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The role of glycine receptors during cerebral cortical development

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List of abbreviations

5-HT_3R	type 3 serotonin receptors
5-HT_6R	type 6 serotonin metabotropic receptors
ACSF	artificial cerebro spinal fluid
Arp	actin-related protein
BDNF	brain derived neurotropic factor
BHLH	basic helix-loop-helix
BLBP	brain lipid-binding protein
BMP	brain morphogenic protein
BRDU	bromodeoxyuridine
BSA	bovine serum albumin
CB	calbindin
CGE	caudal ganglionic eminence
CMTMR	chloromethyl-enzoyl-amino-tetramethylrhodamine
CNS	central nervous system
CP	cortical plate
CR	calretinin
D1R	dopamine receptor type 1
D2R	dopamine receptor type 2
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
EGFP	enhanced green fluorescent protein
EGTA	ethylene glycol tetraacetic acid
FACS	fluorescent activated cell sorting
FGF	fibroblast growth factor
GABA	γ-amino butyric acid
$GABA_A$R	gaba receptors type A
GCS	glycine cleavage system
GDNF	glial cell line-derived growth factor
GFAP	glial fibrillary acidic protein
GLAST	glutamate transporter
GluCl	glutamate-gated chloride channels
GlyR	glycine receptors
GlyT2	glycine transporter 2
HD	homeodomain
HEK	human embryonic kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	hepatocyte growth factor
IPCs	intermediate progenitor cells
IR-DIC	infra-red differential interference contrast
ISVZ	internal sub-ventricular zone
IZ	intermediate zone
KCC2	potassium chloride co-transporter 2

LGE	lateral ganglionic eminence
LTD	long term depression
MCS	multi cloning site
MGE	medial ganglionic eminence
MLC	myosin light chain
MLCK	myosin light chain kinase
mM	millimolar
mRNA	messenger RNA
NDS	normal donkey serum
Ngn1	neurogenin 1
Ngn2	neurogenin 2
NGS	normal goat serum
NIH	national institute of health
NKCC1	sodium potassium chloride co-transporter 1
Nrp	neuropinlin
NT4	neurotrophin-4
OSVZ	outer sub-ventricular zone
PAGE	polyacrylamide gel electrophoresis
Pax6	paired box 6
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PGE2	prostaglandin E2
PP	pre plate
PTN	picrotin
PTX	picrotoxin
PV	parvalbumin
PXN	picrotoxinin
RC2	radial glial cell marker-2
RFP	red fluorescent protein
RGCs	radial glial cell
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
Sema 3A	semaphorins type 3A
SFKs	Src family kinases
Shh	sonic hedgehog
shRNA	short hairpin ribonucleic acid
SP	sub plate
SST	somatostatin
STR	strychnine
SVZ	sub-ventricular zone
Tbr2	t-box brain protein 2
VGCC	voltage gated calcium channels
VZ	ventricular zone

Preface

This study focuses on glycine receptors (GlyR) and explores their possible physiological functions on the control of the migration of cortical interneurons and the generation of projection neurons during brain development.

Chapter one is the introduction and exposes the context for the experimental work presented in the subsequent chapters. This chapter begins with a description of the characteristics of the brain in terms of its anatomy, morphology, cellular structure and functionality. Then, it focuses on general events that occur during brain development and explains, in general terms, how the main characteristics of the brain emerge starting from cycling progenitors to functional neurons. Subsequently, it separately explains the development of the two major types of neurons present in the cortex, the projection neurons and the interneurons. Following that, there is an introduction to GlyR by describing their properties and physiological roles in the functioning of the central and peripheral nervous system. Finally, the most important studies that link GlyR to a possible role on the brain development are presented and discussed.

Chapter two describes the materials and methods that were used during the experimental work.

Chapter three presents the results of the study in relation to the effect of GlyR and interneuron development. Based on the literature we hypothesized that activation of GlyR expressed by interneurons can trigger a sequence of molecular events that ultimately contribute to their tangential migration in the forebrain. To test this hypothesis, we performed patch-clamp recordings on interneurons migrating in brain slice culture and demonstrated the functional expression of alpha two homomeric GlyR. Complementary electrophoretic analyses (western blot) and immunolabelings were performed to: 1) confirm the results and unravel the possible dynamic changes that occurred during development; 2) get an insight in the cellular distribution of the receptors. We further explored the possible effects of GlyR activation on cell migration by using time lapse experiments in slices or explant cultures in combination with *ex-vivo* electroporation. These experiments demonstrated that GlyR activation can change the speed of cell migration by affecting nucleokinesis (translocation of the nucleus towards the centrosome). Therefore, we assessed the effect of GlyR activation on two processes that have been associated with the regulation of nuclear translocation, the spontaneous calcium activity and the phosphorylation of myosin II. Altogether, our results support a mechanism by which GlyR activation depolarizes cell membrane activating voltage gated calcium channels that further contribute to increase intracellular calcium concentration. This increase in intracellular calcium contributes to myosin contraction at the rear of the cell which ultimately controls the frequency of nuclear translocations.

Chapter four presents experimental results obtained during the study of dorsal progenitors and radially migrating projection neurons. Electrophysiological experiments were carried out to identify and characterize GlyR expressed by progenitors and radially migrating neurons at embryonic day 13 (E13). In addition, calcium imaging was performed to get some insight into the cellular and molecular mechanisms downstream GlyR activation. To understand the physiological relevance of GlyR on these cells, we assessed cell proliferation using pharmacological tools and shRNA-mediated knockdown.

Finally, the last two chapters are devoted to a general discussion, the conclusions of the study, and perspectives.

CHAPTER 1

General introduction

1.1 Cerebral cortex

The cerebral cortex is the youngest biological structure of the nervous system. Comparatively, mammals are the animals that present the most complex brain and cerebral cortex. This complexity reaches its highest level in the human cerebral cortex (Molnar et al., 2006), where the decision making task along with fine motor control take place. However, the role of the cerebral cortex is much broader and underlies superior functions of the brain, including the processing of sensations, emotions, feelings, and reasoning.

As its name indicates, the cortex relates to the external part of the brain. This structure is a convoluted layered sheet of tissue, 2-3 mm thick in humans and covers an extensive surface. According to the evolution of species the surface of the brain cortex experience notorious changes. While less evolved brains are lissencephalic, the more evolved brains present sulci and convolutions that drastically expand cortical surface. Large cortical areas in primates, and especially in humans, are believed to be fundamental for the processing capabilities of the brain. The cortex displays different levels of organization. Anatomically, the cortex extends throughout the surface of the brain hemispheres and can grossly be divided into four lobes: the frontal lobe, the parietal lobe, the occipital lobe, and the temporal lobe. In addition, based on functional, cellular and molecular studies, the cortex can be divided in specialized regions that are involved in particular tasks. In general terms, the cortex can be divided in sensory, motor and associative areas. At the cellular level, the cortex presents a distinctive layering and columnar organization (Bystron et al., 2008). The different layers of the cortex are composed of specific type of cells with different morphologies and with different connections within the cortex and with extra cortical structures (figure 1.1). Neurons are organized into layers which are rather conserved among species; however, different sub-classifications of layers have been adopted to account for the more complex layering of primates. In the same way that these layers can have different appearance in different species, they can change depending on the region of the cortex. For example, the regions of the cortex that lack or have a small layer IV are termed agranular or disgranular cortex. In terms of the connectivity in the cortex, as general principle, deeper layer cortical neurons receive extra cortical inputs that are processed and communicated to more superficial layers. Similarly, extra-cortical outputs are sent from layer V. Regarding the columnar organization, this subject is still a matter of debate (Herculano-Houzel et al., 2008, Rakic, 2008), but is generally accepted that the columnar organization is not only an anatomical subdivision, but also a functional compartmentalization (Mountcastle, 1997). It has been noticed that vertically adjacent neurons can have similar functional properties unlike horizontal adjacent cells. The neurons that form the cortex are mainly subdivided in two classes, the excitatory projection neurons, which account for the bulk of cortical neurons, and the inhibitory interneurons that account for

around 20% of the neurons in the mature brain (Cossart, 2011, Sahara et al., 2012). Excitatory neurons are large pyramidal neurons while inhibitory interneurons are generally referred as multipolar local circuit neurons in reference to their morphology characterized by short axons. Excitatory projection neurons and inhibitory interneurons form synapses and generate cortical networks. Pyramidal neurons provide the excitation to the cortical network by releasing glutamate and interneurons control the excitability of the network by releasing GABA. Although interneurons are present in a lower number, they increase in number and complexity relative to projection neurons along with primate evolution (Hendry and Carder, 1993). Additionally, the relevance of inhibitory interneurons for proper functioning of the brain is exemplified by neuropathological conditions. For example, schizophrenic subjects present lower levels of parvalbumin (PV) and somatostatin (SST) (Morris et al., 2008), two interneuron specific proteins, and decreased total number of GAD67/parvalbumin positive interneurons (Hashimoto et al., 2003). Moreover, interneuron dysfunctions have also been reported in autism, epilepsy and mood disorders (Penagarikano et al., 2011, Faux et al., 2012).

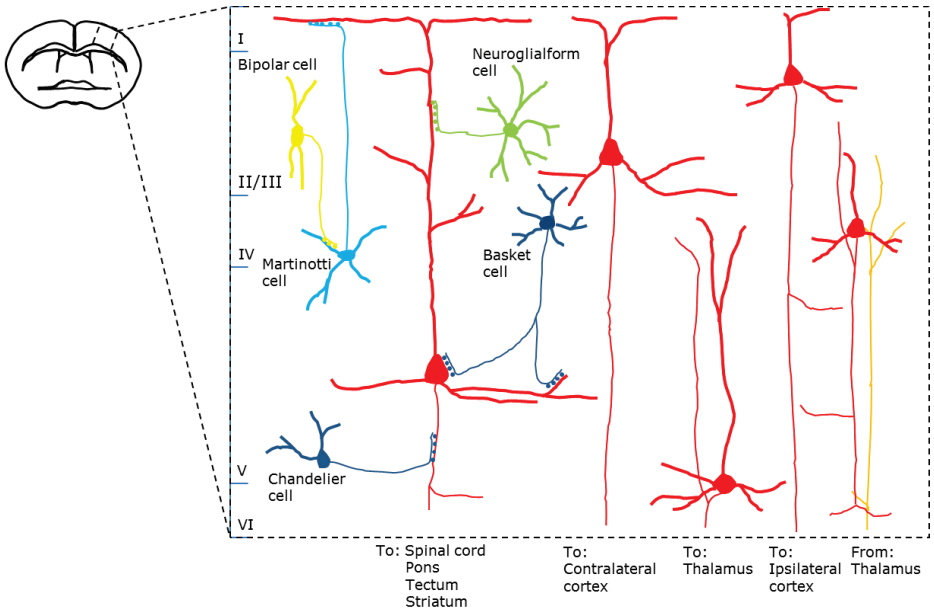

Figure 1.1. Cerebral cortex. The cerebral cortex is organized in several layers of pyramidal neurons (red cells) and interneurons (cells in different colors). Different types of interneurons are classified on the basis of their morphology, neurochemical markers, electrophysiological properties and connectivity. Extra cortical inputs from thalamus are represented in orange.

1.2 General developmental program of the cortex

Cortex development is a long process that starts at early embryonic stages and continues through the first postnatal weeks in the mouse. The cerebral cortex develops from the most anterior part of the neural tube, which is named the prosencephalon. This early structure then divides to form the telencephalon and the diencephalon. The telencephalon is the most anterior part and gives origin to the cerebral cortex from the subsequent development of the dorsal telencephalon and to the basal ganglia from the development of the ventral telencephalon. The initial phase of corticogenesis is characterized by high rate of cell proliferation and intense cell migration, two critical processes that shape the adult cortex and contribute to its functional organization (Bystron et al., 2008). The cortex develops from distinct progenitor cells populations that give rise to excitatory projection neurons, inhibitory interneurons, astrocytes and oligodendrocytes. Five different types of progenitor cells have been identified so far and they are neuroepithelial cells, radial glial cells (RGCs), intermediate progenitor cells (IPCs), short neural precursors (SNP) and the outer subventricular-zone (OSVZ) progenitors (Huttner and Kosodo, 2005, Gal et al., 2006, Attardo et al., 2008, Stancik et al., 2010). Based on their place in the cortical wall, neuroepithelial cells and RGCs are often called apical progenitors while the rest are referred as basal progenitors. The first progenitor cells present in the cortex are neuroepithelial cells. They are organized in a pseudo-stratified epithelium and by differentiation they give origin to neurons and all other progenitors (Malatesta et al., 2003). Radial glial cells have their cell body in the ventricular zone (VZ) and contact the pial surface with their radial processes that also serves as scaffold for migrating neurons. They can directly give origin to neurons (Noctor et al., 2001, Marin and Rubenstein, 2003, Noctor et al., 2004) and, more importantly, to IPCs, which give rise to most of neurons (Takahashi et al., 1995b, Kakita and Goldman, 1999, Arnold et al., 2008, Sessa et al., 2008). Intermediate progenitor cells can be identified based on their cell division which occurs in the SVZ. Although it was originally thought that they were exclusively located in the SVZ, they are also found in the ventricular zone. They have a short leading process rather than a long pial fiber while transiting through the VZ (Noctor et al., 2008), but they also show a multipolar morphology once they move onto the SVZ (Tabata et al., 2009). Short neural precursors are located in the VZ and display distinctive morphological features (Stancik et al., 2010). These progenitor cells have a ventricular endfoot and a basal process of variable length and give rise almost exclusively to neurons (Gal et al., 2006, Stancik et al., 2010). Additionally, outer subventricular-zone progenitors are abundant in the OSVZ in primates and humans and are thought to be involved in the extensive cortical surface expansion present in more evolved brains (Lui et al., 2011). Newly generated projection neurons migrate along radial fibers that guide them out of the proliferative regions to their definitive place (figure 1.2). On the other hand, radial glial cells located outside the cortex, in the medial and caudal ganglionic eminences (MGE and CGE,

respectively), will give origin to cortical interneurons. Interneuron progenitors also proliferate in the ventricular and sub-ventricular zones from the medial and caudal ganglionic eminences. Upon differentiation they give rise to interneurons or more restricted progenitors. It has been proposed that newly generated interneurons initially also follow radial fibers but soon they detach and change their direction of migration heading toward the dorsal cortex, but this is still matter of debate. Although generated outside the cortical wall, interneurons follow an inside-out pattern of placement (Valcanis and Tan, 2003) and settle in the same layers occupied by projection neurons generated at the same stage (Valcanis and Tan, 2003). Thus, projection neurons and interneurons that are born at different places in the VZ and SVZ of the growing telencephalon should get involved in a highly regulated process of differentiation (Guillemot, 2005) and cell migration (Nadarajah and Parnavelas, 2002) before they find their definitive place on the mature cortex (figure 1.2).

