth-cone-like structures (further termed growth cones in the text) of the leading process (Figure 1E). Western blot analyses performed on fluorescence-activated cell sorting (FACS)-purified GFP-positive interneurons from Dlx-GFP embryos further confirmed the specific expression of GlyR  $\alpha 2$  subunits by cortical interneurons at different milestone stages (E13.5, E15.5, and E17.5; Figure 1F).

We further performed whole-cell patch-clamp recordings to test whether  $\alpha 2$  subunits integrate into functional GlyRs. Glycine bath application on cultured brain slices (E13.5 + 1–2 days *in vitro* [DIV]) elicited currents that could be recorded in GFP-expressing interneurons. These currents were characterized by typical fast activation and slow inactivation (Figure 1G). A concentration-response analysis of glycine-mediated currents was best fitted by the Hill equation and yielded a half maximal effective concentration ( $EC_{50}$ ) of  $69 \pm 12 \mu\text{M}$ , a Hill coefficient of  $1.4 \pm 0.3$ , and an average maximal current of  $270 \pm 73 \text{ pA}$ .

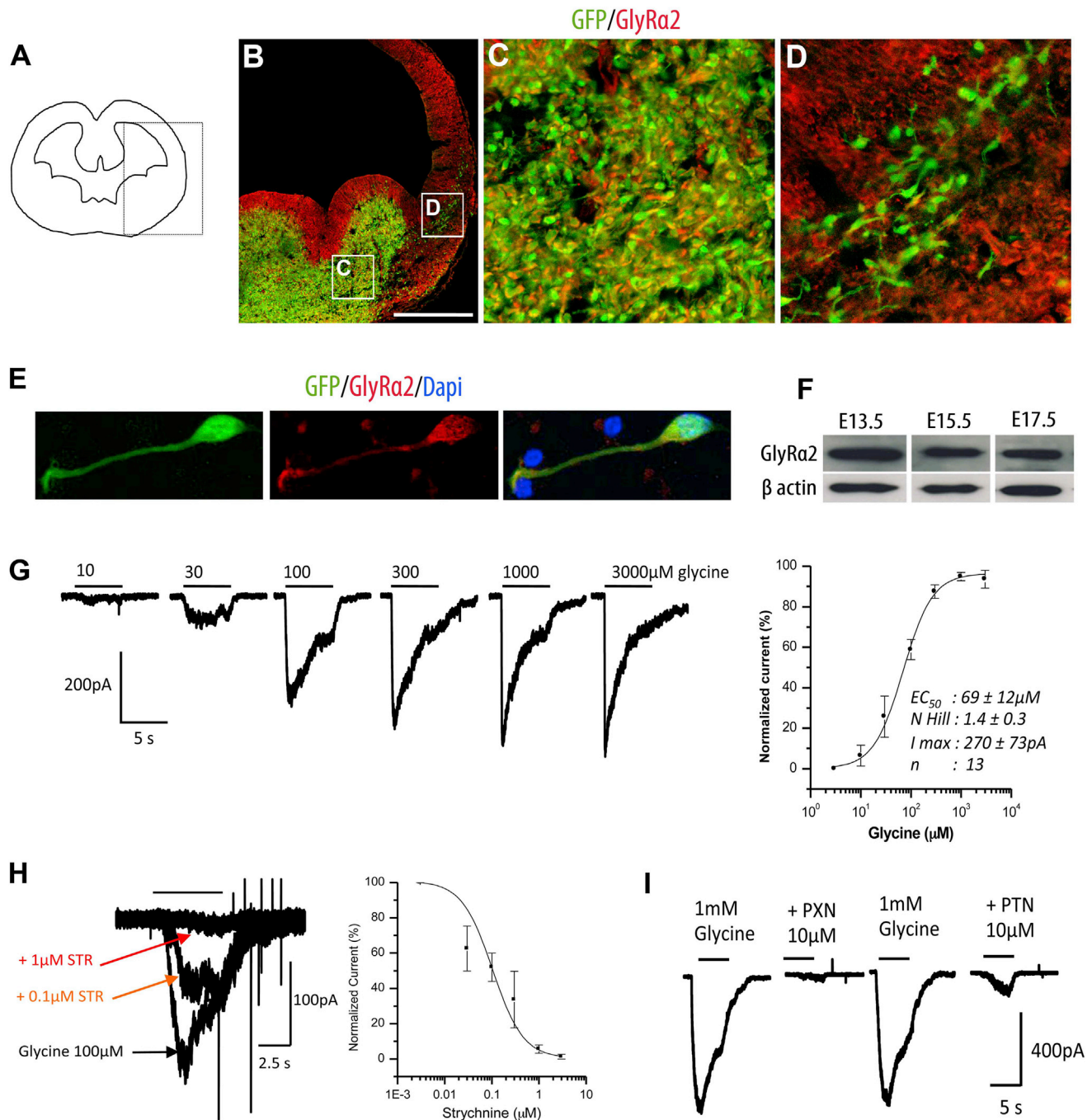
These values were in accordance with those reported previously for  $\alpha 2$  subunit-containing GlyRs in striatal progenitors (Nguyen et al., 2002), embryonic spinal cord neurons (Baev et al., 1990), and CHO cell expression systems (Mangin et al., 2005). Application of the GlyR inhibitor strychnine reversibly blocked glycine-evoked currents (major current inhibition was achieved by application of  $1 \mu\text{M}$  of strychnine) with a half maximal inhibitory concentration ( $IC_{50}$ ) value of  $0.10 \pm 0.02 \mu\text{M}$  (Figure 1H). To gain further insight into the molecular composition of the GlyRs expressed by cortical interneurons, the two components of picrotoxin, picrotin and picrotoxinin, were tested on glycine-triggered currents. While both compounds are known to equally affect  $\alpha 1$  subunit homomers, differential-blocking abilities of these compounds have been reported toward  $\alpha 2$  homomeric GlyRs (30–50 times more sensitive to picrotoxinin) (Wang et al., 2007; Lynch et al., 1995; Yang et al., 2007). Our experiments showed that glycine currents were less inhibited by application of picrotin than picrotoxinin (Figure 1I). Our results also excluded the contribution of  $\beta$  subunits in these GlyRs as  $\beta$  subunit-containing heteromers are insensitive to picrotoxinin application (Pribilla et al., 1992; Mangin et al., 2005). Taken together, our results strongly suggest that the glycine-evoked currents recorded in cortical interneurons are mediated through activation of a homomeric  $\alpha 2$  subunit containing GlyRs.

### Modulation of GlyR $\alpha 2$ Subunit Expression Affects Interneuron Migration

To understand the physiological role of  $\alpha 2$  subunit GlyRs in the developing cortex, loss- or gain-of-function experiments were carried out by the focal electroporation of the MGE mantle zone of cultured brain slices from E13.5 embryos (E13.5 + 3 DIV; Figure 2A). Slices were coelectroporated with Dlx 5/6 enhancer element-driven red fluorescent protein (RFP) reporter construct (Stühmer et al., 2002) and plasmids encoding either GlyR  $\alpha 2$  subunit (GlyR $\alpha 2$ ; or a control, Ctr) or small hairpin RNAs (shRNAs) that target the  $\alpha 2$  subunit (shGlyR $\alpha 2$ ; or a control scrambled shRNA, shSCR) (Young and Cepko, 2004). Electroporation of shGlyR $\alpha 2$  led to complete loss of GlyR function, revealed by the absence of glycine-evoked currents in targeted interneurons (Figure 2B). Quantification of interneuron distribution in the dorsal (cortical wall) and ventral telencephalon suggested that acute loss of GlyR function impaired corticostriatal boundary crossing and interneuron entry into the cortical wall (Figures 2C and 2D). By contrast, overexpression of GlyR  $\alpha 2$  subunits promoted migration of interneurons in the cortical wall (Figures 2E and 2F). Together, these results demonstrate a correlation between the level of GlyR  $\alpha 2$  homomer expression and the migration of cortical interneurons in cultured brain slices.

### GlyR Blockade Reduces Migration Velocity of Cultured Cortical Interneurons

We performed real-time imaging on cultured brain slices to gain further insight into the contribution of GlyRs to tangential migration (Figure 3A). For this purpose, brain slices from E15.5 embryos were maintained in a medium containing glycine, which tonically activates GlyRs (see also Figure 6B). Bath application of strychnine reduced the velocity of migrating interneurons