Every step during cortex development is controlled by the interplay between extracellular signals, which influence almost every aspect of development, and the cell autonomous machinery, that ensure a restricted pattern of gene expression (Hebert, 2005, Borello and Pierani, 2010). Different families of morphogens including Wnt, BMP, FGF and sonic hedgehog (Shh) are expressed at specific signaling centers located in the anterior neural ridge, dorsal midline and ventral midline in the developing telencephalon. In this context, Cajal Retzius cells contribute to maintain the patterning in the cortex by re-enforcing signaling centers and keeping the gradients of extracellular diffusing messengers (Borello and Pierani, 2010). These cells are an specific neuronal cell type that is only present in the developing cortex. They show different molecular identities in different places of the developing brain (Griveau et al., 2010) and are essential for proper layering of the brain by their secretion of reelin (Hevner et al., 2003). In addition, smaller diffusible molecules that are mainly known by their role as neurotransmitters in the adult brain have an important role in controlling proliferation and cell migration during cortex development (Nguyen et al., 2001, Heng et al., 2007).

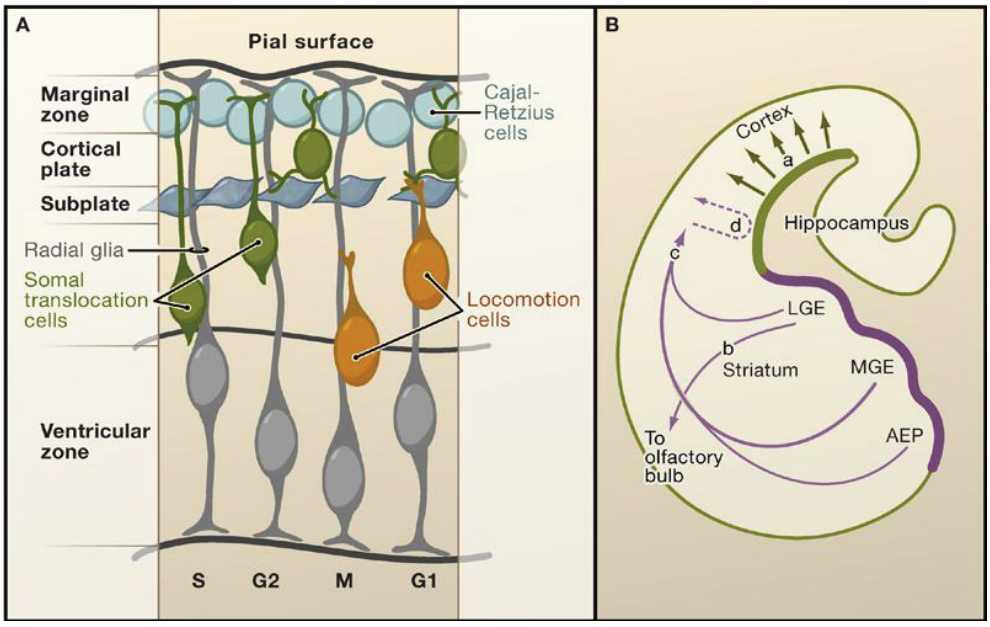

Figure 1.2. Cortex development. (A) Excitatory projection neurons are born from radial glial cells located in the ventricular zone of the dorsal cortex and migrate radially following radial glial fibers. Progenitor cells undergo interkinetic nuclear migration in the ventricular zone possitioning the cell body in different regions of the ventricular zone depending on the cell cycle progression. At this stage, the first neurons that leave the ventricular zone form the preplate. The subsequent neurons split the preplate and generate the marginal zone, which is formed by Cajal-Retzius cells and the subplate. Projection neurons use somal translocation or/and locomotion, two modes of radial migration, to reach their final position. (B) Inhibitory cortical interneurons are mainly born at the medial ganglionic eminence (MGE) and migrate tangentially to populate the cortex through different patways that dynamically change during development. When interneurons arrive at the cortex, some are directed toward the ventricular zone to then radially migrate into the cortex. LGE, lateral ganglionic eminence; AEP, anterior entopeduncular area. Figure taken from Ayala et al., 2007 (Ayala et al., 2007)

1.3 Development of cortical projection neurons

1.3.1 Origin

Progenitors of projection neurons are aligned along the dorsolateral wall of the telencephalon. They arise from neuroepithelial cells and are composed by apical and basal progenitors (Merot et al., 2009). Neuroepitelial cells first divide symmetrically, and then asymmetrically to generate radial glia and neurons (Temple, 2001). Neurons directly arising from neuroepithelial cels will form the pre-plate (Guillemot, 2005, Bystron et al., 2008). Radial glial cells are a more restricted progenitor cell type which self-renew and give birth to IPCs, projection neurons and later to astrocytes and oligodendrocytes (Malatesta et al., 2003, Rakic, 2003, Anthony et al., 2004, Mo et al., 2007). The cell types that will be generated depend on the place and time during development, but as a general rule the neurogenic potential decreases during late development contributing to the generation of astrocytes. Radial glial cells and neuroepithelial cells are characterized by the expression of RC2, nestin and BLBP (Gaiano et al., 2000, Malatesta et al., 2003). Radial glial cells are very similar to neuroepithelial cells, but they differ in some morphological features and the expression of some cellular markers that appears at the onset of corticogenesis such as GLAST, BLBP and GFAP in humans (Malatesta et al., 2003). Similar to what happens with neuroepithelial cells, radial glial cells divisions tend to be symmetrical during early corticogenesis and asymmetrical during later stages of development (Noctor et al., 2008). The division of radial glial cells is characterized by interkinetic nuclear migration, in which the nucleus oscillates between the apical and basal boundaries of the VZ in coordination with cell-cycle progression (figure 1.2). Radial glial cells also serve as scaffold for locomotion of newly generated projection neurons (Rakic, 2003). Intermediate progenitor cells, the other descendant of radial glial cells, characteristically express high levels of Tbr2. Strikingly, it has been found that intermediate progenitors may generate most cortical neurons and have a great impact in the expansion of the cortex (Sessa et al., 2008, Kowalczyk et al., 2009). The generation of IPCs is not that evident at early stages, but it dramatically changes during late development when RGCs give origin almost exclusively to IPCs. As it was previously mentioned, RGCs give rise to SNPs which provide selective amplification to the neurogenic process. Additionally, the primate brain presents other subtypes of basal progenitor cells. There, the SVZ is enlarged and subdivided in the inner SVZ (ISVZ) and the OSVZ (Zecevic et al., 2005). The OSVZ progenitors represents a different population of progenitors which express RGc markers such as GFAP, Pax6 and nestin as well as the basal progenitor marker Tbr2 (Lui et al., 2011). Unlike in the mouse, where basal progenitors quickly divide to generate neurons and astrocytes, humans OSVZ progenitors retain their self-renewal capability for a longer period, adding an amplification step that may contribute to cortical surface expansion in humans (Hansen et al., 2010, Lui et al., 2011). Homologous to primate OSVZ progenitor cells are also sparsely found in the

outer VZ of the mouse cortex. However, they have only limited self-renewal capacities (Shitamukai et al., 2011). After a neurogenic division, radial glial cell-derived neurons undergo four distinct phases of migration: phase one, rapid movement to the SVZ; phase two, migratory arrest in the SVZ; phase three, retrograde migration toward the ventricle; and the phase four, which consist on rapid movement towards the cortical plate (Noctor et al., 2004). Cells in each of these phases display distinctive morphological features. After reaching the SVZ-IZ cells transit through a multipolar stage. Multipolar cells are highly dynamic and extend and retract processes while in the SVZ. In this stage they are loosely attached to radial glial cells and can move tangentially. After that, during the retrograde migration towards the ventricle, neurons extend a main leading process towards the ventricle. Finally, once those neurons have reached the VZ, they reverse their polarity. They transiently adopt a bipolar morphology and start their migration towards the cortex (Kriegstein and Noctor, 2004, Noctor et al., 2004). During radial migration neurons may display two modes of cell migration, somal translocation and locomotion. Somal translocation consists in the progressive movement of the cell body promoted by the retraction of the long leading process that contacts the pial surface. By contrast, cells in locomotion display a short leading process that does not contact the pial surface and has a constant length (Nadarajah et al., 2001). Successive waves of neurons generated in the VZ and SVZ migrate radially and locate themselves more superficially than the previous generated cells, producing the characteristic layered patter of the cortex (Bystron et al., 2008). This pattern of generation is referred as inside-out layering.

Early studies on brain development culminated with the establishment of the concept known as the radial unit hypothesis. This concept offers an explanation about brain development in two parts: (a) the progeny of ventral progenitors expand radially and this leads to the establishment of ontogenic columns. (b) Columns as single processing units could be influenced by external stimuli, such as thalamic innervation, and this would further define cortical areas of variable size, cellular composition, and function (Lui et al., 2011). This explains the expansion of the cortex in the radial dimension, the generation of columns of ontogenically related cells that share a function, and how the ventricular structure is projected in the developing cortex. However, this concept has been proven to be an over simplification because it does not consider the dynamics of proliferation and differentiation displayed by the different progenitors cells.

1.3.2 Transcriptional control of projection neuron development

Proliferation, cell fate determination and cell migration are dependent on the cell autonomous machinery and the activation of several transcription factors (Guillemot, 2007). Homeodomain (HD) proteins, such as Pax6 have been found to play an important role in the neuronal differentiation of human radial progenitors. This factor promotes proliferation of radial glial cells and is needed

for their differentiation towards basal progenitors and neurons (Mo and Zecevic, 2008). A similar function is played by Tbr2 and Sox2 transcription factors that promote the transition from radial glial cells towards basal progenitor cells (Sessa et al., 2008). Additionally, basic helix-loop-helix proteins (bHLH) such as Mash1 or neurogenin 2 (Ngn2) have been found to promote neuronal differentiation of progenitor cells (Guillemot, 2007). More generally, recent evidence suggests that corticogenesis is controlled by the interaction between HD and bHLH transcription factors (Guillemot, 2007).

1.3.3 Mechanisms involved in dorsal progenitor cell proliferation

In addition to the cell autonomous machinery, environment cues present in the developing brain have a profound impact on the control of projection neurons development (Borello and Pierani, 2010). Morphogen gradients are established along the developing brain during early development generated by the coordinated activation of localized sources of soluble factors. Some of the most important signaling pathways involved in the development of the brain are Notch, Wnt, fibroblast growth factor (Fgf), Shh, TGFβ, retinoic acid, BMP (Molnar et al., 2007), and neurotransmitters (Nguyen et al., 2001). All these pathways ultimately regulate intracellular mechanisms and orchestrate molecular networks that control various signal transduction cascades or the epigenetic status of the chromatin (figure 1.3). Specifically, Slit proteins, a soluble extracellular signal partner of Robo receptors, have been shown to influence neurogenesis by a mechanism upstream the Notch pathway that involves transcription of Hes1 and prevents excessive generation of IPCs (Borrell et al., 2012). In a similar manner, Eph/ephrin signaling may influence progenitor cell division through a mechanism involving ZHX_2 nuclear signaling (Wu et al., 2009). Knockout of ephrin-B1 leads to cell cycle exit and loss of progenitor cells (Qiu et al., 2008). Complementary mechanisms have been proposed to involve the activation of regulator protein signaling (RGS) (Qiu et al., 2008) and G beta-gamma (Sanada and Tsai, 2005). Wnt/β-catenin signaling has also been established as a regulator of proliferation and differentiation. Activation of this pathway suppresses neuronal differentiation (Backman et al., 2005, Mutch et al., 2010) and promotes progenitor self-renewal. Moreover, Wnt/β-catenin signaling disruption influences progenitor cell identity along the whole telencephalon (Gunhaga et al., 2003, Backman et al., 2005). It has been shown that β-catenin afect the number of progenitors without increasing cell cycle length, but instead affecting the dession to differenciate (Chenn and Walsh, 2002). At the molecular level, wnt signaling leads to inactivation of GSK3β leading to the accumulation of active N-terminal unphosphorylated β-catenin. Additionally, n-cadherin engagement activates Akt via phosphorylation. Active Akt can leads to increased β-catenin stability indirectly by phosphorylation and inhibition of GSK3β or directly by phosphorylation of β-catenin (Zhang et al., 2010). Downstrem β-catenin, transcriptional activation influences the decision of precursors to proliferate or differentiate (Peifer and Polakis, 2000, Woodhead et al., 2006). Fgf-mediated

signaling is another example of a secreted molecule that controls projection neuron development. Fgf signaling acts by establishing gradients of expression of transcription factors such as Pax6, Sp8 and Emx2 which ultimately influence the rostro-caudal identity in the cortex (Sansom and Livesey, 2009). Moreover, Fgf shortens the G1 phase promoting proliferative divisions (Lukaszewicz et al., 2002). Another signaling pathway that signal directly to the nucleus to exert its effect is the iniciated by transforming growth factor beta (Tgf-β). Ths factor controls cell cycle progression at the G1/S checkpoint through induction of p21 and p57. Activation of Tgf beta receptor 1 (TGFR-1) leads to the activation of Smad 2/3 and subsequently to the activation of Smad4 which translocate to the nucleus and induces the sintesis of p21 which block cell cycle progression (Siegenthaler and Miller, 2005). At the same time Tgf-β promotes differencitation in the presence of Nedd9 that releases the negative feedback imposed by Smads6 and 7 (Vogel et al., 2010). In additon to nuclear singnaling, directly inhibion of cell cycle proteins occur by certain growth factors. It has been shown that IGF-1 controls G1/S cell cycle progression by regulating the expression of cell cycle-related genes such as cyclin D1 and E, $p27^{KIP1}$ and $p57^{KIP2}$ (Mairet-Coello et al., 2009). This effect involves the activation of the PI3K/Akt pathway. Moreover, complementray studies have shown that RhoG triggers the activation of PI3K and GSK-3b which promotes proliferation independently of Rac1, another compontent of the Rho pathway that modulate the expression of cyclin D (Leone et al., 2010). MEK/ERK pathway is also activated but does mediate IGF-1 proliferation effect (Mairet-Coello et al., 2009). A similar mechanism medites the effect of bFGF, which induces a rapid activation of cyclin dependent kinase 2 (CDK2) in a cyclin E dependent way (Li and DiCicco-Bloom, 2004). Cell cycle regulators such as the cyclin-dependent kinase inhibitors (CKI) $p57^{KIP2}$ and $p27^{KIP1}$ have been shown to be inportant regulators of G1 phase and dinamically control apical and basal progenitors cell division (Mairet-Coello et al., 2012). While $p57^{KIP2}$ regulates both RGCs and IPCs cell cycle, $p27^{KIP1}$ controls only IPCs proliferation. Moreover, shortening G1 by overexpression of CDK4 and cyclin D1 delays cell cycle exit and promotes the generation of IPCs while inhibited neurogenesis (Lange et al., 2009). Additionally, G1 shortening may promote RGC asimetric divisions that increase the IPCs pool (Pilaz et al., 2009). One potential explanation to the effect of cell cycle length in the regulation of cell differentiation is the control of proteins involved in cell differentiation. Several signaling molecules can have a direct influence on the regulation of gene expression (Sansom and Livesey, 2009). This is the case of Notch, which controls signaling pathways in contacting cells, down-regulating the expression of the pro-neural gene, neurogenin-2 (Ngn2) and stabilizing the radial glial phenotype (Gaiano et al., 2000, Itoh et al., 2003, Kageyama et al., 2008, Shimojo et al., 2008). This is a dynamic signaling cascade that presents oscillatory expression of several of its components during cell cycle. In this way, cell fate determination is precisely controlled by a biological clock mechanism (Hirata et al., 2002, Hatakeyama et al., 2004). Direct phosphorylation of Ngn2 occurs by CDK which block Ngn2 ability to

promote the transcription of pro-neural genes and cause differentiation. Conversely, during a longer G1 phase, where CDK levels remain low and CDK inhibitors accumulate, un-phosphorylated Nrg2 remains available and promotes differentiation (Ali et al., 2011).