**Figure 1. Migrating Interneurons Express GlyR  $\alpha 2$  Subunits during Embryonic Cortical Development**

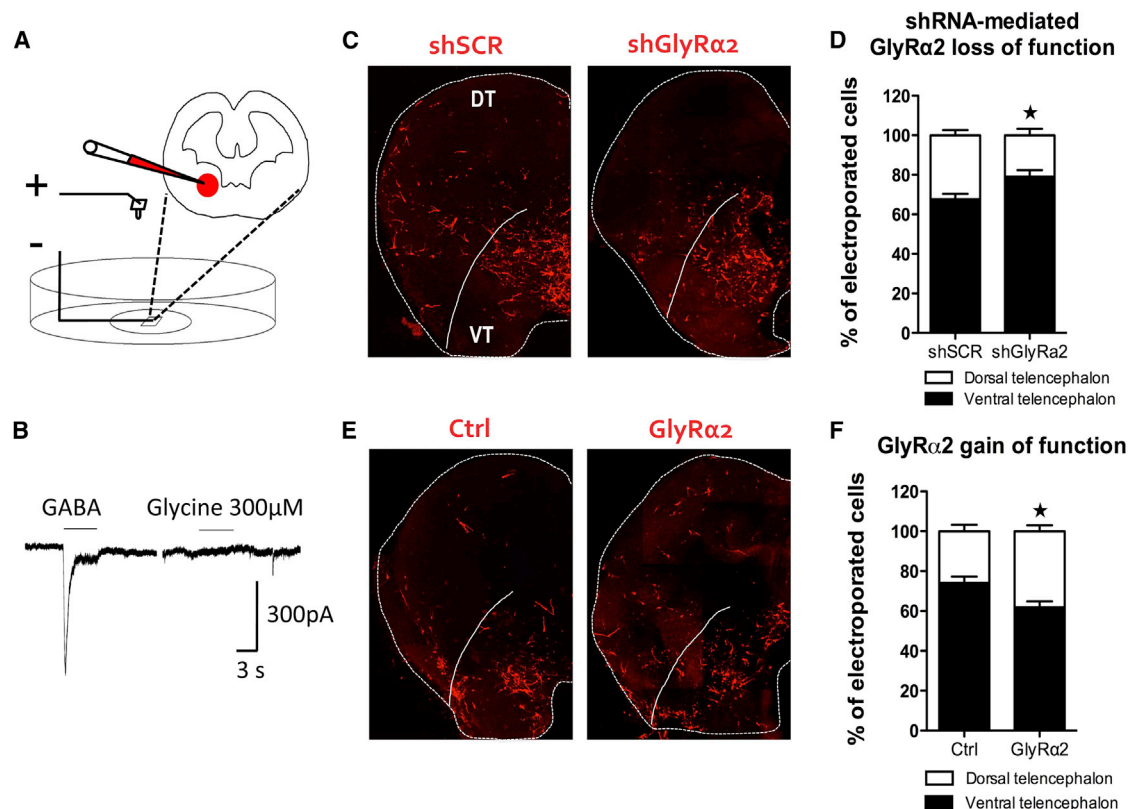
(A–D) Immunolabeling performed on a brain slice from an E13.5 Dlx-GFP embryo showing partial overlap for GlyR  $\alpha 2$  subunit (GlyR $\alpha 2$ , red) and GFP in interneurons (green).

(E) Individual interneurons migrating out of MGE explants (GFP, green) show subcellular expression of GlyR  $\alpha 2$  (red) at the soma and growth cone. Nuclei are in blue (DAPI).

(F) Western blot analysis showing expression of GlyR  $\alpha 2$  in protein extracts from Dlx-GFP FACS-isolated cortical interneurons at different embryonic stages.

(G–I) Whole-cell patch-clamp analyses performed on in-vitro-labeled cortical interneurons in acute E13.5 Dlx-GFP brain slices (+1–2 DIV). (G) Concentration-response curve obtained from successive glycine applications ( $n = 13$  cells; 100% of recorded cells were sensitive to glycine and GABA application; mean  $\pm$  SEM). (H) Representative traces of strychnine (STR) inhibition and inhibition curve (H) ( $n = 5$  cells; mean  $\pm$  SEM). (I) Picrotoxinin (PXN) and picrotin (PTN) induce inhibition of glycine-elicited currents.

Scale bar in (B) represents 300  $\mu m$ .



### Figure 2. Modulation of GlyR $\alpha 2$ Subunit Expression Affects Interneuron Migration

(A) Scheme depicting focal electroporation of a plasmid solution (red) in the MGE of a cultured brain slice.

(B) Lack of glycine-evoked currents in cortical interneurons after shRNA-mediated (shGlyR $\alpha 2$ ) knockdown of the GlyR  $\alpha 2$  subunit (n = 4 cells).

(C–F) GlyR  $\alpha 2$  expression modulation by loss or gain of function experiments on E13.5 cultured brain slices. Representative pictures of electroporated brain sliced cultured 3 days in vitro. Electroporated neurons express either RFP (red) and shSCR or shGlyR $\alpha 2$  (C) or RFP and Ctrl or GlyR  $\alpha 2$ . Interneurons that cross the corticostriatal boundary (solid white line) enter the cortex (E). Telencephalic distributions of electroporated cells with various plasmids, as indicated on histograms in (D) and (F).

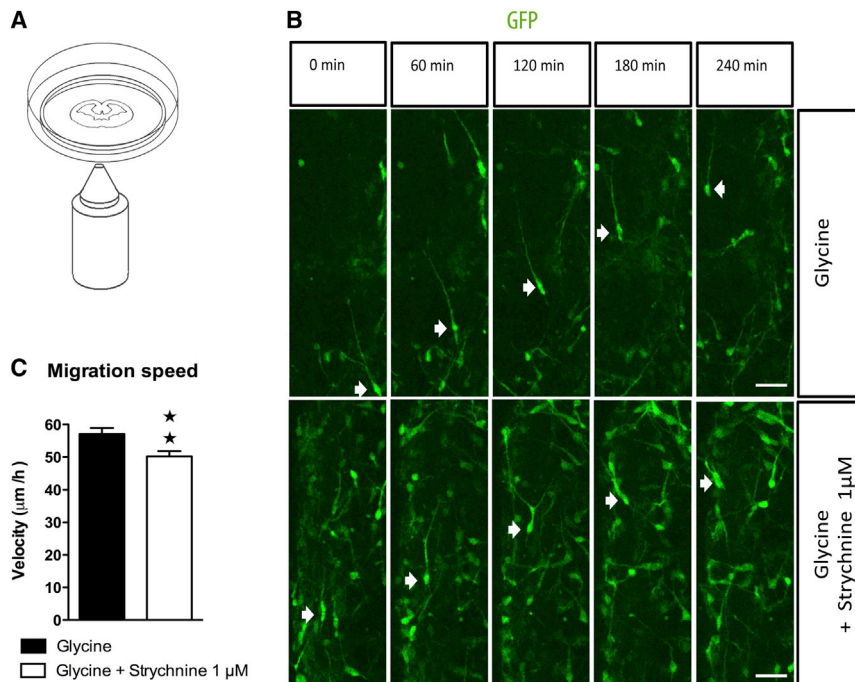
\*p < 0.05, t test; n = 4–12 slices per condition, mean  $\pm$  SEM.

by 15% compared to controls (Figures 3B and 3C). A more pronounced effect of strychnine was observed when glycine was omitted from the culture medium (Figures S1A and S1B), supporting the endogenous production and release of a GlyR agonist by cortical cells (Figures S1C and S1D). It is worth noting that reduction of migration velocity was less pronounced after strychnine application in brain slices from older embryos (E17.5; data not shown), which likely reflects either a predominant motility role of other neurotransmitters (e.g., GABA; Behar et al., 1996; Bortone and Polleux, 2009) or glutamate (Komuro and Rakic, 1993; Manent et al., 2006; Métin et al., 2000) over glycine via activation of their cognate receptors in cortical interneurons, or a progressive change of the chloride gradient through upregulation of KCC2 (Bortone and Polleux, 2009) in older migrating interneurons.

Taurine is also present in the developing cerebral cortex (Flint et al., 1998; Benítez-Díaz et al., 2003), and taurine deficiency is associated with cortical development defects (Sturman, 1988). Due to the high levels of taurine concentration in the developing brain, this transmitter has been proposed to act as an endoge-

nous GlyR agonist in the developing cortex (Flint et al., 1998; Yoshida et al., 2004). However, glycine has also been detected in the developing brain (Benítez-Díaz et al., 2003), and our immunolabeling experiments suggest that cortical plate neurons represent a potential source of endogenous glycine (Figures S1C). We conducted time-lapse experiments performed in the presence of taurine, and in combination with strychnine, to test the ability of this neurotransmitter to drive interneuron migration through activation of GlyRs. While taurine could not substitute for glycine in these experiments, taurine did promote interneuron cell migration independently of GlyR activation (Figures S1A and S1B). This result is in agreement with a recent study that suggested that taurine may act via a different target, the K<sup>+</sup>-Cl<sup>-</sup> cotransporter 2 (KCC2) (Inoue et al., 2012). Taken together, these results demonstrate that glycine is the main endogenous GlyR agonist that acts on interneurons during corticogenesis.

One of the main features of tangential migration of interneurons is the discontinuous translocation of their nucleus toward the centrosome, a process termed nucleokinesis (Bellion et al.,



### Figure 3. GlyR Blockade Reduces Interneuron Migration Speed in Brain Slices

(A) Scheme illustrating a brain slice processed by confocal microscopy for real-time imaging.

(B) Time-lapse sequence of GFP-expressing interneurons migrating in E15.5 Dlx-GFP brain slices incubated in medium containing glycine with or without supplementation of strychnine. White arrows denote one representative migrating interneuron for each condition.

(C) Migration speeds of interneurons recorded in E15.5 Dlx-GFP brain slices incubated in the media described above. \*\* $p < 0.01$ , t test;  $n = 161$ – $162$  cells from six different brains; mean  $\pm$  SEM. See also [Figure S1](#).

SVZ stream (Figures 5F and 5G). It is worth noting that the amplitude of nucleokinesis remained unaffected in all migration corridors (Figure 5H). Together, these results suggest that the functional expression of GlyR  $\alpha 2$  subunit homomers is critical for proper migration of interneurons in the SVZ. Our results also support the distinctive nature of cortical migratory paths (Tiveron et al., 2006), since interneurons in the SVZ stream are clearly dependent in vivo on GlyR activation.