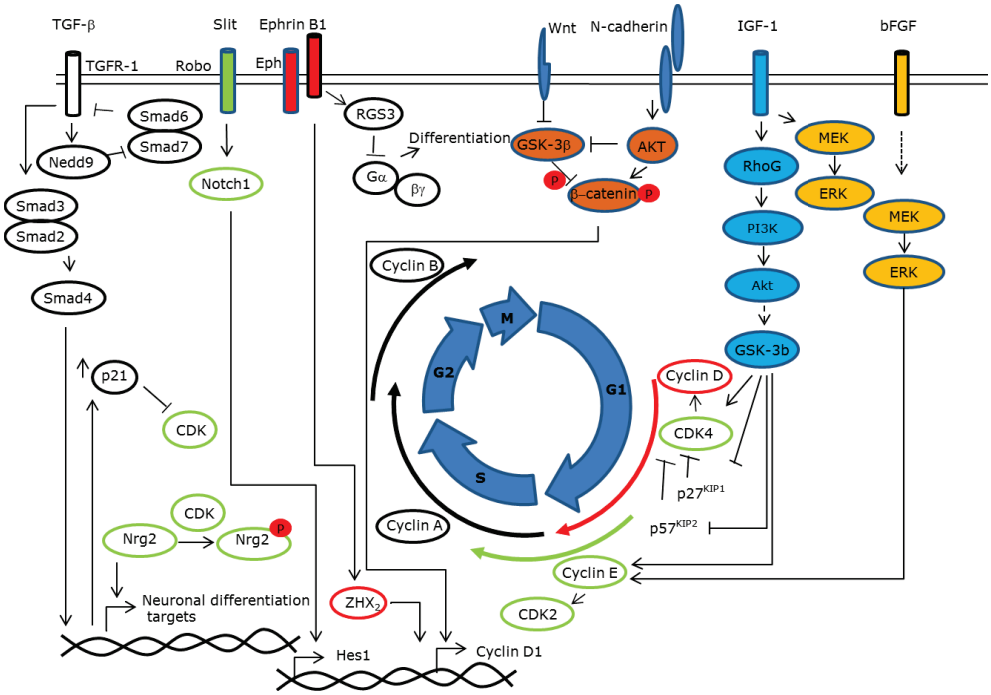

Figure 1.3. General mechanisms involved in dorsal progenitor cell proliferation. Progression through cell cycle is driven by cyclin dependent kinases (CDK) that require cyclins to be enzymatically active. Regulation of the G1-S transition is critical for dorsal neurogenesis. Different signaling cascades are activated in response to extracellular cues present in the extracellular environment of cortical progenitor cells and modulate their cell cycle progression.

1.4 Development of cortical interneurons

1.4.1 Origin

In most species, cortical interneurons are born in the ventral telencephalon (Cobos et al., 2001, Tanaka et al., 2011). Most cortical interneurons are generated in the medial and the caudal ganglionic eminences (MGE and CGE, respectively). Additionally, the lateral ganglionic eminence (LGE) is considered as a minor source of cortical interneurons (de Carlos et al., 1996, Anderson et al., 1997, Tamamaki et al., 1997, Xu et al., 2004, Wonders and Anderson, 2006) dedicated to populate the olfactory cortex and olfactory bulb (Wichterle et al., 1999). Analysis of the expression of the Lim homeodomain gene *Lhx6*, a marker of MGE and CGE-derived cells (Grigoriou et al., 1998), shows an expression pattern that marks a stream of interneurons invading the neocortex (Lavdas et al., 1999). Consequently, in vivo transplantation experiments have confirmed that the MGE is the main source of tangentially migrating neurons destined for the developing neocortex (Wichterle et al., 1999). In addition, the interference with Nkx2.1 expression, which specifies MGE-derived interneuron phenotype, almost completely depleted the neocortex in interneurons (Sussel et al., 1999). However, the cortical interneurons deficit is less pronounced than in the case of the disruption of Dlx1/2 transcription factors, which are more widely expressed in the LGE, MGE and CGE (Anderson et al., 1997, Anderson et al., 2001), suggesting that there should be other major source of cortical interneurons. More recently, the CGE (McGahon et al., 2007) was also found to be a source of cortical interneurons (Nery et al., 2002, Nery et al., 2003). Besides their contribution to the cortex, all the eminences also contribute to interneurons in other regions of the brain (figure 1.2). The MGE can also give origin to hippocampal and striatal interneurons (Marin et al., 2000), while the CGE mainly gives origin to hippocampal interneurons (Pleasure et al., 2000) although in a lower proportion compared to the LGE and MGE (Nery et al., 2002). An exception to the general ventral origin of interneurons is the primate brain, where interneurons are also generated in the VZ and SVZ of the dorsal forebrain (Letinic et al., 2002, Petanjek et al., 2009). More recently, fate mapping experiments have identified radial glial cells present in the SVZ as the progenitor cells that give rise to these primate specific interneurons (Yu and Zecevic, 2011). Cortical interneurons are a very diverse population of neurons, and different subclass identities have been established based on immunocytochemistry, electrophysiology, positioning and relationship to projection neurons (Flames and Marin, 2005, Gelman and Marin, 2010, Anastasiades and Butt, 2011). Concerning their origin, interneurons can be roughly classified by the differential expression of selected calcium binding proteins (Wonders and Anderson, 2005). Specifically, MGE derived interneurons are recognized for being calretinin (CR) negative (Lavdas et al., 1999), calbindin positive (Sussel et al., 1999), and most of them are also parvalbumin (PV) and somastostatin (SST) positive (Wichterle et al., 1999, Xu et al., 2004, Zhao et

al., 2008). Complementary, CGE derived interneurons are mostly positive for CR (Lopez-Bendito et al., 2004, Xu et al., 2004).

Cortical interneurons are generated in the VZ of the ventral telencephalon following the emergence of ganglionic eminences (around E9.5) and move independently towards the surface of the ventral pallium (de Carlos et al., 1996). At E13, radial glial cells appear in the ventral telencephalon and along with this, the first ventral radial glia-descendent interneurons. Resembling the generation of projection neurons, interneurons follow radial glial fibers in close contact while they migrate in the ventral telencephalon (de Carlos et al., 1996), but then, they detach and migrate independently towards the cortex. Ventral radial glial cells also give rise to BLBP positive basal progenitors (Anthony et al., 2004), which subsequently may generate migratory interneurons. Recent evidence suggests that neurogenic basal progenitors for interneurons are first found in the tangential migratory stream and later on in the dorsal telencephalon, where they contribute to the intra-cortical generation of interneurons (Wu et al., 2011). The generation of the different subgroups of interneurons occurs early at the MGE and CGE during the time of cell fate determination (Wonders and Anderson, 2006). Studies using transplantation have shown that MGE or CGE-derived interneurons retain the ability to be specified into subgroups, expressing specific neuronal markers (Xu et al., 2004), and displaying defined layer identity (Valcanis and Tan, 2003) independently of the environment and extracellular cues. Remarkably, these transplantation studies confirmed the strong tendency of CGE derived cell to generate CR positive interneurons while MGE derived cells mainly give rise to SST and PV expressing cells. Similar results were found using electrophysiological approaches, where MGE and CGE produced distinct populations of interneurons, according to the idea that phenotype is established early at the proliferative regions (Butt et al., 2005).

1.4.2 Signaling molecules involved in interneuron generation

In general, the environmental cues that influence interneuron development are not different from the ones that control the development of excitatory neurons, and the development of other organs. One of these general cues is Wnt, which promotes interneuron differentiation (Gordon and Nusse, 2006, Paina et al., 2011). In fact, the expression of Wnt5a is regulated by Dlx2 (Paina et al., 2011). Notch signaling is also involved in the generation of interneurons. Experimental evidence supports the idea that increased Notch signaling blocks cell differentiation in progenitors (Yun et al., 2002). Specifically, it has been shown that the interplay between Dlx1/2 and Mash1 controls the level of activation of Notch signaling and this is how they exert their functions (Yun et al., 2002). Sonic hedgehog signaling (Shh) is another powerful influence for interneuron generation. The coordinated action of shh and FGF8 influences cell fate determination by affecting Nkx2.1 patterning (Kohtz et al., 1998, Fuccillo et

al., 2004, Storm et al., 2006). Reduction of Shh results in less Nkx2.1 positive cells and, as a direct consequence of this, affects the generation of PV and SST interneurons in cortex (Xu et al., 2005, Xu et al., 2010). The influence of Shh in interneuron generation has been proposed to occur by antagonizing Gli3R first (Rallu et al., 2002, Gulacsi and Anderson, 2006) and BMP signaling later during development (Gulacsi and Lillien, 2003). On the other hand, Shh expression is induced by the coordinated expression of Lhx6 and Lhx8 establishing a control loop for interneurons generation (Flandin et al., 2011). Another pleiotropic signaling molecule involved in the process is retinoic acid which also down-regulates the expression of Nkx2.1 (Marklund et al., 2004). Specifically, it has been described that retinoic acid can affect interneuron generation (Chatzi et al., 2011), their diversity, and their organization in the cortex (Crandall et al., 2011). Growth factors can also influence interneuron generation and development. GDNF can change interneuron development in multiple ways, promoting the GABAergic phenotype, their morphological differentiation and stimulating their motility (Pozas and Ibanez, 2005). Thus, cell autonomous and non-autonomous signaling cascades are both needed for proper interneuron development (Lupo et al., 2006).

1.4.3 Transcriptional control involved in interneuron generation

In parallel to the extensive information about the cell autonomous machinery and the transcriptional control of dorsal neurogenesis, there is extensive evidence regarding the transcription factors involved in interneuron specification, differentiation and migration (Wonders and Anderson, 2006). One of the most important transcription factors that control the generation of interneurons is Mash 1. Mash 1 mutant embryos show abnormal development of the ventral brain characterized by the lack of MGE delimitations, decreased progenitor proliferation and misspecification of cell types (Horton et al., 1999). These defects have been explained by the interplay of Mash1 and Dlx2 in the control of Notch signaling (Yun et al., 2002) and other important transcription factors (Long et al., 2007). This illustrates the interaction between transcription factors, which is a general phenomenon in the control of interneuron development (figure 1.4) (Long et al., 2009). Additionally, Dlx genes can function independently of *Mash1*. Dlx 1 and Dlx2 genes are expressed in the LGE, MGE, the septum of the embryonic brain (Bulfone et al., 1993) and the CGE (Nery et al., 2003). These genes have a direct influence on the generation of interneurons and this is exemplified by the Dlx-1/2 mutant mice where the number of GABA-reactive cells is significantly reduced in the cortex (Anderson et al., 1997). It has been shown that these transcription factors promote the differentiation towards the interneuron phenotype (Anderson et al., 1999), and that they inhibit oligodendrogenesis via Olig2 repression (Petryniak et al., 2007). Dlx 1 is also cell-autonomously needed for tangential migration (Anderson et al., 2001) and in the long term, Dlx genes also control interneuron survival (Cobos et al., 2005). A similar role is played by the transcription factor Nkx2.1. In the

absent of this factor only a few interneurons populate the cortex (Sussel et al., 1999), and there is a total lack of PV and SST expressing cells (Du et al., 2008). Alternatively, MGE-derived interneurons acquire characteristics of LGE derived interneurons that do not migrate into the cortex (Sussel et al., 1999). More recently, it has been found that this factor contributes to the generation of MGE derived, but not CGE derived interneurons (Nery et al., 2003). As a consequence, a subset of interneurons are completely absent in the hippocampus (Pleasure et al., 2000). Another important transcription factor for interneuron specification and migration is the LIM-homeobox gene Lhx6, which is a characteristic marker of MGE (also in CGE but not LGE), and is expressed soon after cell cycle exit in migrating interneurons derived from this eminence (Lavdas et al., 1999, Liodis et al., 2007). This factor controls the generation of parvalbumin and somatostain expressing interneurons (Denaxa et al., 2012) and is a direct target of Nkx2.1. Lhx6 overexpression can rescue the neurochemical and morphological aspects of Nkx2.1 knockout (Du et al., 2008). As interneurons advance towards their final destination, the expression of the mentioned transcription factors evolves and lead to the establishment of subsets expressing DLx1, Lhx6 or a combination of both (Cobos et al., 2005, Wonders and Anderson, 2006). In humans, the same transcription factors Mash1, Nkx2.1, Dlx1,2, and Lhx6 are important for interneuron development. However, their expression is not restricted to the ventral brain, but extends to the dorsal cortex supporting the idea that, in humans, interneurons are born dorsally and ventrally from several progenitor sources (Jakovcevski et al., 2011).

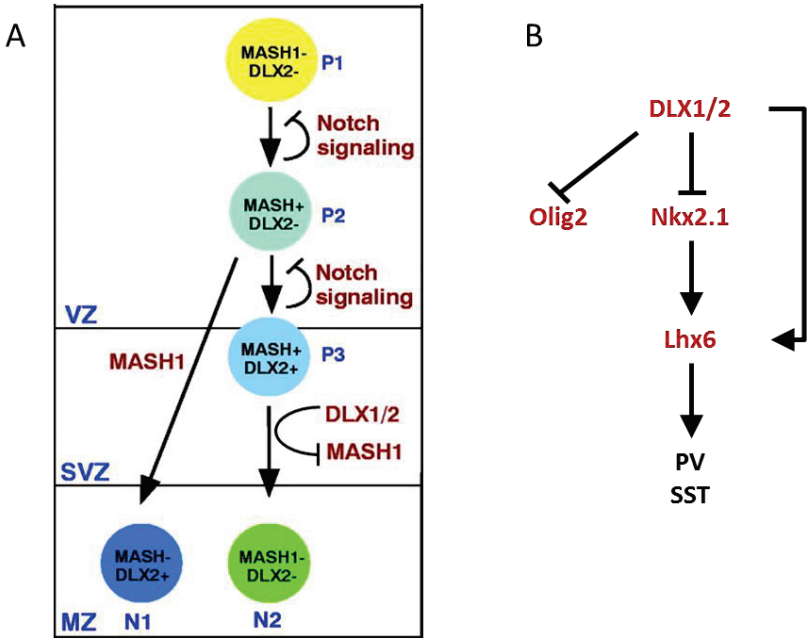

Figure 1.4. Interneuron progenitors are affected by a complex interaction between transcription factors.
A) Progenitor cells (P1-P-3) in ventral telencephalon generates immature neurons (N1, N2). MASH1-/DLX2- neuroepithelial progenitors (P1) give rise to MASH1+/DLX2- (P2) and MASH1+/DLX2+ (P3) progenitors around E10 and to MASH1-/DLX2+ (N1) neurons at E10.5 and to MASH1-/DLX2- neurons after E11.5 (N2). *Mash1* is required cell-autonomously to generate P2 and N1 cells. *Mash1* is also required non cell-autonomously, via Notch-dependent lateral inhibition, to prevent neighboring cells from acquiring the P3 fate prematurely. A potential mechanims to explain the generation of MASH1-/DLX2- neurons is the repression of *Mash1* by *Dlx1/2* genes. Ventricular zone (VZ), sub-ventricular zone (SVZ) and mantle zone (MZ). Figure adapted from Long *et al.*, 2009. B) *Dlx1/2* genes repress Nkx2.1 and Olig2, but promote Lxh6 expression. Additionally, NKX2.1 activates Lhx6 to control terminal differentiation of somatostatin (SST) and parvalbumin (PV) expressing interneurons.