2005, Schaar and McConnell, 2005). To examine the involvement of GlyRs on this process, MGE explants from E13.5 Dlx-GFP embryos were grown for 1 day on homochronic cortical mixed feeder (Figures 4A and 4B) (Bellion et al., 2005, Godin et al., 2012). Strychnine application reduced migration speed in this assay (Figure 4C). Under control conditions, nuclear translocation frequency was  $1.37 \pm 0.07$  events per hour and was constant during the whole experimental time period (Figures 4E and 4F). Exposure to strychnine reduced this activity to  $1.11 \pm 0.06$  events per hour (Figure 4D). Similar analyses were conducted on acute slices and the effect of strychnine was comparable (data not shown). Reduction of both velocity of migration and nucleokinesis frequency upon strychnine application strongly suggests that GlyR activation controls interneuron migration by modulating nucleokinesis.

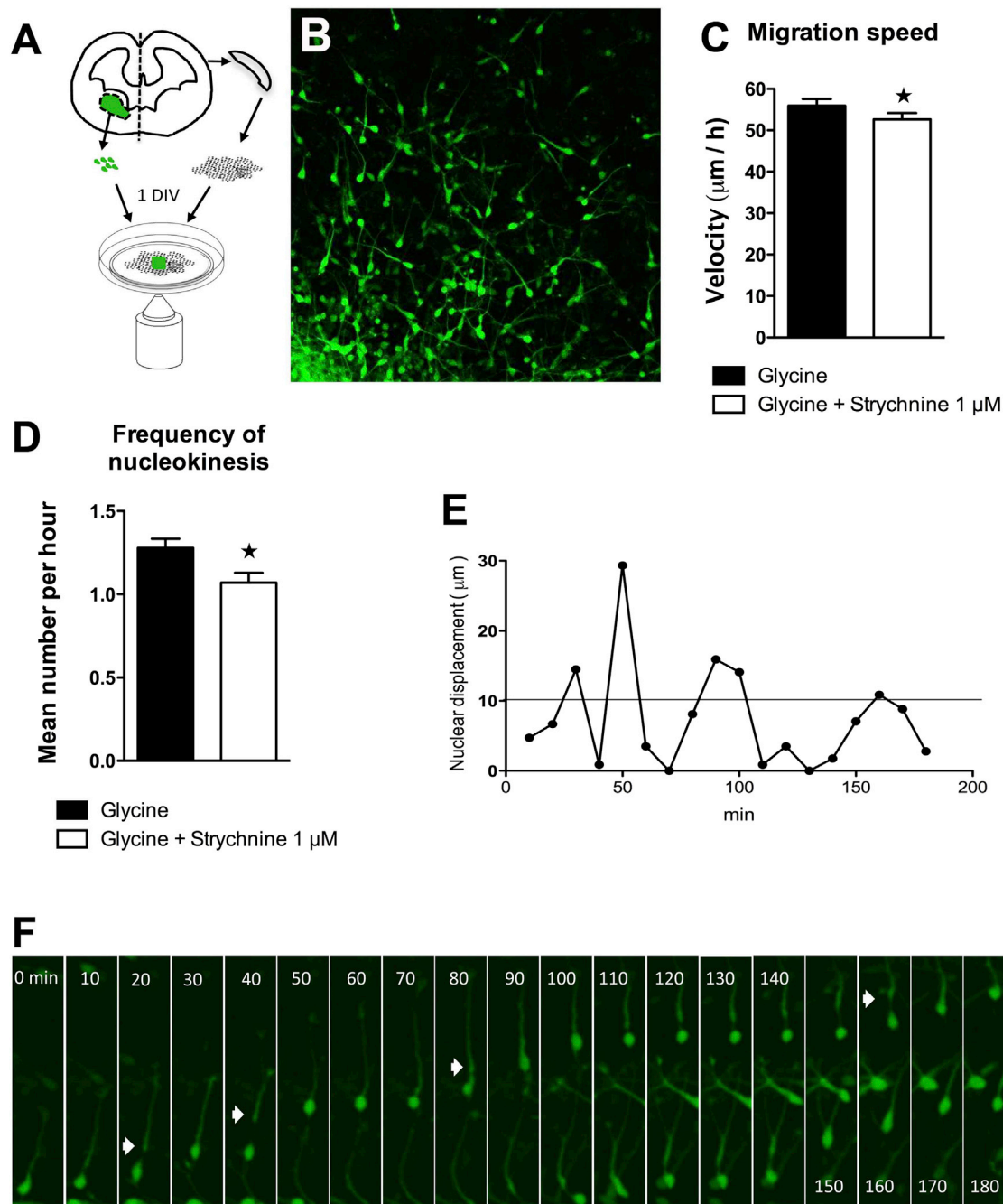
### Genetic Disruption of the GlyR $\alpha 2$ Subunit Impairs Interneuron Migration in Vivo

To further assess the contribution of GlyRs containing the  $\alpha 2$  subunit to cortical interneuron migration in vivo, we engineered a *Glr2* knockout mouse line (see [Experimental Procedures](#) and [Figure S2](#)) where exon 7, containing the membrane spanning domains M1–M3, was deleted. Cortical interneurons that lack GlyR  $\alpha 2$  subunit homomers were assessed in embryos arising from Dlx-GFP/*Glr2* knockout mouse line (Figures 5A and 5B). Histochemical analyses performed on E15.5 embryos showed an overall reduction of the number of GFP-expressing cortical interneurons migrating into the cortical wall. However, only neurons traveling in the SVZ migration stream were affected (Figures 5C–5E; [Movies S1](#) and [S2](#)). Time-lapse recordings confirmed a reduction of both velocity of migration and frequency of nuclear translocation for interneurons traveling in the

### Interneuron Migration Is Controlled by Endogenous Glycine-Mediated Calcium Oscillations

Spontaneous calcium oscillations occur in migratory interneurons and are needed for proper cell migration (Bortone and Polleux, 2009; Martini and Valdeolmillos, 2010). Most importantly, nucleokinesis requires calcium transients (Martini and Valdeolmillos, 2010). GlyR activation leads to membrane depolarization of immature neurons and, hence, opening of VGCCs that control intracellular calcium dynamics (Flint et al., 1998; Young-Pearse et al., 2006). This suggests that glycine could also affect spontaneous calcium oscillations in migratory interneurons. To test this hypothesis, we performed calcium imaging on migratory interneurons from Dlx-GFP slices loaded with Fluo4 AM. Focal application of glycine led to intracellular calcium increases in cortical interneurons (Figure 6A). In addition, spontaneous calcium oscillations were recorded in interneurons (Figure 6B) that were modulated by strychnine (Figures 6C–6E). The power spectrum, an unbiased representation of the frequencies that dominate the calcium traces, showed a significant decrease of slow intracellular calcium transients (0.003–0.03 Hz) after strychnine application (Figures 6F and 6G). It is also noteworthy that coapplication of gabazine, a specific GABA<sub>A</sub>R blocker, further inhibited calcium oscillations in interneurons.

In order to decipher whether calcium oscillations triggered by GlyR activation contribute to cell migration, we performed real-time imaging in presence of various calcium channel blockers. Omega-conotoxin and calciseptine were used as specific blockers of N-type and L-type calcium channels, respectively. Application of either of these antagonists decreased the speed



**Figure 4. Nucleokinesis in Migrating Interneurons after GlyR Blockade**

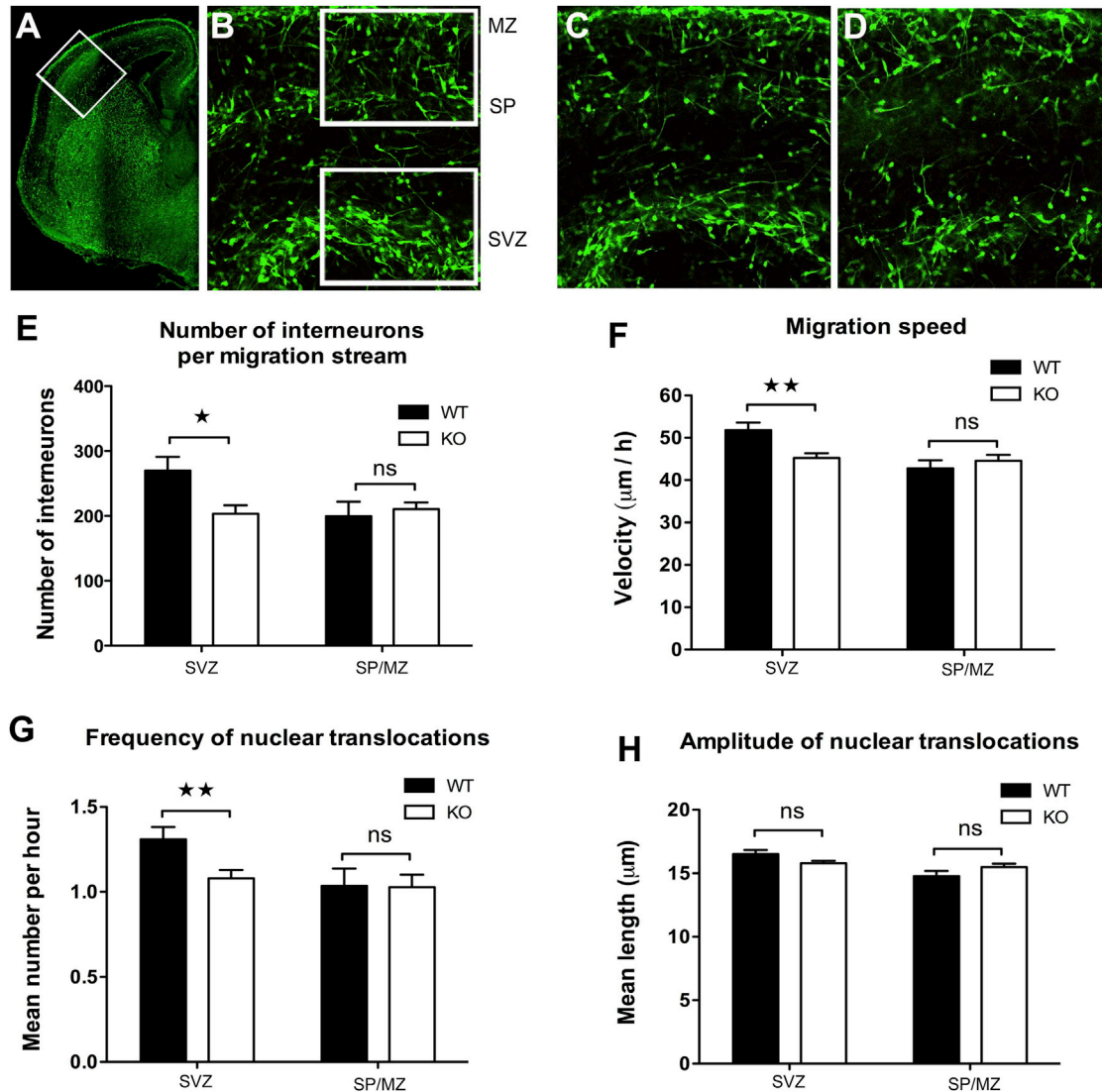
(A and B) Time-lapse recordings on E15.5 Dlx-GFP MGE explants cultured on homochronic mixed cortical feeder from wild-type (WT) embryos (right half coronal section separated by dotted white line) (A). GFP-expressing interneurons (green) that migrate out of the MGE in culture (B).