1.4.4 Tangential migration

After cell fate determination, immature interneurons engage in tangential migration to travel a long journey from the basal forebrain towards the cortical wall. The earliest generated interneurons migrate at around E11 and rapidly move to the pre-plate at the surface of the developing cortex. MGE-derived interneurons further invade the cortex by migrating through specific paths in the upper cortical plate (CP), the intermediate zone (IZ) and SVZ (figure 1.2). In the same way, MGE derived interneurons widely spread to the anterior and posterior cortex while CGE derived interneurons are mainly directed toward the caudal cortex (Yozu et al., 2005). Consequently with the differential destination of interneurons, LGE-derived interneurons migrate rostrally without entering into the cortex (Wichterle et al., 1999). As it was mentioned, interneurons are positioned in an inside-out pattern according to their birthdate (Miller, 1985, Fairen et al., 1986, Peduzzi, 1988). To accomplish this, interneurons must change their direction once they have reached the cortex. It has been described that the first step in this change is a ventricle-directed movement (Nadarajah et al., 2002). After that, they remain in the VZ for some time and then migrate radially (Hevner et al., 2004)(figure 1.2). Today, it is known that interneurons reach the cortical wall by migration on various tangential and multidirectional paths that are dynamically remodeled during embryogenesis (Marin and Rubenstein, 2001, Metin et al., 2006, Tanaka et al., 2006, Wonders and Anderson, 2006, Ayala et al., 2007, Inada et al., 2011).

During migration, interneurons regularly split their leading process to produce new branches. Then, the generated branches grow in different directions. Like if sensing the environment, only one of the branches is stabilized while the others disappear. In this way, the cell is able to change direction continuously (Martini et al., 2009, Valiente and Martini, 2009). On the other hand, the nucleus at the rear of the cell displays an intermittent movement, translocating from its place towards the swelling that forms in between the nucleus and the growth cone. The transient swelling contains the centrosome and the Golgi outpost. The generation of the swelling and the translocation of the centrosome precede each nuclear translocation. Growth cones stop their migration and often split during nuclear translocation.

1.4.5 Molecular mechanisms involved in the control of interneuron migration

First attempts to understand the mechanisms that guide interneuron migration suggested that they may follow cortico-thalamic axons while sensing the extracellular environment (Parnavelas, 2000). Since then, several attractive or repulsive guidance cues have been shown to influence interneuron migration. These cues are believed to be sensed by growth cones. Consequently, most of

the molecules involved in axon guidance have been found to influence interneuron migration (Figure 1.5). However, the specific cellular and molecular mechanisms that drive the migration of interneurons have just begun to be unveiled (Bellion et al., 2005, Schaar and McConnell, 2005, Ayala et al., 2007, Tanaka et al., 2009, Marin et al., 2010, Courtes et al., 2011, Inamura et al., 2012).

Tangential migration is probably initiated by repulsion factors present at interneuron birth place (Marin, 2013). Migrating interneurons express EphA4 receptors which are activated by ephrin-A5 and have a repulsive effect in the MGE (Zimmer et al., 2008, Rudolph et al., 2010). This effect depends on Src family kinases (SFKs) activity, which can further inactivate contacin and lead to a reduced activity of the actin-related protein (Arp) 2/3 complex. Reduction on the activity of this complex triggers the breakdown of the actin cytoskeleton that is essential for cytoskeletal remodeling (Huang et al., 1997). Complementarily, semaphorins 3A (Sema 3A) and 3F prevent interneurons from entering the striatum (Marin et al., 2001). This occurs via the activation of neuropilin receptors (Nrp1 and Nrp2) and their signal transduction subunit, plexin, which are expressed in cortical interneurons. At the same time, Robo1 has been shown to interact with Nrp1 receptors and control their expression level and thus semaphoring signaling (Hernandez-Miranda et al., 2011). However, in vivo experiments have not detected migratory defects in Robo ligands, Slits and Robo deficient mice (Marin et al., 2003). Recently, it has been demonstrated that activation of Nrp1 results in the activation of LIM kinase (Limk) 2, which phosphorylate the actin-depolymerizing protein Cofilin (Andrews et al., 2013). Phosphorylation of Cofilin stabilizes actin filaments decreasing the amount of free monomers and limiting migration. Additional studies have suggested that interaction of Rnd1 with Plexin-A1 leads to a conformational change that activates RhoA upstream Limk (Zanata et al., 2002).

New born interneurons respond to several factors that enhance their motility. Migration is stimulated by brain-derived neurotropic factor (BDNF) and neurotrophin-4 (NT4) (Polleux et al., 2002, Levitt et al., 2004). These two factors have been involved in controlling migration of CB and CR positive interneurons. BDNF and NT4 act via similar signaling cascades causing auto phosphorylation of TbrkB receptors in a calcium dependent process that leads to activation of PI3-kinase and subsequently to the phosphorylation of AKT (Polleux et al., 2002). An additional growth factor involved in promoting interneuron migration is the hepatocyte growth factor (HGF). This factor activates MET receptor and its disruption gives results that are consequently with migration delay (Powell et al., 2001, Martins et al., 2011). MET receptors have tyrosine kinase activity and may trigger activation of Ras and through this or independently of PI3K. Evidence suggest that Nrg1 can act as a short and long range attractant factor and modify the routes of tangential migration by creating a permissive corridor (Flames et al., 2004, Martini et al., 2009, Marin et

al., 2010), which leads to the integration of interneurons into specific cortical circuits (Marin, 2012). However, other studies have shown that Nrgs are expressed in patterns that outline the paths of the migrating ErbB4-expressing interneurons, suggesting that the ErbB4-expressing interneurons avoid the Nrg expressing domains and this signal works as a repellent (Li et al., 2012). The glial cell line-derived neurotrophic factor (GDNF) has also been found to stimulate interneuron migration and act as a powerful chemoatractan. Animals lacking GDNF receptors show an altered distribution of CB-expressing migrating interneurons at E15 suggesting an effect on stream specification (Pozas and Ibanez, 2005). This signaling molecule acts via the GPI-anchored receptor GFRa1 that has been shown to trigger ERK1/2 and AKT phosphorylation.

Besides repulsing and stimulating cues, certain factors provide fine control over directionality. This is the case of the stromal cell-derived factor-1 (SDF1) which regulates intracortical migration of interneurons influencing their layer positioning and preventing premature invasion of the cortex (Stumm et al., 2003, Lopez-Bendito et al., 2008). This effect is achieved by decreasing branching frequency and is mediated by the activation of CXCR4 receptors. These receptors function as Gi-coupled receptors that inhibit adenosine cyclase, lowering cAMP levels and decreasing PKA activity (Lysko et al., 2011). Additionally, branching dynamics is influenced by Rho/ROCK signaling (Martini et al., 2009, Valiente and Martini, 2009). It is worth noticing that Rho-family GTPases mediate different responses, sometimes opposite, depending on the cascade involved (Lowery and Van Vactor, 2009). This pathway was demonstrated to act upstream myosin light chain kinase (MLCK) that phosphorylate myosin II (Godin et al., 2012) during control of interneuron migration. Non-muscle type II myosins are primarily regulated by phosphorylation at Ser-19 and Thr-18 of the myosin light chain (MLC). Additionally, this phosphorylation can take place in a calcium calmodulin dependent process (Emmert et al., 2004). The saltatory pattern of migration displayed by interneurons requires actomyosin contractions in the rear part of the cell (Bellion et al., 2005, Schaar and McConnell, 2005, Martini and Valdeolmillos, 2010). Myosin accumulates periodically in the trailing process and, in interaction with the actin filaments, propels the nucleus toward the centrosome. This cycle of relaxation and contraction must be tightly regulated to ensure proper migration.

Termination of interneuron migration has been proposed to occur via intrinsic and extrinsic mechanisms (Inamura et al., 2012). Intrinsic mechanisms involve the increase in intracellular chloride and activation of $GABA_AR$, an event that modulates calcium influx (Bortone and Polleux, 2009); while extrinsic mechanisms are thought to be mediated by the extracellular environment (Inamura et al., 2012). Other neurotransmitters have also been involved in interneuron migration and this will be discussed more extensively.

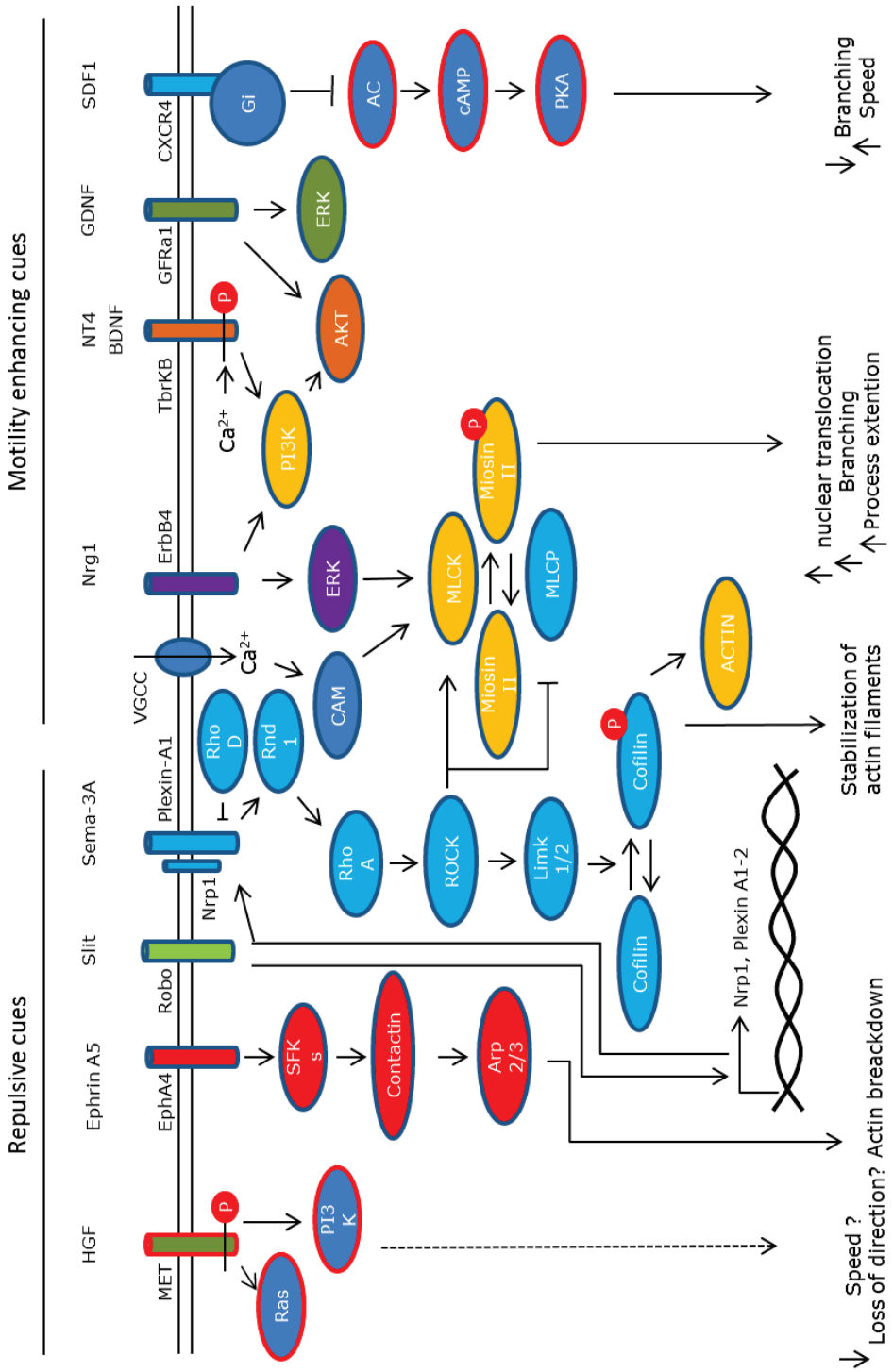
Repulsive cues
Motility enhancing cues
HGF
Ephrin A5
Slit
Sema-3A
Nrg1
NT4
BDNF
GDNF
SDF1
MET
EphA4
Robo
Nrp1
Plexin-A1
ErbB4
TbrKB
GFRa1
CXCR4
Gi
AC
cAMP
PKA
Branching
Speed
ERK
AKT
PI3K
Ca2+
VGCC
Rho D
Rnd 1
CAM
MLCK
MLCP
Miosin II
nuclear translocation
Branching
Process extention
Ras
PI3 K
SFK s
Contactin
Arp 2/3
Rho A
ROCK
Limk 1/2
Cofilin
ACTIN
Stabilization of actin filaments
Nrp1, Plexin A1-2
Speed ?
Loss of direction? Actin breackdown

Figure 1.5. Signaling pathways involved in the control of interneuron migration. During migration, a variety of extracellular molecules activates different signaling pathways that ultimately control the cytoskeleton remodeling. Depicted components have been specifically investigated on interneurons. Components of the same pathway are shown in the same color. Some pathways such as the initiated by GDNF are still only partially understood in the context of interneuron migration. Dotted arrow indicates a possible connection.

1.5 The role of neurotransmitters during brain development

These small molecules have a remarkable impact during projection neuron development. Although, they are generally known for their contribution to neurotransmission, they are also present during early embryonic development before the formation of active synapses (Nguyen et al., 2001)(figure 1.6). GABA and glutamate are two of the most studied neurotransmitter systems during development and they contribute to several aspects of corticogenesis (Represa and Ben-Ari, 2005). Pioneer electrophysiological experiments demonstrated the presence of GABA (Owens et al., 1999) and glutamate receptors in cortical progenitor cells (LoTurco et al., 1990, Lo Turco and Kriegstein, 1991, LoTurco et al., 1991). GABA and glutamate display a differential effect promoting cell proliferation in the VZ, while inhibiting it in the SVZ (Haydar et al., 2000). More detailed experiments have demonstrated that these two neurotransmitter are able to depolarize the cell membrane (Owens et al., 1996) and inhibit DNA synthesis in the VZ (LoTurco et al., 1995). Evidence suggests that this effect on DNA synthesis is most probably due to the depolarization and subsequent activation of voltage gated calcium channels (VGCC) as has been suggested for GABA in the control of proliferation of postnatal striatum precursor cells (Nguyen et al., 2003).