(C–E) Histograms of migration speed ( $n = 182\text{--}312$  cells from seven explants; mean  $\pm$  SEM) (C) and nucleokinesis frequency ( $n = 104\text{--}112$  cells from seven explants; mean  $\pm$  SEM) (D) of MGE-derived Dlx-GFP interneurons incubated in the media described above. (E) Nuclear movements plotted against time. Threshold of detection for nuclear translocation is 10  $\mu\text{m}$  (solid black line).

(F) Time-lapse sequence showing nuclear translocation displayed by an interneuron in a control MGE culture. Centrosome containing swelling is denoted by white arrows at different time points. \* $p < 0.05$ , t test.

of migration, suggesting that both N-type and L-type channels play an active role in controlling cell motility, in addition to their described roles in cell migration termination (Bortone and Pol-

leux, 2009) (Figure 6H). Bath coapplication of these blockers with strychnine showed that omega-conotoxin had an additive effect on cell migration inhibition. This result suggests that



**Figure 5. Genetic Targeting of *Glra2* Impairs Interneuron Migration in the SVZ**

(A–D) In vivo analyses of interneuron migration in the *Glra2* knockout line. Two-photon imaging performed on E15.5 *Glra2*;Dlx-GFP shows interneurons migrating in the subventricular zone (SVZ), subplate (SP), and marginal zone (MZ) streams. Neurons migrating in the SP and MZ were analyzed together (common boxed area). (C and D) Magnified area of the migratory corridors boxed in (B).

(E–H) Histograms showing absolute numbers (E), velocity (SVZ,  $n = 178$ –344 cells; SP/MZ,  $n = 68$ –161 cells; from four brains per genotype) (F), frequency of nucleokinesis (SVZ,  $n = 97$ –195 cells; SP/MZ,  $n = 39$ –86 cells; from 4 brains per genotype) (G), and amplitude of nuclear translocation (SVZ,  $n = 107$ –198 cells; SP/MZ,  $n = 35$ –94 cells; from 4 brains per genotype; mean  $\pm$  SEM) (H) of interneurons (GFP, green) navigating in SVZ or SP/MZ. WT, wild-type embryo littermates from *Glra2*;Dlx-GFP mouse line; KO, knockout embryos from *Glra2*;Dlx-GFP mouse line.

See also [Figure S2](#) and [Movies S1](#) and [S2](#).

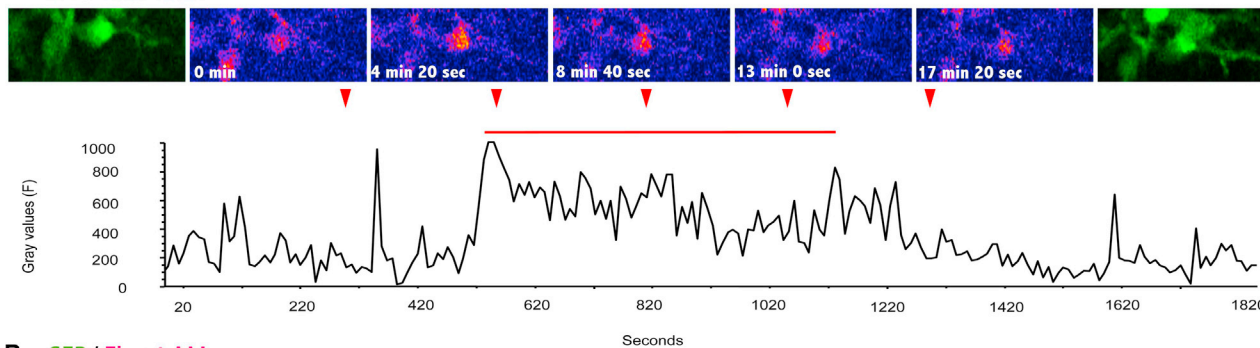
strychnine and calciseptine share the same pathway. Interestingly, similar results have been reported for the effect of GABA, which mainly acts via the activation of L-type calcium channels ([Bortone and Polleux, 2009](#)).

#### Myosin II Phosphorylation Acts Downstream of Glycine Receptor Activation in Interneuron Migration

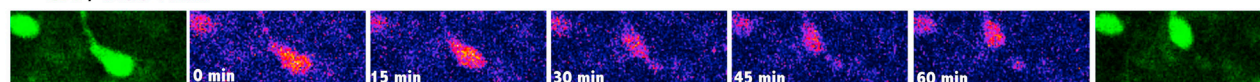
The saltatory pattern of migration displayed by interneurons results from the dynamic accumulation and contraction of actomyosin fibers at the rear of the nucleus ([Bellion et al.,](#)

[2005](#); [Godin et al., 2012](#); [Schaar and McConnell, 2005](#)). Myosin II activation happens periodically at the rear of the nucleus where it promotes actomyosin contractions to push the nucleus toward the leading process ([Godin et al., 2012](#)). This cycle of relaxation and contraction is tightly regulated to ensure proper tangential migration. The non-muscle-type II myosin complex is primarily regulated by phosphorylation at Ser-19 and Thr-18 of its myosin light chain (MLC). This phosphorylation requires either activation of the calcium-calmodulin-dependent myosin light chain kinase (MLCK) or signaling through the Rho kinase-signaling

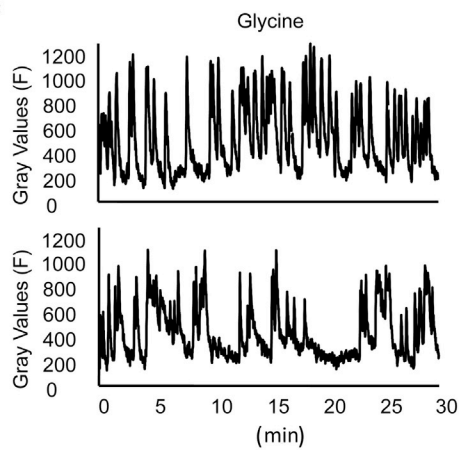
**A** GFP/Fluo4 AM



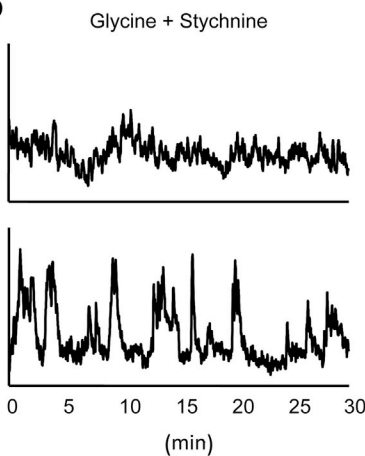
**B** GFP/Fluo4 AM



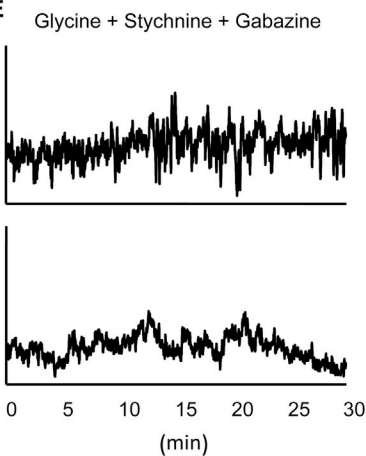
**C**



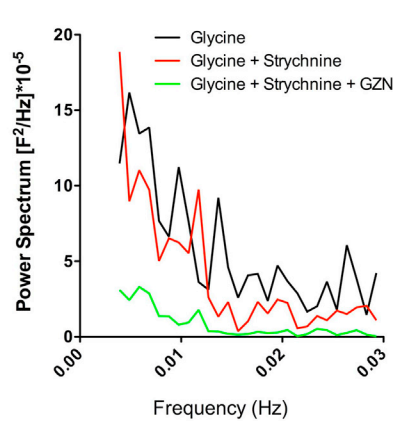
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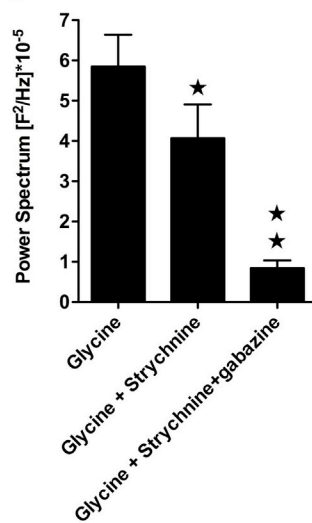
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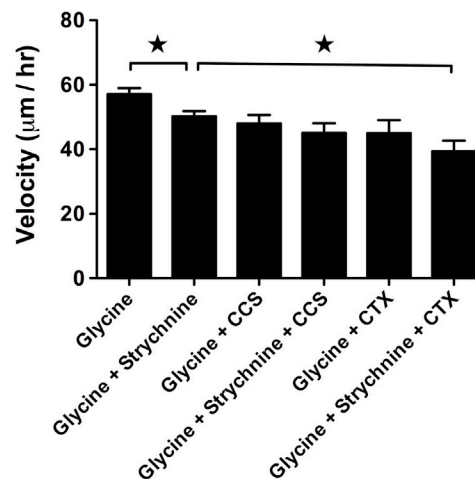
**F**



**G**



**H**



**Figure 6. GlyR Activation Controls Intracellular Calcium Dynamics**

(A and B) Intracellular calcium oscillations recorded in Dlx-GFP interneurons loaded with Fluo4 AM. Time-course of glycine-elicited (A) or spontaneous (B) intracellular calcium oscillations in cortical interneurons.

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pathway (Emmert et al., 2004). Interestingly, nucleokinesis correlates with calcium oscillations (Martini and Valdeolmillos, 2010). Therefore, we decided to test whether changes in calcium transients after GlyR activation control myosin II activity. Western blot analyses performed on dissected MGEs cultured in a medium containing glycine, with or without strychnine, demonstrated a significant change in phosphorylation of the myosin light chain (pMLC) (Figure 7A). Treatment with ML-7 (a specific MLCK blocker), which reduces pMLC levels (Godin et al., 2012), mimicked strychnine application (Figures 4C and 4D) and affected both migration velocity and nuclear translocation (Figures 7B and 7C). Similar results were obtained with application of blebbistatin, a drug that prevents ATP loading on myosin II heavy chains (data not shown). Coapplication of ML-7 (or blebbistatin, data not shown) with strychnine did not lead to additive inhibitory effects, suggesting that GlyR activation ultimately controls myosin II activity in interneurons (Figures 7B and 7C). To further investigate the dynamic changes of actomyosin activity, we performed real-time imaging of migratory interneurons transfected with Utroph-GFP (Burkel et al., 2007). It has been shown that actin and myosin II follow similar dynamic patterns in migrating interneurons during nuclear translocation (Martini and Valdeolmillos, 2010) and that inhibition of myosin II activity by ML-7 results in modification of Utroph-GFP signals (Godin et al., 2012). MGE-isolated interneurons were cotransfected with RFP to compensate for the differences in electroporation efficiencies and changes in cell shape during the analysis (Figure 7D). Under control conditions, Utroph-GFP signal was detected as a nonhomogeneous signal with an intensity peak behind the nucleus, in the trailing edge, and at the rear of the nucleus during nuclear translocation. Strikingly, strychnine-treated neurons displayed a homogeneous signal with a reduction of Utroph-GFP accumulation at the rear of the cell (Figures 7E and 7F). Taken together, these results demonstrate that GlyR activation controls migration velocity and nucleokinesis by triggering a molecular pathway that ultimately tunes myosin II activity and, hence, actomyosin contractility behind the nucleus.

## DISCUSSION

Neurotransmitters have roles beyond neurotransmission, particularly during the development of the CNS (Nguyen et al., 2001). While glutamate and GABA have been shown to control early steps of neurogenesis, including cell proliferation and cell migration (Heng et al., 2007), the role of glycine and GlyRs has remained elusive. Our results demonstrate that functional GlyRs are expressed by cortical interneurons in the developing forebrain. These GlyR are homomers, composed of  $\alpha 2$  subunits,

and tonic activation of these receptors by endogenous glycine tunes tangential migration. The molecular pathway triggered by GlyR activation involves membrane depolarization and voltage-dependent calcium-channel-mediated calcium oscillations. These oscillations are required for proper phosphorylation of MLC, which in turn controls activation of the myosin II complex. This complex contributes to contractile actomyosin fibers that accumulate at the rear of the nucleus of cortical interneurons in order to propel them forward during nucleokinesis (Bellion et al., 2005, Godin et al., 2012). We also illustrate that interfering with GlyR activation disturbs the fine regulation of nucleokinesis and tangential migration of interneurons in the developing cortex.

### GlyRs Are Expressed by Cortical Interneurons during the Early Stages of Corticogenesis

Previous studies support the expression of  $\alpha 2$  subunit GlyRs in immature cells during cortical development (Malosio et al., 1991). However, to date, functional cortical GlyRs have only been described in immature projection neurons (Flint et al., 1998; Young-Pearse et al., 2006) and Cajal Retzius cells (Okabe et al., 2004). By combining immunohistochemistry and western blot analyses, we demonstrated that immature cortical interneurons express GlyRs at several developmental milestones (E13.5, E15.5, and E17.5). GlyR immunoreactivity was not restricted to the cell body and was also detected in the growth cone of the leading process. Patch-clamp analyses performed on GFP-expressing interneurons navigating in slice culture supported the functional expression of GlyR  $\alpha 2$  homomers. The EC<sub>50</sub> value for glycine in cortical interneurons was 69  $\mu$ M, in close agreement with previous studies (Flint et al., 1998; Okabe et al., 2004; Kilb et al., 2002). The sensitivity of these GlyRs to picrotoxin suggests that the  $\beta$  subunit does not contribute to the formation of these GlyRs (Pribilla et al., 1992). While acute knockdown of GlyR  $\alpha 2$  subunits prevented glycine-elicited currents in cultured brain slices, we cannot completely rule out the presence of other GlyR subunits (e.g.,  $\alpha 3$  or  $\alpha 4$ ) in vivo. However, the latter have, to our knowledge, never been located in the developing telencephalon. Importantly, blockade of GlyR-mediated cellular effects by strychnine could be mimicked by  $\alpha 2$  subunit loss of function experiments, suggesting that activation of GlyRs containing the  $\alpha 2$  subunit are central to cell migration.

In addition, our study supports glycine, but not taurine (Flint et al., 1998), as the predominant endogenous GlyR ligand. Although taurine may indirectly affect GlyR-mediated effects by modulating KCC2 (Inoue et al., 2012), the gating efficacies of glycine and taurine at GlyRs are different. A small concentration of glycine is more effective than a high concentration of

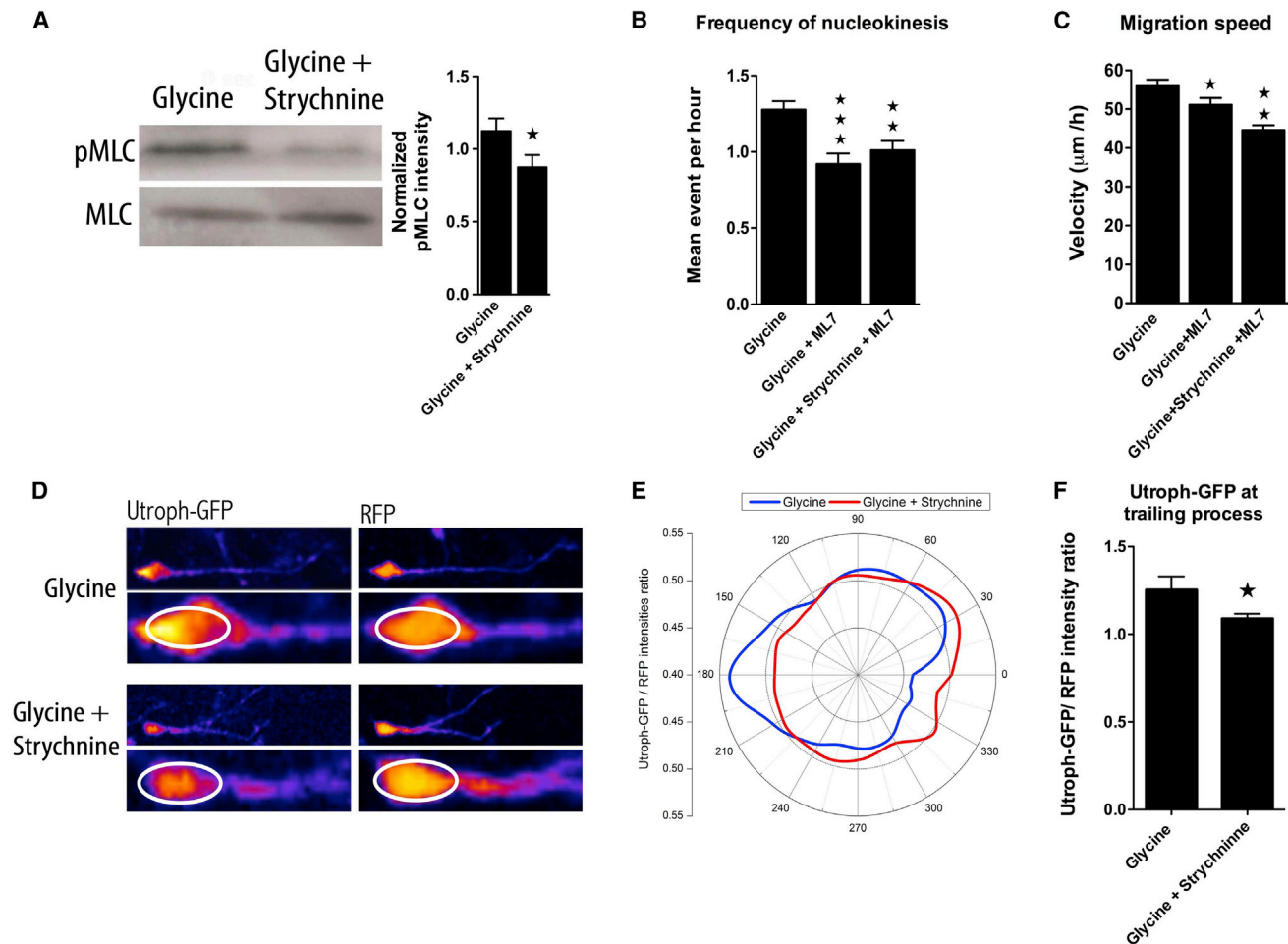
(C–E) Representative traces of spontaneous calcium oscillations in interneurons cultured in glycine-containing medium with application of various pharmacological antagonists, as indicated (n = 5–12 spiking cells per condition).