In neurons, the potassium-chloride cotransporter 2 (KCC2) actively reduces the intracellular concentration of chloride, transforming the opening of a chloride channel into a hyperpolarizing stimulus (Lee et al., 2005, Zhu et al., 2005). Conversely, the sodium-potassium-chloride co-transporter (NKCC1) exerts the opposite effect and thus increases the intracellular concentration of chloride transforming the ion channel-mediated chloride flux into a depolarizing signal. Thus, the expression levels and functionality of one or the other chloride co-transporter will influence the direction of the chloride flux. During development, chloride gradients evolve in function of the expression of chloride transporters. Electrophysiological measurements, using the perforated patch clamp technique, indicate that the intracellular chloride concentration is 25 mM until the first postnatal week and then it falls to 10 mM during the second week (Yoshida et al., 2004). Considering that the resting membrane portential of the cell remains constant at about -60 mV, this change in chloride concentration is enough to reverse the chloride flux. In consequence, $GABA_AR$ activation during embryonic and early postnatal development induces the depolarization of the cell membrane which in turn may activate VGCC. Compared to glutamate-mediated depolarization, chloride efflux is thought to be less harmful to the cell since there is no risk to induce excitotoxicity. This is due to the fact that the equilibrium potential of chloride is closer to the resting membrane potential of the cell which generates small chloride currents compared to the amplitude of glutamate elicited sodium currents.

The effect of neurotransmitters is not only restricted to the control of cell proliferation as they also have an influence on cell migration (figure 1.6). It has been reported that GABA receptor antagonists can retard radial migration in the cortical plate (Behar et al., 2000) and hippocampus (Manent et al., 2005). In consequence, *in vivo* experiments have demonstrated that activation of GABA receptors type A ($GABA_AR$) is needed for proper lamination of the cortex. Failure of proper $GABA_AR$ activation leads to the formation of heterotopic clusters formed by glutarmatergic and GABAergic neurons (Heck et al., 2007), interfering also with the morphological maturation of neurons (Cancedda et al., 2007). The effect of $GABA_AR$ activation on cell migration starts early during cortex development, first promoting cell migration and then, inhibiting this process in the SVZ at postnatal stages (Bolteus and Bordey, 2004). The effects of GABA on cell migration are not only restricted to the activation of $GABA_AR$, as are also mediated by the activation of the metabotropic type B GABA receptors ($GABA_BR$) (Behar et al., 2001). The molecular mechanisms downstream the activation of $GABA_AR$ and $GABA_BR$ have both been linked to changes in the intracellular calcium dynamics, which is known to be important for proper cell migration (Komuro and Rakic, 1998). Specifically, the effects of $GABA_AR$ activation are linked to the depolarizing effect of the efflux of chloride mediated by this channel. Membrane depolarization triggers the activation of VGCC and hence allows the entrance of extracellular calcium ions. Besides this, there is evidence that $GABA_AR$ activation could also be involved in the control of calcium release from intracellular stores independently of membrane depolarization (Bolteus and Bordey, 2004). While GABA signaling has been involved in various developmental regulations, mice defective for GABA synthesis do not show major cortical defects (Ji et al., 1999). This unexpected result has been explained by the possible compensation exerted by other neurotransmitters such as glutamate or glycine. Much less is known about the role of other related neurotransmitters during projection neurons development. Interestingly, it has been reported that glutamate acting via NMDA receptors is a potent chemoattractant signal that affect radial migration from the VZ and SVZ (Behar et al., 1999). Indeed, it has been shown that glutamate is more potent than GABA in the modulation of this process. Moreover, glutamate is more concentrated in the outer layers of the cortex and is distributed in a gradient along the radial axis (figure 1.6).

Specifically in the case of interneurons, it has been found that neurotransmitters actively control cell migration, have a strong influence on cell morphology (Maric et al., 2001, Nguyen et al., 2001), and have a crucial role in the establishment of the first synaptic connections (Represa and Ben-Ari, 2005). Non cell-autonomous signals in the form of extracellular guidance cues are distributed along migratory paths and are sensed and integrated to ultimately control cytoskeleton remodeling and ensure the dynamic cell shape changes required for migration. Neurotransmitters act as signaling molecules in the surrounding of migrating interneurons (Nguyen et al., 2001, Soria and Valdeolmillos, 2002,

Heng et al., 2007). Among them, the role of GABA has been characterized by several groups (Lopez-Bendito et al., 2003, Cuzon et al., 2006, Cuzon Carlson and Yeh, 2011). Activation of $GABA_AR$ has been shown to promote interneurons motility at initial phases of migration, but blocks radial migration at later stages when interneurons reach their final position in the developing cortex (figure 1.6). This mechanical switch implies the inversion of the chloride gradient in interneurons that reach the cortical plate (Bortone and Polleux, 2009). In line with that, *in vivo* experiments have demonstrated that GABA facilitates tangential migration only when there is a high level of NKCC1 (Inada et al., 2011). Additionally, it has been shown that tangentially migrating neurons express functional AMPA receptors (Metin et al., 2000) and that their activation lead to neurite retraction suggesting that AMPA receptor activation might be a complementary control mechanism for interneurons migration (Poluch et al., 2001). Moreover, type D1 and type D2 dopamine receptors (D1R and D2R) have been found to differentially modulate interneuron migration and their entrance into the cortex (Crandall et al., 2007). While the activation of D1R promotes cortical interneuron migration, activation of D2R inhibits this process. These results were confirmed by electroporation, and by using D1R and D2R deficient-animals. Furthermore, serotonin has also been found to impact interneurons migration by activating type 6 serotonin metabotropic receptors ($5\text{-}HT_6R$) (Riccio et al., 2009). Interestingly, CGE-derived interneurons selectively express type 3 serotonin receptors ($5\text{-}HT_3R$) (Lee et al., 2010, Vucurovic et al., 2010). However, these receptors do not seem to be involved in the control of cell migration (Riccio et al., 2009).

Once migration has been completed, synaptic connections start to be made. The first synaptic connections that appear during development are between interneurons, or between an interneuron and a pyramidal cell. These first synapses are purely GABAergic. At this age, GABA provides the initial excitation to the network generating the earliest oscillations (Rheims et al., 2008). The depolarization induced by GABA has been suggested to be an important event to remove the Mg^{2+} blockade of NMDA receptors and promote its involvement in new excitatory synaptic contacts. Once a sufficient number of glutamatergic synapses have been generated, the effect of GABA becomes inhibitory and fine tunes the network oscillations (Ben-Ari et al., 2004). The effect of GABA in synaptogenesis has preferentially been studied in the hippocampus where the network remains intact after slice preparation. The fact that GABA has an excitatory effect during embryonic development seems to be the key factor for the generation of the first networks. Once the network have reached a certain level of excitation, the developmental switch in the expression of NKCC1/KCC2 transforms GABA signaling into the inhibitory signal typically present in adults (Ben-Ari et al., 2004). However, the excitatory effect of GABA during postnatal development has recently been debated as experimental conditions may change the normal chloride gradient and lead to erroneous conclusions. Slicing procedure has been suggested to be pathological rather than physiological

model to study synaptic function where injured cells fail to effectively control the chloride gradient. Additionally, the inadequate solution composition, which may lack adequate energy sources (Holmgren et al., 2010), or the modality used during electrophysiological measurements, which may expose the cells to non-physiological concentration of ions (Tyzio et al., 2003), can also induce a change in chloride homeostasis or create a technical artifact. This is an important consideration as there is abundant evidence that demonstrate an inhibitory role of GABA *in vivo* during synapse development (Khakhalin, 2011, Bregestovski and Bernard, 2012).

Before and after the generation of the first synapses the cell morphology dynamically changes. Sometimes these changes are part of the developmental program and sometimes they emerge as a consequence of the activity of the network, or as an adaptation in response to external stimulus. It has been shown that GABA can facilitate neurite outgrowth and acts as a trophic factor by promoting the calcium entry upon depolarization in interneurons (Maric et al., 2001).

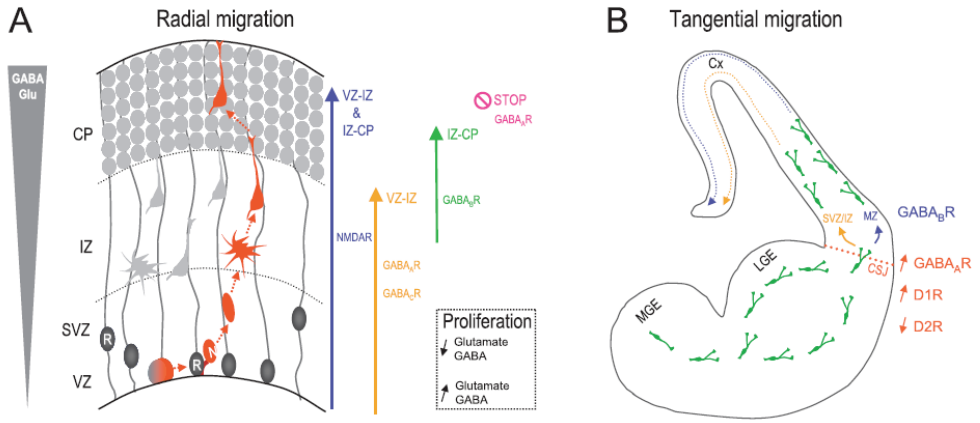

Figure 1.6. Cell migration and the role of neurotransmitters in the developing brain cortex. (A) Schematic diagram showing the cortical wall of an E13.5 mouse brain. This diagram illustrates the process of radial migration and the functions undertaken by neurotransmitters and their cognate receptors in the regulation of progenitor cell proliferation and migration of newborn projection neurons within the cortical wall. (B) GABAergic interneurons (green cells) are mainly generated in the medial ganglionic eminence (MGE) and follow distinct tangential migratory routes to integrate into the cerebral cortex (Cx). Different neurotransmitters can influence this process. Figure taken from Heng et al., 2007 (Heng et al., 2007)

1.6 Glycine receptors and neuronal development

1.6.1 Generalities about GlyR

Glycine receptors (GlyR) are part of the ligand gated ion channel family that also includes the nicotinic acetyl-choline receptor (nAChR), $GABA_AR$, $GABA_CR$, insect glutamate-gated chloride channels (GluCl) and $5HT_3R$. All these receptors share structural and functional characteristics that are related to their molecular organization, activation, and ion flux control (figure 1.7). Specifically, the GlyR is a transmembrane protein complex formed by the assembling of five subunits arranged symmetrically around a central pore. There are five types of subunits, four alphas and 1 beta (figure 1.7). The GlyR can be formed either as alpha homomer or as a heteromer that incorporate the beta subunit. Furthermore, its composition can vary depending on the alpha subunit subtype, although some combinations have been more studied such as the only alpha homomers, the alpha1/alpha2 or the alpha1/beta heteromers (Lynch, 2009). Currently, the atomic structure of the GlyR has not been determined yet, but there are reasonable assumptions about its topology based on its homology to nAchR (Unwin, 2005), and to the acetyl choline binding protein (Brejc et al., 2001). The accepted model postulates that each subunit presents a large extracellular domain, which is the site of ligand binding, four trans-membrane alpha helices named M1 to M4, where the M2 form the pore lining domain, and a long intracellular loop linking the M3 and M4 domains, which is a site for phosphorylations and protein-protein interactions (figure 1.7).

Besides glycine, GlyR can also be activated by other ligands, such as taurine and alanine. Each of these molecules can bind to the extracellular domain and promote the opening of the central pore that allows the selective flux of chloride and bicarbonate ions. In the same way as for $GABA_AR$, GlyR-mediated ion flux is solely dependent on the electrochemical gradient established across the cell membrane for these ions. Ionic current flowing through GlyR can be blocked by alkaloids such as strychnine and picrotoxin (PTX). These drugs can be used to discriminate between the molecular compositions of the receptors due to the differential efficacy with which different subunits of the receptor are affected. Indeed, the two components of PTX, picrotin (PTN) and picrotoxinin (PXN), exert differential effects on alpha 2 homomeric receptors while they have the same potency to block alpha 1 homomeric GlyR. Moreover, PXN is only active on receptors lacking the beta subunit (Pribilla et al., 1992, Okabe et al., 2004). Together with strychnine and PTX there is an extensive list of endogenous molecules and pharmacological compounds, such a as cannabinoids, neurosteroids, hormones, alcohols and anesthetics, ginsenosides, ginkgolides, beta-carbolines and others, that interact with GlyR and can potentially been used for research or pharmacological treatment in GlyR-associated pathologies (Webb and Lynch, 2007). Finally, it should be noted that a sub-threshold depolarization, mediated by GlyR, may be inhibitory if it results in the clamping

of the membrane voltage at a more hyperpolarized potential lower than the threshold for action potential generation.