(F) Power spectral analysis calculated from traces shown in (C)–(E).

(G) Power spectral analysis for low (0.003–0.03 Hz) frequencies for neurons cultured in various conditions, as indicated.

(H) Histogram summarizing the effect of the following drugs: 1  $\mu$ M strychnine (STR), 10  $\mu$ M calciseptine (CCS) (an L-type calcium channel blocker), and 1  $\mu$ M omega-conotoxin (CTX) (an N-type calcium channel blocker) on migration velocity of E15.5 Dlx-GFP interneurons (n = 161 cells, STR; n = 44, CCS; n = 48, STR-CCS; n = 24, CTX; n = 28, STR-CTX; mean  $\pm$  SEM).

\*p < 0.05, Dunn's multiple comparison test.



**Figure 7. GlyR Activation Controls Actomyosin Contractility during Nucleokinesis**

(A) Western blot analysis showing phosphorylation levels of myosin light chain (MLC) in MGE dissected from slices cultured in glycine-containing medium with or without strychnine ( $n = 4$  different brains).

(B and C) Histograms of nucleokinesis frequency ( $n = 77$ – $115$  cells from seven explants) (B) and migration speed ( $n = 182$ – $200$  cells from seven explants) (C) of E15.5 Dlx-GFP cortical interneurons migrating out to MGE explants after treatment with various drugs, as indicated.

(D and E) Time-lapse recording of interneurons transfected with Utroph-GFP (fire, picture set on the left; the upper pictures represent an individual interneuron undergoing nucleokinesis) and RFP (fire, picture set on the right; the upper pictures represent an individual interneuron undergoing nucleokinesis) expressing plasmids (D). The Utroph-GFP/RFP intensity ratio was calculated from the addition of intensities from the center to the periphery of cell and plotted in a polar graph (E). Values around 180 degrees correspond to the trailing edge of the cell. The blue line represents the normalized distribution of GFP signal in the control, while the red line represents distribution in response to strychnine exposure.

(F) Focal Utroph-GFP/RFP intensity at the trailing process of the cell ( $n = 13$ – $16$  cells per condition; mean  $\pm$  SEM).

\* $p < 0.05$ , t test.

taurine on GlyRs containing the  $\alpha 2$  subunit. Indeed, the reported differences in  $EC_{50}$  are up to 100 times higher for taurine as compared to glycine for the same neurons (e.g.,  $EC_{50}$  of  $406 \mu\text{M}$  for taurine and  $32 \mu\text{M}$  for glycine), and the sensitivity of cortical neuron GlyRs to taurine is very low after birth ( $EC_{50}$  of  $7.7 \text{ mM}$ ) (Hussy et al., 1997; Schmieden et al., 1992; Yoshida et al., 2004).

#### GlyR Activation Controls Tangential Migration of Cortical Interneurons

Developmental functions of GlyRs have already been demonstrated in the retina, where they contribute to photoreceptor

generation (Young and Cepko, 2004), as well as in the spinal cord, where they promote neuronal wiring (Scain et al., 2010). A recent study also suggested that GlyRs contribute to radial migration during late embryonic development. However, this effect was only seen in vitro after drastic pharmacological treatment of cultured slices with excess of glycine and in presence of glycine transporter blockers (Nimmervoll et al., 2011).

Our work unveils a function for GlyR activation in the tangential migration of cortical interneurons. Real-time imaging demonstrated that blockade of GlyRs by strychnine application impaired both nucleokinesis and migration velocity of cortical interneurons in culture. In addition, gain- and loss-of-function

experiments confirmed the cell-autonomous nature of GlyR-regulation of interneuron migration. Previous analyses of different *Gla2* mice did not report major cortical defects (Young-Pearse et al., 2006). However, we decided to perform a more in-depth analysis to reinvestigate this issue. The genetic deletion of *Gla2* in our knockout line led to interneuron migration defects, which were restricted to those migrating in the deep SVZ stream. We currently do not have any experimental evidence explaining why migration of interneurons that travel in the MZ and the SP corridors do not depend on GlyR  $\alpha 2$  subunit expression. However, since the molecular composition of those migratory streams is different (Tiveron et al., 2006), we postulate that interneurons selectively entering the cortex by the SVZ corridor are clearly affected by the loss of GlyR  $\alpha 2$  subunit homomers. This could be explained by the existence of compensatory mechanisms such as the expression of different GlyRs that do not incorporate  $\alpha 2$  subunits in interneurons navigating the MZ and SP streams. Alternatively, a lack of functional GlyRs might have less impact on the migration of MZ and SP interneurons if they specifically express other LGICs that trigger membrane depolarization linked to a distinct set of neurotransmitters. These issues require further investigation. Preliminary data suggest that the delays in interneuron migration observed in *Gla2* knockout E15.5 embryos correlate with a reduction in number but not laminar distribution of cortical interneurons at birth (A.A., unpublished data). A reduced number of cortical interneurons will affect cortical wiring and potentially cause changes in behavior or defects in learning. In addition, considering the remarkable function of interneurons in controlling excitability of cortical circuits, a reduced number of cortical interneurons may also favor status epilepticus under specific conditions (Cobos et al., 2005).

The role of GlyR activation may be complementary to the one exerted by GABA<sub>A</sub>Rs, which controls cell migration termination (Bortone and Polleux, 2009). It has been reported that GABA influences not the speed of migration of cortical interneurons, but only the pausing time (Bortone and Polleux, 2009). Thus, GlyRs and GABA<sub>A</sub>Rs could act on different cellular processes during interneuron migration, despite their common ability to trigger membrane depolarization in migrating interneurons as a result of their high intracellular chloride content. To understand these effects, comparative studies must be performed on different interneuron compartments. Spatial segregation of GABA and glycine responses on interneurons could differentially influence local calcium modifications that affect actomyosin.

### Cellular and Molecular Mechanisms Acting Downstream of GlyR Activation

Recent studies have demonstrated that GABA has opposite effects on the control of cell migration depending on the intracellular chloride concentration. This is consistent with a mechanism whereby the initial depolarization activates VGCCs that finally change the frequency of intracellular calcium oscillations (Bortone and Polleux, 2009). However, the downstream signaling pathways and the final effect on cytoskeletal dynamics associated with migration remain unclear. Here, we demonstrated that GlyR activation promotes opening of VGCC and changes in intracellular calcium oscillations. It is noteworthy

that the promotion of migration induced by GlyR activation was exclusively mediated through activation of L-type VGCCs. Compartmentalization and association of ion channels might be responsible for the selective L-type calcium channel activation. Nevertheless, the selective dependence on L-type calcium channels is in strong accordance with the mechanism proposed for the contribution of GABA<sub>A</sub>Rs to neuron migration (Bortone and Polleux, 2009). Interestingly, slow calcium transients are needed for nuclear translocation during interneuron cell migration (Martini and Valdeolmillos, 2010). Time-lapse experiments have shown that actomyosin contractions at the rear of the cell contribute to nucleokinesis by pushing the nucleus toward the centrosome in a calcium-oscillation-dependent process (Martini and Valdeolmillos, 2010). We found an exact temporal correlation between interneuron migration velocity and nucleokinesis frequency after blocking GlyR activation or the downstream intracellular signaling pathway. Western blot analyses showed a reduction of MLC phosphorylation after treatment of MGE explants with strychnine. In addition, bath-applied ML-7, which reduced MLC phosphorylation, and hence myosin II activation, mimicked the effect of GlyR blockade on migration. While we did not observe differences in nucleokinesis frequency between bath-applied ML-7 alone or in combination with strychnine, the latter strengthened the reduction of migration velocity. Previous work from our laboratory demonstrated that the migration rate of interneurons depends on both the frequency of nucleokinesis and the dynamic branching activity of the growth cone (Godin et al., 2012). While GlyR  $\alpha 2$  subunits were detected in both the soma and growth cone of cortical interneurons (Figure 1E), our data suggest that regulation of actomyosin contractility is dependent on GlyR activation at the rear of the nucleus rather than the growth cone (Figure 7D), where other mechanisms may promote the phosphorylation of MLC. Real-time imaging of Utrroph-GFP signals supported this hypothesis by showing specific defects of F-actin condensation at the rear of the cell during nuclear translocation after selective inhibition of GlyR activation. This led us to propose a molecular model whereby membrane depolarization induced by GlyR activation changes intracellular calcium oscillations to promote actomyosin contractions, fine-tuning the frequency of the nuclear translocations promoting migration of immature interneurons. In conclusion, by combining in vitro cultures and time-lapse experiments with in vivo analyses, we have demonstrated that GlyRs containing the  $\alpha 2$  subunit control cortical interneuron migration, thus expanding the known physiological functions of glycine and GlyRs in cerebral cortex development.

### EXPERIMENTAL PROCEDURES

#### Animals

All animal experiments were performed following the guidelines of the local ethical committee at both Hasselt and Liège Universities. For timed pregnant mice, E0.5 was identified by the presence of a vaginal plug the next morning after mating.

Dlx5,6:Cre-IRES-EGFP transgenic mice expressing EGFP under the control of the Dlx5,6 enhancer element (Dlx-EGFP) were maintained in MF1 background and housed at both the GIGA mouse facility and the transgenics and BIOMED institute animal facility. The mouse GlyR  $\alpha 2$  subunit gene (*Gla2*) was selectively disrupted by homologous recombination using the *cre*-Lox

gene targeting system (Kühn et al., 1995). A replacement-targeting vector was used for homologous recombination in the embryonic stem cell (ESC) line PC3 (Figure S2). This generated a targeted allele containing loxP sites flanking exon 7 of *Glr2* (which encodes the membrane-spanning domains M1–M3) and the neomycin (neo) cassette. The PC3 ES cell line is derived from the 129/SvJae strain and contains a transgene driving expression of cre recombinase via the protamine 1 promoter (O’Gorman et al., 1997). Chimeras generated with such ESCs express cre recombinase exclusively in the male germline during the terminal haploid stages of spermatogenesis. Thus, cre-mediated excision of exon 7 and/or the neo cassette was induced by mating *Glr2* chimeras with wild-type mice. Targeted disruption of *Glr2* in the progeny was confirmed by PCR and Southern blotting using probes adjacent to both arms of the *Glr2* targeting construct. As females homozygous and males hemizygous for the *Glr2* deleted allele were viable and fertile, mice with this allele were used for further breeding and phenotyping. Mice containing the deletion allele were genotyped by PCR. These animals were backcrossed onto the MF1 background, mated with *Dlx5,6:Cre-IRES-EGFP*, and housed at the BIOMED institute animal facility.

### Time Lapse

Migration experiments were performed on brain slices or cultured MGE explants. Imaging was performed after 6 hr of recovery at 37°C in 5% CO<sub>2</sub> for brain slice imaging. Explants were kept in the incubator for 20 hr before imaging and only 1/3 of medium was replaced before imaging. Image acquisition was carried out using a Zeiss 200M inverted microscope coupled to a LSM510M confocal scanner (Zeiss, Germany) connected to a MaiTai Titanium-Sapphire laser (Spectra physics) for two-photon imaging. Excitation of EGFP was achieved by using 900 nm and a 20×, 0.5NA, long working-distance objective. z stacks spanning for 30  $\mu$ m from the surface of the slice were acquired every 5 min. Analyses were done using ImageJ software (NIH) and the Mtrack plugin for semiautomated cell tracking (Meijering et al., 2012). Frequencies of nuclear translocations were measured from the graphs of nuclear displacement versus time. Every peak above 10  $\mu$ m was considered as an independent event (Bellion et al., 2005). All values are expressed as mean  $\pm$  SEM.

For additional details on the materials and methods used in this study, please see the [Extended Experimental Procedures](#).

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, two figures, and two movies and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2013.07.016>.

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## EXTENDED EXPERIMENTAL PROCEDURES

### Time-Lapse Imaging with Two-Photon Microscopy

#### Pharmacological Experiments

The microscope was surrounded with a temperature controlled incubator chamber supplied with 5% CO<sub>2</sub> (Pecon). All pharmacological compounds were kept in stock solution and dissolved in the medium just before imaging. Strychnine and blebbistatin (Sigma-Aldrich) were dissolved to 10 and 50 mM, respectively, in DMSO, ML7 (Sigma-Aldrich) was dissolved to 20 mM in 50% ethanol, and conotoxin (Sigma-Aldrich) and calciseptine (Latoxan, France) were dissolved to 10 mM in water. Pharmacological compounds were chosen according to their photochemical stability. Accordingly, nifedipine was not suitable for two-photon imaging and was replaced by calciseptine. Control conditions included equal amounts of the respective solvents.

#### Calcium Imaging

Calcium imaging was performed using Fluo-4 AM (Life technologies) in slices from Dlx-EGFP transgenic animals. Discrimination between the EGFP and Fluo-4 AM was possible thanks to their differential two-photon excitation spectra (selectively excitation of Fluo-4M at 820 nm, and EGFP at of 900 nm). It is noteworthy that switching from 900 nm to 820 nm required adjustment of the laser power. Loading was done at 37°C for 30 min in agitation without holding insert. For the assessment of spontaneous calcium transients, one single plane was acquired every 500 ms for 30 min (12 bits). Only spiking cells were considered in the analysis. Individual spikes were considered as fluctuations above 10% of the base line. Responses evoked by glycine or GABA were recorded in ACSF at 37°C. Agonist application was achieved by semi focal slice perfusion. Spontaneous activity was recorded in culture media, which was changed just before imaging. Image intensity analyses were done using ImageJ software (NIH), defining regions of interest and analyzing its change of intensity with time. Numeric values of intensity were then converted in traces using Clampfit (Harvard Apparatus). The same software was used for baseline correction and power spectrum calculations.

#### Immunolabelings

E13 embryonic brains were fixed 30 min at 4°C with a solution containing 4% PFA for, cryoprotected, frozen and further sectioned at 20 μm in a cryostat (Leica). Prior to the staining, sections were washed with NH<sub>4</sub>Cl for 30 min, to reduce auto-fluorescence, and then with PBS for another 30 min. Blocking was made in 10% normal donkey serum (NDS) added with 1% BSA dissolved in PBS for 1 hr at room temperature. GlyRs detection was performed using alpha 2 specific antibodies (N18, 1:100; Santa Cruz Biotechnologies) diluted in PBS and containing 3% NDS plus 1% BSA, overnight at 4°C. Secondary antibodies were donkey anti-goat labeled with A647 (1:500; Invitrogen). Nuclear counterstaining was performed using DAPI (1:100 Life Technologies, Belgium).

#### Tissue Processing

##### Slice Preparation

E13-E15 embryos were dissected in cold PBS containing 25 mM glucose. Embryonic brains were embedded in 3% low melting point agarose (Fisher Scientific), rapidly cooled down, sectioned at 300 μm and cultured on MILLICELL-CM 0,4 μm inserts (PICMORG50, Millipore) in a semi-dry condition at 37°C and 5% CO<sub>2</sub> for the needed time. Culture medium was composed of Neurobasal (Cat. No.: 12348-017, Life Technologies) supplemented with N2, B27, penicillin-streptomycin and L-glutamine (Life Technologies). In the case of free glycine medium, this was customized by removing glycine from the commercial Neurobasal medium (Life Technologies).

##### Explant Cultures

They were prepared on homochronic mixed cortical feeder from WT embryos on poly-ornithine (1 mg/mL, 45 min, 37°C) and laminin (0.05 mg/mL, 1 hr, 37°C)(Sigma-Aldrich) coated glass bottom multi well plates (MatTek Corporation, USA). The big advantage of this method was that it allowed us to perform simultaneous imaging in multiple explants seeded in separated wells. This increased the efficiency of the procedure and the output of the experiments, while it decreased at the same time the error in the measurements and the need for more embryos.

#### Electrophysiology

Interneurons were labeled in vitro with CMTR-coated tungsten particles placed on the surface of the MGE of E13.5 slices. Slices were maintained in culture for 1 to 2 days to allow interneuron migration. Whole-cell patch-clamp recordings were performed using an Axon 200B patch clamp amplifier (Harvard Apparatus). All the experiments were done at room temperature, in voltage-clamp mode at a V<sub>H</sub> = -60 mV. The internal solution was composed of KCl 130, NaCl 5, CaCl<sub>2</sub> 1, MgCl<sub>2</sub> 1, HEPES 10, EGTA 11, NaATP 2 and NaGTP 0.5 (mM) and the external ACSF was composed of NaCl 125, KCl 2.5, MgCl<sub>2</sub> 1, CaCl<sub>2</sub> 2, NaHCO<sub>3</sub> 25, NaH<sub>2</sub>PO<sub>4</sub> 1.25, Glucose 25 (mM). The amplitude of glycine-elicited currents was assessed by brief applications that lasted for 5 s. All ligands and blockers were focally applied, at a distance around 150 μm from the surface of the slice, using a Warner perfusion system that allowed an exchange time of less than 20 ms (Fast-Step, Warner Instrument Corp.). Data acquisition and analysis were performed using pClamp and Clampfit softwares respectively (Harvard Apparatus).