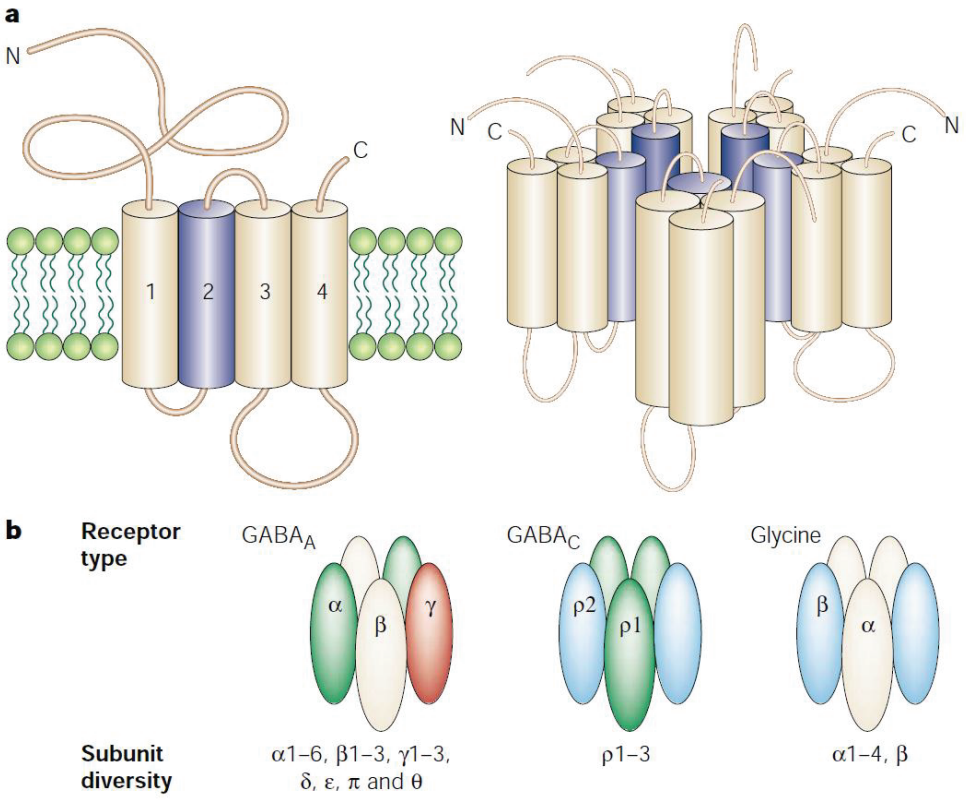

Figure 1.7. Ligand gated chloride channels. A, It has been proposed that each receptor subunit has four hydrophobic transmembrane domains, where the second is believed to be exposed to the inside wall of the ion channel pore. Functional ion channels are formed by five subunits arranged around the central pore. The large extracellular domain contains the ligand and some modulators binding sites while the cytoplasmic domain contains consensus sites for intracellular regulation. **B**, Proposed stoichiometric arrangement of subunits for GABA and GlyR. Additionally, GlyR can form homomeric channels composed of only alpha subunits. Figure taken from Moss and Smart, 2001. (Moss and Smart, 2001)

1.6.2 Physiological function of GlyR

GlyR were first detected in the spinal cord, from where they were isolated thanks to the affinity of strychnine (Pfeiffer et al., 1982, Graham et al., 1983). Spinal cord contains the highest amount of GlyR and was thus used as a model tissue to learn more about the physiological role of GlyR. In adult animals, the alpha 1 and beta subunits are abundant in spinal cord whereas the less abundant alpha 2 subunits, are exclusively detected in the brain. While the expression of the beta subunit increases during adulthood and becomes widely distributed in the brain, alpha 2 subunits harbor a more restricted expression in hippocampus and layer VI of the cerebral cortex. The alpha 3 GlyR subunits are less abundant and show a restricted expression pattern in the CNS including the cerebellum, the olfactory bulb and the spinal cord (Malosio et al., 1991). Finally, although there is no clear mapping of alpha 4 subunit distribution functional receptors containing this subunit have been detected in the mouse and chicken (Harvey et al., 2000), but not in human (Simon et al., 2004).

1.6.2.1 GlyR and spinal cord neurotransmission

The importance of the physiological function of GlyR in the spinal cord is exemplified by the dysfunction of the motor-reflex circuit as observed in the hereditary disorder named hyperekplexia or startle disease where GlyR function is impaired. This disorder is characterized by an exaggerated response to an acoustic or a tactile stimulus. Patients manifest uncontrolled muscle contractions in seizure like episodes that are triggered by any tactile or acoustic stimulus that in normal conditions would not elicit any reaction. The disease somehow mimics sub-lethal strychnine poisoning. Its origin is most of the times a mutation in the GlyR alpha 1 subunit, which results in its reduced expression, low glycine affinity or channels with smaller conductance. In addition, other related proteins can also contribute to the disease. This is the case of gephyrin, which interacts with the intracellular domain of GlyR and keeps them clustered at synapses. Mutations that affect expression of the protein have been associated with human startle disease (Rees et al., 2003). Another example is the dysfunction of the glycine transporter 2 (GlyT2), which is in charge of loading synaptic vesicles with glycine. A mutation of this transporter can have a direct impact on its functionality (Gimenez et al., 2012), thus directly impairing glycinergic neurotransmission. This last report is in line with findings of others that report several missense mutations in GlyT2 that are associated with hyperekplexia (Gomeza et al., 2003). The different recessive mutations in the GlyT2 gene are considered as the second major cause of startle disease (Carta et al., 2012).

GlyR are also involved in pain perception. Its major influence on this process takes place in superficial layers of the dorsal horn of the spinal cord where nociceptive afferents terminate. It has been demonstrated that GlyR alpha 3 subunits, present in the postsynaptic terminals of dorsal horn synapses in the

spinal cord, are involved in perception of pain derived from mechanical and thermal activation (Zeilhofer, 2005). Specifically, bath-applied prostaglandin E2 (PGE2), which induces sensitization to pain, reversibly reduces the amplitudes of GlyR-mediated inhibitory postsynaptic currents (Ahmadi et al., 2002). More exhaustive studies demonstrated that this inhibition resulted from alpha 3 subunit phosphorylation of its intracellular domain (Harvey et al., 2004). Consequently, knockout mice for GlyR alpha 3 show no response to intrathecal sensitization caused by PGE2 (Harvey et al., 2004). In line with this, knockout animals for prostaglandin receptors show a similar phenotype. These studies demonstrated the involvement of GlyR in the perception and processing of painful stimuli and proposed the alpha 3 GlyR as a new pharmacological target for idiopathic pain (Lynch and Callister, 2006).

In situ hybridization analyses of spinal cord sections (Jonsson et al., 2012) revealed that GlyR alpha 2 is the most abundant subunit expressed during embryogenesis, while alpha 1 and beta subunits are predominant after birth (Watanabe and Akagi, 1995). This change in expression is gradual and alpha 1 progressively replaced alpha 2, which decreases until being undetectable in the ventral horn of mature animals (Watanabe and Akagi, 1995). The developmental expression of alpha 3 subunit increases before birth and then remains stable. Nevertheless, physiological levels of alpha 3 subunits are very low (Malosio et al., 1991). Expression of GlyR beta subunit parallels the expression of alpha 1 ones, suggesting that before and after birth the glycinergic synaptic currents are mainly mediated by activation of alpha2/beta and alpha1/beta containing receptors, respectively. Alpha 2 homomeric receptors are considered too slow to support synaptic transmission in addition to the fact that they lack the ability to interact with gephyrin and, therefore, the ability to cluster at synaptic sites. The first synaptic activity in the spinal cord appears at E12.5 and is initially purely GABAergic. One day later, the synaptic protein gephyrin starts to be expressed and interact with both $GABA_AR$ and GlyR at the synaptic sites (Colin et al., 1998). At that time glycinergic synapses can be detected for the first time (Scain et al., 2010), preceded by the spontaneous opening of extrasynaptic GlyR (Scain et al., 2010). The blockade of GlyR at this developmental stage significantly slows down the rostro–caudal propagation of electrical activity (Scain et al., 2010).

1.6.2.2 Glycine receptors and cerebral neurotransmission

Although expression of GlyRs in the adult brain was described two decades ago (Frostholm and Rotter, 1985, Probst et al., 1986, van den Pol and Gorcs, 1988, Malosio et al., 1991), there is little information about their function. Contrary to what has been shown for the spinal cord, the most abundant GlyR subunits are beta, alpha 2, alpha 3, and alpha 1 is only marginally found (Jonsson et al., 2012). It is believed that GlyR mainly mediates extrasynaptic inhibition and that they are composed by alpha 2/3 heteromeric channels in the hippocampus

(Aroeira et al., 2011). Interestingly, extrasynaptic receptors are involved in synaptic plasticity and play a role in long term depression (LTD) (Song et al., 2006, Chen et al., 2011). On the other hand, GlyRs are also involved in neurotransmission and glycinergic synapses have been morphologically identified in excitatory and inhibitory neurons using immunofluorescence and electron microscopy (Danglot et al., 2004). Impaired GlyR alpha 3 subunit alternative splicing has been detected in patients suffering of temporal lobe epilepsy (Eichler et al., 2009). In this case, the short splice variant, which showed a lower tendency to form clusters at synapses, was significantly upregulated, possibly decreasing total GlyR contribution to synaptic transmission (Eichler et al., 2009).

1.6.2.3 Glycine receptors and non-neuronal signaling

Finally, GlyR are also expressed in non-neuronal tissues (den Eynden et al., 2009). However, there is only limited information about their physiological function there (den Eynden et al., 2009). Perhaps, the most well-known example of a non-neuronal tissue is the spermatozoa, where GlyR play a central role in acrosome reaction. Specifically, it has been shown that glycine can trigger this reaction in a strychnine-sensitive manner (Sato et al., 2000, Llanos et al., 2001) and that spasmodic mutant mice, that harbor a mutation in the GlyR alpha 1 gene, show a decreased fertility (Meizel and Son, 2005).

1.6.3 The role of GlyR during embryonic brain development

Glycine receptor expression starts early during brain development. This was first studied by Malosio and colleagues who used in situ hybridization to assess the presence of various GlyR subunits in the embryonic rat brain (Malosio et al., 1991). In this study, the authors found abundant expression of both, alpha 2 and beta subunits during prenatal stages. The messenger RNA (mRNA) encoding alpha 2 subunit was widely detected starting from E14 onwards in all cortical layers during the whole embryonic period (Malosio et al., 1991). GlyR alpha 2 mRNAs were detected in the diencephalon and the midbrain at E14, and in the thalamus and the cerebellum primordium at E19. Beta subunit mRNAs were mainly found in the cortex at E14, where they were homogenously distributed. At E19 their expression pattern became more restricted and only layers I and II were labeled. Interestingly, at E19 the cerebellum was also selectively enriched for beta subunit mRNAs. No other GlyR subunits were detected, but it is important to note that the expression of the GlyR alpha 4 subunit was not studied.

To examine when functional GlyR first appear during cortical neurogenesis, Flint and colleagues recorded glycine and taurine elicited currents in different zones of the embryonic cortex at E19 (Flint et al., 1998). Interestingly, all recorded neurons in the CP and IZ showed GlyR-mediated responses. In contrast, none of

the cells located in the VZ seemed to have functional GlyR (Flint et al., 1998). These results were confirmed by immunostainings, which showed intense labeling in the IZ and CP, but not in the VZ. Single cell resolution was achieved in the IZ where GlyR expression was localized in the soma and apical leading process. Therefore, GlyR expression seem to first appear in migratory projection neurons at this age (Flint et al., 1998). Complementarily, *in vitro* experiments, carried out at E17 in rats, demonstrated that new born projection neurons already express GlyR, along with $GABA_AR$ and voltage gated ion channels at this age, by the end of their radial migration (Noctor et al., 2004). The published representative current trace showed that 5 mM of glycine elicited a small current of 10 pA in immature projection neurons recorded under symmetrical chloride concentration. Nevertheless, glycine elicited currents were only four times smaller than GABA elicited currents (Noctor et al., 2004). For comparison, the measured normalized basal conductance is about 10 $pS/\mu m^2$ in immature cortical neurons (Huguenard et al., 1988) and the reported GABA elicited conductance is 7.4 times larger than that at the same age (Shen et al., 1988). Calcium measurements provided further evidence about the presence and function of GlyR in the embryonic cortex. Consistent with a depolarizing effect, glycine application triggered a massive calcium influx in the upper-layer pyramidal neurons at E17. This effect was blocked by strychnine and totally absent in the GlyR-knockout (KO) animals (Young-Pearse et al., 2006). The same effect was recorded very early during cortex development, at E13 (Platel et al., 2005). At this age, GlyR were shown to have a unique role in pre plate (PP) neurons, which are depolarized in response to GlyR activation. This membrane depolarization activates voltage-gated sodium channels that subsequently activate sodium-sensitive calcium transporters. This complex channel association leads to a rise in intracellular calcium and promotes vesicular release of glutamate (Platel et al., 2005). Importantly, the activation of voltage-gated sodium channels and the subsequent activation of sodium-sensitive calcium transporters were exclusively induced by GlyR and were independent of the activation of $GABA_AR$, which are also expressed in PP neurons at this age. Moreover, released glutamate amplified GlyR-mediated signaling, and triggered calcium influx in the VZ as well (Platel et al., 2005). In humans, the embryonic brain also contains GlyR. Specifically, it has recently been shown that differentiated human midbrain-derived cells express functional GlyR for which glycine has low affinity and can trigger calcium influx. Embryonic cells were obtained from 10-16 weeks human fetuses and kept in culture for 1-3 weeks. After that period, abundant expression of beta and alpha 2 subunits was evident. GlyR expression significantly increased from the first to the third week in culture. To understand the physiological function of GlyR in these cells, different markers were used to assess GlyR influence on the differentiation process. However, GlyR expression had only a limited impact on their neurogenic capability and differentiation towards dopaminergic neurons (Wegner et al., 2012).

Despite the functional expression of GlyR during embryonic brain development, the lack of GlyR does not seem to greatly affect brain development. GlyR alpha 2 knockout animals (GlyRa2$^{-/-}$) did not show any gross morphological abnormality in the cortex at postnatal day 0 (P0) (Young-Pearse et al., 2006). In these animals, Notch1, Id2, Btg2, TUG1, and $GABA_AR$ subunit 6, expression were normal around birth, suggesting, according to the authors, that GlyR alpha 2 subunit is not required for acquisition of the main morphological and biochemical features of the cortex. (Young-Pearse et al., 2006). The absence of morphological defects may arise through compensatory up-regulation of other GlyR or $GABA_AR$ subunits in GlyRa2$^{-/-}$ animals. In this regard, it is interesting to note that GlyRa2$^{-/-}$ animals displayed glycine elicited responses at P7 (Young-Pearse et al., 2006), suggesting that other subunits of GlyR could be still present during postnatal development, or that this is a retarded compensatory mechanism in response to the disruption of alpha 2 subunit expression. Recently, a short report has suggested that GlyR could have an influence on radial migration during late embryonic development. However, this effect is only seen after a drastic pharmacological treatment of slices with excess of glycine and in the presence of two blockers for glycine transporters (Nimmervoll et al., 2011).

Endogenous activation of GlyR in the developing cortex occurs through non-synaptic release of neurotransmitters and involves paracrine / autocrine mechanisms (Le-Corronc et al., 2011). Early studies suggested that taurine could be the endogenous ligand acting on immature GlyRs in the developing cortex. This suggestion was based on the presence of this amino acid in cortical plate neurons (Flint et al., 1998), and on the detection of taurine and glycine in adult cortex (van den Pol and Gorcs, 1988). However, more recent studies have shown that levels of neurotransmitters can drastically change during the development of the brain and are essentially different from the concentrations found in the adult. While the levels of GABA in the parietal cortex of young adults are 2.6 folds higher than glycine, the levels of this amino acid are 3-4 folds lower than glycine in the embryonic brain. The concentration of taurine progressively increases during embryogenesis, reaching its maximum around birth in the cortex. Comparatively, E13 levels of taurine are 10- and 20-folds higher than the levels of glycine and GABA, respectively (Benitez-Diaz et al., 2003). However, it has been shown that cortical GlyR (Okabe et al., 2004, Kilb et al., 2008), and specifically homomeric GlyRa2, (Schmieden et al., 1992) are 10 times less sensitive to taurine, which in addition, is only a partial agonist of GlyR (Schmieden et al., 1992). This suggests that both neurotransmitters, glycine and taurine, could act as ligands of GlyR at various time points and locations during cortical development.