#### Electroporation

Gain and loss of GlyR function experiments for the assessment of interneuron migration were performed on in vitro cultured slices electroporated with the wild-type form of GlyR alpha 2 or with a shRNA targeted to the 3'UTR region of the GlyR alpha 2. Successfully

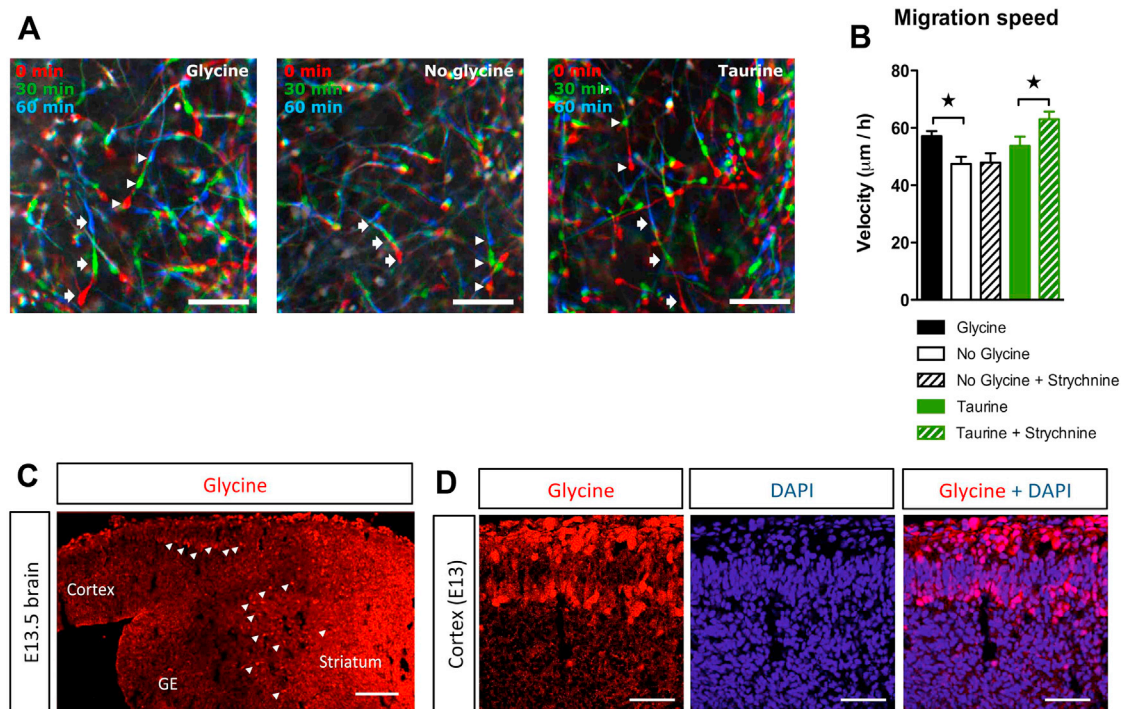
electroporated interneurons were identified by co-electroporation with a Dlx5,6 enhancer driven RFP reporter plasmid. Briefly, slices were injected in the MGE with a mixture of DNA and Fast Green, placed on a slice electroporation chamber and focally electroporated delivering 5 pulses of 100 Volts, lasting for 10ms, every 1 s using an ECM 830 square pulse electroporation device (Harvard Apparatus). DNA concentration was 1.4  $\mu\text{g}/\mu\text{L}$  for each plasmid. Three days after electroporation, slices were fixed in PFA 4% for 30 min, washed with PBS and imaged under confocal illumination.

### Fluorescence-Activated Cell Sorting

Telencephalic vesicles from Dlx-EGFP transgenic embryos (E13.5, E15.5, and E17.5) were microdissected and EGFP positive cells were isolated with a FACS Aria II cell sorter. Post-sorting analysis showed above 95% purity. Immediately after sorting, cells were pelleted at 300 g for 10 min at 4°C and incubated in lysis buffer for 15 min more. After that period, small aliquots were frozen at -80 until the time of the Western blot.

### Western Blot

For the detection of GlyR on migratory interneurons, protein samples derived from the lysis of FACS isolated cells were quantified by the BCA method (Pierce). Ten micrograms of protein were mixed with loading buffer, incubated for 5 min at 90°C and used for SDS PAGE. Separated proteins were transferred to PVDF membranes that were blocked for 1 hr in PBS-Tween with 5% dry milk. Primary antibody (N18, Santa Cruz biotechnology) was used at 4 $\mu\text{g}/\text{ml}$ , dissolved in blocking solution (1:50) and incubated for 1 hr at RT. To assess myosin phosphorylation, E13 slice cultures were allowed to recover for 6 hr and then treated with strychnine 1  $\mu\text{M}$  or an equivalent amount of the vehicle for 18 hr. After this period, MGEs were microdissected and processed for protein extraction. Ten micrograms of protein were loaded per sample lane onto the SDS-PAGE gel. Phosphorylation of myosin light chain (MLC) was assessed by using anti-mouse phospho-MLC (P-MLC) antibody (Cell signaling) dissolved in PBS-T at 1:1000, and mouse MLC antibody (Abcam) at 1:1000 dissolved in PBS-T/5% milk, overnight. Final detection of HRP reaction was achieved by using the ECL method (Pierce). For quantification purposes, films were analyzed with the built in tool present in ImageJ (NIH) and the signal of the phosphorylated protein was normalized by the total amount of MLC.

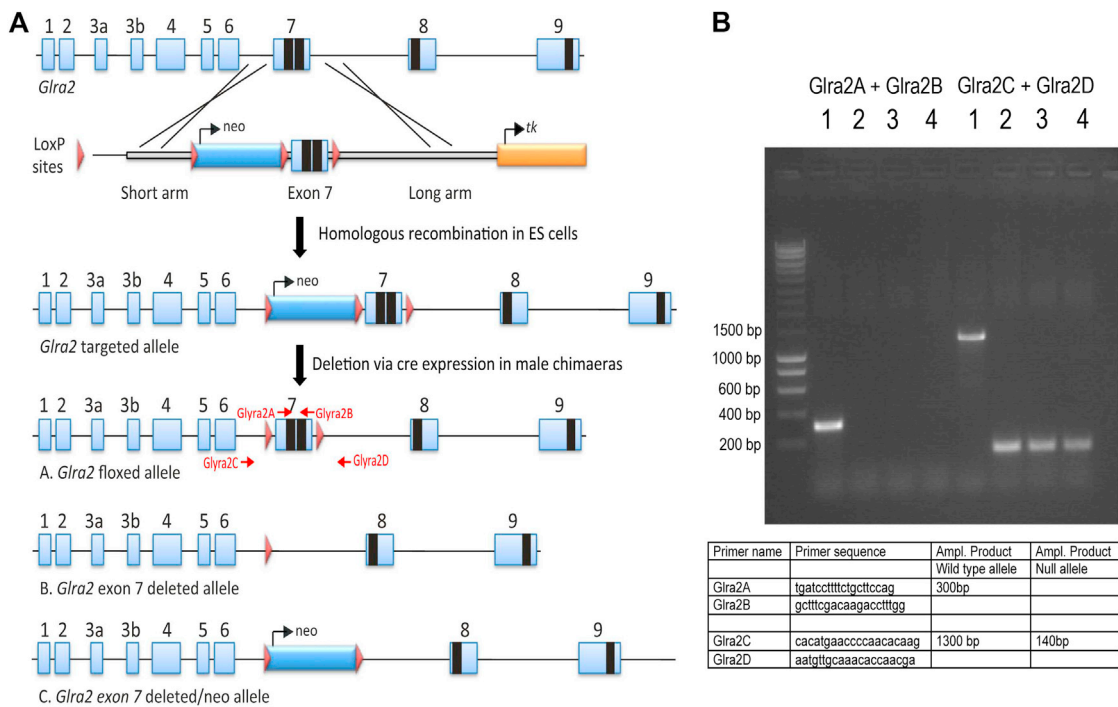


**Figure S1. Glycine Is the Main Endogenous GlyR Alpha 2 Agonist that Promotes Interneuron Migration in the Developing Cortex, Related to Figure 3**

(A and B) Time-lapse recordings of interneurons migrating in E15.5 Dlx-GFP slices incubated in medium containing specific drugs, as illustrated. Color-coded pictures show different time points during the experiment (A). Arrows and arrowheads point to distinct tangentially migrating interneurons. Scale bar: Histogram showing interneuron velocity after bath application of various molecules, as indicated (glycine,  $n = 162$  cells from 6 independent brain slices; no glycine,  $n = 79$  cells from 3 independent brain slices; no glycine + strychnine,  $n = 54$  cells from 3 independent brain slices; taurine,  $n = 77$  cells from 3 independent brain slices; taurine + strychnine  $n = 84$  cells from 3 independent brain slices; ANOVA-1, Dunn's multiple comparison post-test  $*p < 0.05$ ) (B).

(C and D) Glycine immunoreactivity (red) in E13 mouse telencephalon. White arrows point to strongly immunoreactive glycine containing cells (C). Glycine accumulates within the cortical plate. Nuclei are in blue (Dapi) (D).

Scale bars are 55 μm (A), 100 μm (C), and 50 μm (D).



**Figure S2. Generation of the *Glra2* Knockout Mouse Line, Related to Figure 5**

The mouse GlyR  $\alpha 2$  subunit gene (*Glra2*) was disrupted by homologous recombination using the *cre*-Lox system in the ES cell line PC3. This generated a targeted allele containing loxP sites flanking exon 7 of *Glra2* and the neomycin (*neo*) cassette. Three different outcomes were obtained in offspring of chimeras mated to C57BL/6J. Outcome A is the floxed allele, while outcome B is the deleted allele - studied in this manuscript. The allele for outcome C (a deleted allele still containing the *neo* cassette) was not utilized (A). PCR genotyping using specific primers to detect WT or null *Glra2* alleles, as indicated in the table. Animal 1 is WT and the others (2–4) are *Glra2* knockout animals (B).