While the identity of the endogenous GlyR ligand remains unclear, both glycine and taurine seem to have an important effect during neuronal development. The concentration of glycine is influenced by the glycine cleavage system (GCS) that

catalyzes the degradation of glycine and provides the developing brain with other metabolites, such as 5,10-methylenetetrahydrofolate which is essential for DNA synthesis. Failure in GCS activity leads to serious malformations, such as agenesis of the corpus callosum, gyral malformation and cerebellar hypoplasia. Interestingly, the GCS appears to be highly expressed in cortical progenitor cells (Ichinohe et al., 2004). On the other hand, the deprivation of taurine during pregnancy leads to abnormal cortical development in kittens (Sturman, 1988, 1991)

1.6.4 The role of GlyR during postnatal brain development

By the day of birth, GlyR alpha 2 and beta transcripts are abundant in the cortex and other brain structures. However, during the first two postnatal weeks of development, there is a dynamic change in the levels of expression of these mRNAs (Malosio et al., 1991). At the first postnatal day, the alpha 2 subunit continues to be widely expressed and the most abundant subunit but, during the following weeks it gradually becomes more restricted to the deep layers of the cortex and adopts the adult distribution pattern. At the same time, the beta subunit displays a wide expression distribution pattern and can be localized along the entire brain. Just after birth, the alpha 2 subunit mRNA is detected throughout the whole cortex (Malosio et al., 1991) and it seems to be homogeneously distributed in the CP and Cajal Retzius cells, which also express beta subunits (Okabe et al., 2004). At P5, alpha 2 subunits are only detected in the layers I/II and IV while the beta mRNA transcript displays a preferential labeling of layers I/II and VI. Additionally, the beta subunit can also be found in the hippocampus and thalamus at this age. This pattern keeps on changing during the next 10 days, and by P15 it reaches the adult distribution. While the alpha 2 transcripts display strong labeling in layer VI, the beta transcripts are detected in all the layers of the cortex, hippocampus and cerebellum (Malosio et al., 1991). Around P15, the transcripts for the alpha 1 and alpha 3 subunits are also marginally detected in the brain. In attention to the drastic decrease of GlyR alpha 2 subunit in the brain it has been suggested that alpha 2 subunit containing receptors could gradually been replaced by alpha 1/beta heteromers, similarly to what happens in the spinal cord. However, recent analyses, at the protein level have shown that the increase in alpha 1 subunit is so small that it could hardly replace the expression of the alpha 2 subunit, which seems to minimally decrease after birth (Jonsson et al., 2012).

Several electrophysiological experiments have explored the functionality and pharmacological properties of GlyRs in the postnatal cortex (Flint et al., 1998, Kunz et al., 2012). Specifically, by using patch clamp experiments, it has been shown that both Cajal Retzius cells and CP neurons express functional GlyR. Interestingly, GlyR-elicited currents were 3 times larger in Cajal Retzius cells compared to CP neurons at the same age (Okabe et al., 2004). Despite such difference, there were no other pharmacological or molecular differences in

terms of GlyR response or subunit composition, which suggested that GlyR in both cell types may consist of alpha 2 / beta heteromeric receptors at least during the first postnatal days (Okabe et al., 2004). Functional GlyR have also been described in subplate (SP) neurons, where cells respond with less affinity to taurine compared to glycine and beta-alanine in the same way as it has been shown for CP and Cajal Retzius cells (Kilb et al., 2008).

Activation of GlyR in postnatal brain can cause different biological effects depending of the cell type. During early postnatal age, application of glycine to CP neurons in voltage clamp mode induces a sustained current along with intense postsynaptic currents events (Flint et al., 1998). This neurotransmitter release facilitation seems to persist in the developing visual cortex after two weeks (Kunz et al., 2012). In contrast to these studies, the application of glycine to Cajal Retzius cells induces a shunting inhibition of evoked action potentials, hence blocking synaptic transmission (Kilb et al., 2002). All together, these results show that GlyR can actively modulate synaptic transmission in neonatal brain cortex and likely affect synaptogenesis. A key factor during the establishment of the first synapses in the cortex is the arrival of extra cortical inputs carried by thalamo-cortical axons. These axons primarily innervate SP neurons, which subsequently transfer the information to the rest of the layers in the cortex. Interestingly, GlyR are also present in SP neurons during early postnatal development (Kilb et al., 2008). It has been shown that GlyR present in those cells display properties similar to those of receptors present in the cortical plate, and upon activation they depolarize the cell lowering the threshold for the generation of action potentials (Kilb et al., 2008). Therefore, these findings provide more evidence to support the involvement of GlyR in the generation of the early patterns of activity in cortical networks.

During postnatal development, high performance liquid chromatography measurements have described another shift in the concentration of inhibitory neurotransmitters. According to these measurements, after a peak around birth, the levels of taurine remain high and rather constant for the first postnatal week. Then, taurine concentration decreases but at P15 it remains 2 and 5 folds higher than GABA and glycine, respectively (Sturman, 1988, Benitez-Diaz et al., 2003). Functional experiments carried out at P6 demonstrate that taurine mainly activates glycine and not GABA receptors in the cortical plate (Yoshida et al., 2004). The 2-fold increase in the level of glycine during embryogenesis, reaches a peak around birth and gradually decreases during the first two weeks of postnatal development to about 60 % of its original peak value. Interestingly, at this age certain components of the GCS are highly expressed in cortex, cerebellum, and hippocampus (Ichinohe et al., 2004). This suggests that GABA and glycine receptors could have an important role during a period of intense synaptogenesis. In addition, the fact that taurine levels remain high after the first two weeks of postnatal development, when GlyR are activated almost exclusively by glycine (Yoshida et al., 2004), suggest that taurine could serve

other functions independently of the sole activation of glycine or GABA receptors in the postnatal brain. Indeed, it has been shown that taurine could play a role in the membrane integrity or indirectly affect the chloride gradient by inhibiting KCC2 (Inoue et al., 2012).

1.7 Aims of the study

The preceding evidence demonstrates the presence of GlyR during early stages of brain development and suggests a role for this receptor in the control of certain aspects of this process. Additionally, GlyR dysfunction may be associated to the development of neuro-developmental associated diseases. It is therefore plausible to assume that GlyR activation could have a modulatory effect on the cellular physiology of the developing brain. The present work attempts to understand the function of GlyR during selected stages of embryonic brain development. Moreover, this work may provide a new insight in the molecular and cellular effects exerted by neurotransmitters during early development and contribute to a better understanding of the effects of ion channels and the electrical excitability during a developmental period that precedes synaptic transmission. Thus, our hypothesis is the following:

Glycine receptor influences brain development by regulating interneuron migration and the generation of projection neurons in the cortex.

To test this hypothesis we have defined the following general objectives:

1) To assess the expression and function of GlyR in migratory interneurons, dorsal progenitors and radially migrating cells.
2) To study the possible influence of GlyR on the migration of cortical interneurons.
3) To characterize the molecular mechanisms downstream the activation of GlyR in migrating interneurons.
4) To investigate the possible influence of GlyR on the proliferation of dorsal progenitors and the generation of projection neurons.

CHAPTER 2

Materials and methods

2.1 Animals

All animal experiments were carried out following the guidelines of the local ethics committee at Hasselt University (protocol number 201247,201261 and 201262). Dlx5,6:Cre-IRES-EGFP transgenic animals (Stenman et al., 2003) expressing EGFP under the control of the Dlx5,6 enhancer element (Dlx-EGFP) were kept in MF1 background and mated with wild type MF1 females purchased from Harlan laboratories. Dlx-EGFP mice were used as a reporter line where most of cortical interneurons are labeled with EGFP. For timed pregnant mice, the E0 day was identified by the presence of a vaginal plug the next morning after mating. The mouse GlyR α2 subunit gene (*Glra2*) was selectively disrupted by homologous recombination using the *cre*-Lox gene targeting system (Kuhn et al., 1995). These animals were constructed by Prof. Dr. Robert J Harvey and Prof. Dr. T. Neil Dear, who provided us with this mouse line. Briefly, a replacement-targeting vector was used for homologous recombination in the ES cell line PC3 (Figure 3.6). This generated a targeted allele containing loxP sites flanking exon 7 of *Glra2* (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). Chimaeras generated with such ES cells express cre recombinase exclusively in the male germ line during the terminal haploid stages of spermatogenesis. Thus, cre-mediated excision of exon 7 and/or the neo cassette was induced by mating *Glra2* chimeras with wild-type mice. Targeted disruption of *Glra2* in the progeny was confirmed by PCR and Southern blotting using probes adjacent to both arms of the *Glra2* targeting construct. As females homozygous and males hemizygous for the *Glra2* 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 BIOMED institute animal facility.

2.2 Slice culture

Time mated pregnant MF1 females mice, carrying embryos from embryonic day 13 to 14 (E13 to E14), were euthanized by cervical dislocation accordingly to FELASA guidelines. After that, the abdominal cavity was opened and the uterine horns were exposed and removed from the animal. The uterus was placed in a large Petri dish while the embryos were quickly removed and decapitated. Then, individual heads were kept in cold PBS plus 25 mM glucose on top of a cold surface for the extraction of the brain. This procedure was performed under a stereo microscope and using sterile instruments. Once the intact brain was removed from the head, they were embedded in 3% low melting point agarose (Fisher Scientific), rapidly cooled down and kept for 1 hour on ice to ensure the complete polymerization of the agarose. After that period, the embedded brains

were fixed to the vibratome holder (Microm, Termo Scientific) by using cyanoacrylate glue (Ted Pella). Sectioning proceeded at a speed of 10 with a frequency of 0.7 and an amplitude of 60 arbitrary units, accordingly to the vibratome settings set by the manufacturer. Sectioning was done in a coronal orientation and the thickness was set to 300 µm. Individual slices were picked up from the vibratome cutting chamber and transferred to a Petri dish containing 1 ml of medium under a MILLICELL-CM 0.4 µm inserts (Millipore). A minimum of 1 and a maximum of 5 slices were placed on every insert. For the analysis of interneuron migration, only MGE containing slices were used. Petri dishes and inserts were kept on ice during the time of sectioning. After sectioning, the excess of medium from the top of the slices was removed to ensure that the culture proceeds in a semi-dry condition with access to nutrients from below and to oxygen from the top of the slice. Slice cultures were kept for different time periods, depending on the experiment, at 37°C in humidified atmosphere with 5% of CO_2. Culture medium was composed of serum free Neurobasal medium (Life Technology) supplemented with 1 % of penicillin-streptomycin, N2 supplement, B27 supplement and glutamine (Life technologies). Free glycine medium was obtained by buying custom made Neurobasal medium (Life Technologies).

2.3 Immunolabelings

For detection of GlyRs in the developing cortex, E13 embryonic brains were fixed in 4% paraformaldehyde (PFA) for 30 minutes at 4°C, cryoprotected by overnight incubation in 30% sucrose solution, frozen in TissueTek embedding medium (Sakura Instruments) and sectioned at 20 µm in a cryostat (CM-3050-S, Leica). Prior to the staining, sections were washed with NH_4Cl for 30 minutes to reduce unspecific binding, and then with normal phosphate buffered saline (PBS) for other 30 minutes. Blocking was made in 10% normal donkey serum (NDS) plus 1% BSA dissolved in PBS for 1 hour at room temperature. Glycine receptor detection was performed using the alpha 2 specific antibody N18 (1:100; Santa Cruz Biotechnologies, Heidelberg, Germany) diluted in PBS containing 3% NDS plus 1% BSA, overnight at 4°C. Excess of antibody was removed by 3 washing steps of 10 minutes in PBS. The secondary antibody was donkey anti-goat labeled with A647 (1:500; Life Technologies, Gent, Belgium) and it was dissolved in the blocking solution. Finally, nuclear counterstaining was performed using DAPI (1:100; Life Technologies, Gent, Belgium) and slides were mounted using fluorescent mounting medium (Dako, Heverlee, Belgium).

Immunolabeling of glycine in the developing brain was performed on E13 brains fixed for 1 hour in 4% PFA. In a way similar to the method used for GlyR detection, brains were cryoprotected, frozen and sectioned at 10 µm by using a cryostat. To ensure rehydration and for the removal of embedding medium, slides were washed twice for 5 minutes in PBS. After that, they were permeabilized in 0.2% Triton X-100 (Sigma-Aldrich) supplemented with 1%

bovine serum albumin dissolved in PBS for 20 min at room temperature. Then, the slides were washed once for 5 min in PBS and blocked in 10% NGS plus 1% BSA in PBS for 30 min. The primary antibody against glycine was diluted at 1:500 (ImmunoSolutions, Australia) in 3% NGS plus 1% BSA dissolved in PBS and incubated overnight at 4°C. After that period, the slides were incubated for 1 hour with the secondary antibody diluted at 1:500 in 3% NGS plus 1% BSA dissolved in PBS. Secondary antibody solutions were centrifuged before use to reduce aggregates. Finally, the slides were washed 5 times for 5 minutes with PBS and mounted using Vectashield mounting medium with DAPI (Vector Laboratories) and a cover slip.

Bromodeoxyuridine (BrdU) staining for assessing proliferation in embryonic brain slices was performed in 20 µm re-cut cryosections. These sections were left at room temperature for at least 15 minutes after removal from the freezer to allow equilibration. Then, they were washed twice for 5 minutes in PBS for rehydration of the samples. After that, DNA denaturation and antigen exposure was forced by incubation of the slides in 2M HCl for 30 minutes at 37°C. Following the denaturation, the slides were washed 3 times for 10 minutes in PBS to wash and neutralize samples. Blocking of unspecific binding was performed by incubating the slides in 3% NDS (Tremecula), 0.1% triton X100 (Sigma, Bornem, Belgium) dissolved in PBS for 1 hour. Excess of blocking solution was removed by a 10 minutes washing step with PBS. The primary monoclonal anti-BrdU antibody (IgG1 BMC 2318 clone, Roche diagnostics GmbH) was diluted at 1:200 in PBS and slices were incubated in this solution for 2 hours or overnight (200µl per slide with coverslip). Secondary donkey anti-mouse Alexa 555 or 488 (Life Technologies, Gent, Belgium) dissolved in blocking solution was used at 1:500 for 1 hour at room temperature. Finally, slices were washed 3 times for 10 minutes each in PBS to remove the excess of secondary antibody and they were mounted with DAPI- or PI-containing mounting medium (Vector Laboratories) for nuclei counterstaining.

2.4 Confocal imaging for immunolabelings

Images of immunolabled brain sections were acquired with a Carl Zeiss 200B motorized microscope attached to a confocal laser scanner system LSM 510 META (Carl Zeiss). Images were taken with Plan Apochromat 20X/0.75 or Plan-Neofluar 40X/1.3 Oil DIC objectives. The Alexa fluor 488 fluophore was visualized by excitation with the 488 nm emission line of an Ar-Ion laser while The Alexa Fluor 555 and Alexa Fluor 647 dyes were visualized using the 543 nm spectral line of the He-Ne laser and the 633 nm spectral line of a second He-Ne laser, respectively. DAPI excitation was achieved by two-photon excitation at 700 nm with the light produced by a mode locked MaiTai laser (Spectra Physics).

2.5 CMTMR in vitro labeling for migrating interneurons

In vitro cell labeling was performed by positioning dye-coated particles onto the MGE. The fluorescent dye was taken up by neurons. Thus, interneurons that migrate out of the MGE were fluorescently labeled after 1 to 4 days *in vitro* allowing their recognition for patch-clamp experiments. Dye-coated particles were prepared by dissolving 5-(and-6)-(((4-Chloromethyl)Benzoyl)Amino)Tetramethylrhodamine (CMTMR, Life Technologies, Gent, Belgium) in DMSO at a concentration of 10mM. The solution was further diluted in ethylene dichloride at a concentration of 1mM final. In parallel, 50 µg of tungsten particles (0.8 µm, Bio-Rad) were spread onto a glass slide. Then, 100 µL of CMTMR ethylene dichloride solution were added to the particles and the solvent was let to evaporate. Coated particles were then collected from the glass slide and stored at 4°C until the day of the labeling (Alifragis et al., 2002). E13 slices were prepared according to the described procedure for *in vitro* slice culture. After 4 hours of recovery, slices were taken out of the incubator and CMTMR-coated particles were placed on the MGE by using a stereo-microscope and sharp dissection instruments (Fine Science Tools). Slices were kept in culture for 1 to 4 days to allow interneuron migration.

2.6 Retrovirus preparation

EGFP expressing retroviruses were prepared using three separated plasmids, one encoding for the EGFP, and two for the assembling and packing of the virus. These viruses were prepared using HEK cells and lipofection (Tashiro et al., 2006). The first day of the procedure, 5 millions of cells were seeded per 100 mm plates for a total of 12 plates. Each plate had a final volume of 10 ml. The next day (or until they reach more than 70 % of confluence), the plates were transfected using lipofectamine 2000 (Life Technologies, Gent, Belgium). Each plate was transfected by mixing 1.6 mL of Optimem (Life Technologies, Gent, Belgium) with 16-32 µl of lipofectamine and 15 µg of DNA. The amount of DNA for each plasmid was 7.5µg of CAG-EGFP, 5 µg of CMV-gp and 2.5 µg of CMV-vsvg. The preparation of the transfection mixture was done in two steps. First, 800 µL of Optimem (Life Technologies, Gent, Belgium) were mixed with lipofectamine while the other 800 µl were mixed with the DNA. After 5 minutes of incubation at room temperature, the two tubes were mixed and rested at room temperature for 30 additional minutes. After that step, the complete volume of the mixture was added to one of the 100 mm plates. The transfection mixture was added drop wise while gently shaking the plate. Virus isolation was performed after two days of transfection, when EGFP expressing HEK cells were already visible. At this moment, all the supernatants of every plate were collected in 50 ml falcon tubes. The medium was then centrifuged at 2000 rpm for 2-3 minutes to remove cellular debris. After that, the supernatant was filtered through a 0.22 µm syringe filter and collected in 15 ml tubes. Then, the viruses were collected by centrifugation at 21000 g, at 4°C for 2 hours. After

that, the supernatant was discarded and the pellet re-suspended for 20 – 30 minutes with 700 µl of PBS for each tube. All tubes were pooled together and transferred to one 15 ml falcon tube. The original 15 ml tubes were washed one more time with 700 µl of PBS, which were also transferred to the re-suspended viruses. At this point, 1000 µl of 20% sucrose were added to the viruses and a second centrifugation at 21000 g, at 4°C for two hours took place. Finally, the pellet was re-suspended in 200 µl of PBS by vortexing for 30 seconds and then pipetting. Small aliquots were stored at -80 until the time of the experiment.

The titer of the virus was determined by infection of HEK cells (Tashiro et al., 2006). For this purpose, 100,000 cells were seeded per well in a 24 well plate. The next day, a serial dilution of the viruses (10^2,10^3, 10^4 and 10^5) was performed and 10 µl of each were added to one well so that the final dilution series was 10^1,10^2, 10^3 and 10^4. After 2-3 days, the number of fluorescent clusters was counted under a fluorescent microscope and the virus titer was calculated by multiplying the number of clusters by the dilution factor divided by 10 resulting in a number of colony-forming units (c.f.u.) per ml. Typically, the titer was 10^4 c.f.u./µl.

2.7 Electrophysiology

Electrophysiological assessment of migratory interneurons was performed in two different ways. The first approach used an *in vitro* labeling strategy where interneurons were labeled in their place of origin, *i.e.* the MGE, and were then kept in culture allowing their migration. The second approach made use of transgenic animals where interneurons are already labeled by the expression of EGFP under the control of an interneuron specific promoter. Brain slices were generated according to the same procedure for the generation of *in vitro* slice cultures, but following the slicing step, they were transferred into artificial cerebro-spinal fluid (ACSF) saturated with 95% O_2/5% CO_2 at 32 °C. Slices were kept for 1 hour in this condition to allow recovery and then kept at room temperature for the rest of the experiment. ACSF solution was composed of NaCl 125, KCl 2.5, MgCl2 1, CaCl2 2, NaHCO3 25, NaH2PO4 1.25, Glucose 25 (mM). Interneuron-targeted electrophysiology was performed using a combination of imaging techniques. First, the selected slice was placed under an upright FN1 microscope (Nikon) equipped with an epifluorescent illumination attachment and a monochromatic camera (Hamamatsu). The epifluorescent illumination attachment included filter cubes optimized for EGFP and TRITC detection. Once the appropriated cell was selected by its fluorescent signal, the imaging mode was changed to infra-red differential interference contrast imaging (IR-DIC) by placing the DIC prisms and the polarizer and analyzer into the transmitted light path. After identification, the patch-clamp electrode was approached to the cell under the control of electronic micromanipulators (Burleigh). Recording electrodes were made of borosilicate glass capillaries pulled using a P1000 micropipette puller (Sutter Instruments, USA). Every micropipette was fire-

polished using a micro-forge (Narishige). Pipette filling solution (internal solution) was composed of KCl 130, NaCl 5, $CaCl_2$ 1, $MgCl_2$ 1, Hepes 10, EGTA 10 , NaATP 2 and NaGTP 0.5 (mM). The resistance of the pipette filled with internal solution was around 10 MΩ. Recordings were performed at room temperature using an Axon 200B Axoclamp patch-clamp amplifier (Molecular Devices) and an Axon digitizer connected to a personal computer (Microsoft OS). The cells were voltage-clamped at a holding potential of -60 mV. The amplitudes of glycine-elicited currents were assessed by brief applications that lasted for 5 seconds. All ligands and blockers were focally applied, at a distance of approximately 150 µm from the surface of the slice using a Warner perfusion system that allowed exchanging times of less than 20 ms (Fast-Step, Warner Instrument Corporation). Data acquisition and analysis were performed using the pClamp and Clampfit software, respectively (Harvard Apparatus).

Whole-cell recording of dorsal progenitors or radially migrating cells was performed on a similar way to the procedure used with interneurons. However, a few steps were changed based on the differences between these cell types. Dorsal progenitors or radially migrating cells were labeled by infection of E13 slices with EGFP encoding retroviruses, which labeled proliferating cells. Application of retroviruses was performed 4 hours after slicing and was restricted to the ventricles. One microliter of the diluted viral suspension was applied to each hemisphere. Following this procedure, EGFP positive cells were already visible lining the ventricles after 24 hours. For the detection of glycine-elicited currents, recordings were performed after one or two days *in vitro* in the ventricular and sub-ventricular zones, respectively. The concentration-response curve was built exclusively from radially migrating cells present in the sub-ventricular zone after two days *in vitro*.

2.8 Time-lapse videomicroscopy

Migration experiments were done directly in slices or in *in vitro* culture explants. In the case of slices, imaging was performed after 6 h recovery at 37°C in 5% CO_2. Medium was fully exchanged prior to imaging by conditioned medium containing different pharmacological compounds or control medium containing the appropriate amount of solvents present in the conditioned medium. Explant cultures were prepared onto poly-ornithine- (1 mg/ml, 45 min, 37°C) and laminin- (0.05mg/ml, 1 hour, 37°C)(Sigma-Aldrich, Bornem, Belgium) coated glass bottom multi-well plates (MatTek Corporation, USA) (Bellion et al., 2005, Godin et al., 2012). The big advantage of this method is 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 and at the same time decreased the measurement errors and the need for more embryos. Explants were kept in the incubator for 20 hours before imaging and only 1/3 of medium was replaced before imaging. All pharmacological compounds were kept as concentrated stock solutions and dissolved in the

medium immediately 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 10mM in water. Whenever possible, the pharmacological compounds were chosen with attention to their photochemical stability to prevent unwanted reactions due to the excitation light. This is the reason why nifedipine was not suitable for two-photon imaging and needed to be replaced by calciseptine. Control conditions included equal amount of the corresponding solvents. 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, Irvine, USA) for two-photon illumination. Unlike normal confocal microscopy, two-photon imaging seemed to be less harmful to the cells and apparently made interneurons migrate faster (data not shown). However, although these observation are in agreement with the reported by other, they were made while still optimizing other parameters such temperature and CO_2 level and we can not make clear conclusions. To keep the environment of the slices as close as possible to the physiological conditions during imaging, the microscope was surrounded by a temperature-controlled incubator chamber supplied with 5% CO_2 (Pecon, Germany). Excitation of EGFP was achieved by using 900 nm and a 20X, 0.5 NA, long-working distance objective. Z stacks spanning for 30 µm from the surface of the slice where acquired every 5 minutes. Stack acquisition compensated for any drift in the focus. Emitted fluorescence was collected using non-de scanned detectors located after an HQ 475-565 filter. Excitation light was selected using a HFT KP 650 filter and an NDD KP685 dichroic.

2.9 Image analysis

All analyses were performed using ImageJ (NIH) freeware and the Mtrack plugin for semi-automatized cell tracking (Meijering et al., 2012). The series of images were transformed to hyperstacks in four dimensions for cell tracking. The procedure followed the methodology described in the technical specifications of the MTrack plugin (Meijering et al., 2012) where the soma was the reference for the tracking. In average 30 cells were tracked for each time lapse. In the case of slices, cells were randomly chosen among those exhibiting tangential migration. Explant cell tracking used a similar approach where 30 cells were chosen at random from those cells moving away from the explants. To simplify the analysis of the data obtained from explants, the z dimension was compressed to have one single picture. In some cases where there was tissue or explant drift, all coordinates were corrected individually by subtracting the coordinates obtained from the tracking of an immobile object that moved only due to the tissue or explant drift during the whole length of the experiment. Cells present in the field of view for less than two hours were not considered for analysis. To get an appreciation of the time course of the effects, the values of speed measured from one frame to the next, were averaged in three groups according to whether

the cell was tracked for the first 2 h, for the second 2 h or for the last 2 h. In this way, we obtained the average speed of migration at different time points during the experiment. The tracks measured from cells that were recorded for more than two hours or for the whole length of the experiment were divided accordingly to obtain the average speed that contributed to each time point. Frequencies of nuclear translocations were measured from the graphs of nuclear displacement versus time. Every peak above 10 μm was considered as an independent event. The established criterion for the detection of nuclear translocations from the nuclear displacement versus time graphs was validated by visual inspection of the first 60 tracked cells. Moreover, when there was doubt about one single nuclear translocation event, this was solved by visual inspection of the series of images. Once again, frequencies were measured at different time points to obtain information about the time course of the experiment.

2.10 Electroporation

2.10.1 Plasmid amplification

Recombinant DNA was amplified by transformation of top 10 chemo-competent *E. coli* (Life Technologies, Gent, Belgium). Bacteria stocks were kept at -80C° until the time of transformation. Upon removal from the freezer, bacteria were placed on ice. After that period, DNA (20 ng to 1 μg) was added to the bacteria and they were gently re-suspended by typing the tube a few times. Bacteria and DNA were incubated on ice for 30 minutes. Then, the mixture was heat-shock treated by placing it at 42 °C for 90 seconds in a heat block or in a water bath. After that, the mixture was placed back on ice for 2 minutes and seeded in agar plates containing the appropriate selection antibiotic. Fourteen to 16 hours later, one single and isolated colony was inoculated in a 5 ml starter culture LB medium (Life Technologies, Gent, Belgium), which was kept in agitation for 8 h at 37°C. The starter culture was diluted 500 times in 100 ml. Incubation was performed under strong agitation at 37°C for 14 to 16 hours. The next day, transformed bacteria were pelleted at 5000 g for 15 minutes in a refrigerated centrifuge at 4°C. Medium was removed and pellets were stored at -20°C for one or two days until the moment of plasmid isolation. DNA purification was perfomed by using DNA preparation kit (Qiagen, Venlo, Netherlands) according to the instructions of the manufacturer. DNA was eluted from the columns using water. Quantification of the DNA was done using a regular spectrophotometer or a nanodrop machine. In addition, linearization of the plasmid for 2 hours, at the appropriate temperature, using a restriction enzyme was carried out in order to perform agarose gel electrophoresis and have a visual identification of the restriction pattern of the plasmid. Agarose gels were made of 1% agarose containing 0.5 μg/ml of ethidium bromide.

2.10.2 Plasmid construction

The identification of electroporated interneurons during gain and loss of function experiments was achieved by co-electroporation of a fluorescent Dlx reporter plasmid. This plasmid was constructed based on the pDsRed 2.1 plasmid (Clontech Laboratories) and the plasmid encoding the *Dlx 5/6* enhancer (p1230 dlx enh1), kindly provided by Dr. Rubenstein (UCL, San Francisco, USA). The design of the construct was such that the Dlx 5/6 enhancer sequence was downstream the beta globin minimal promoter so that both sequences were driving the expression of DsRed on Dlx1, Dlx2 and/or Dlx5 expressing interneurons (Zerucha et al., 2000, Stuhmer et al., 2002)(figure 2.1).

Initially, both plasmids were digested with BamHI and ApaI according to the instructions of the manufacturer (New England BioLabs) to set a double digestion with these enzymes. In this way, the pDsRed 2.1 was opened in the multi cloning site while the p1230 dlx enh1 released the sequence encoding the beta globin promoter and the dlx 5/6 enhancer with complementary end sequences. After digestion, both reaction mixtures were loaded on a 1% agarose gel for the separation of the generated fragments using electrophoresis. The bands of the fragments of interest were visualized under UV light, cut from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen, Venlo, Netherlands). To assess the purity of the isolated DNA, small samples of the purified fragments were loaded on a 1% agarose gel for electrophoretic analysis in the presence of a SmartLadder and quantified with the program Quantity One. Once the vector and insert were obtained, a ligation reaction was set to a final volume of 10 µl. Several conditions were tested for this procedure and a 3:1 ratio was found to be the most appropriate for the relative amount of insert to vector. In addition to the DNA, the reaction mixture was composed of 75 ng of vector, 1 µl T4 DNA ligase (Fermentas) and 1 µl 10x T4 DNA ligase buffer (Fermentas). The ligation reaction was carried out for 4 h at room temperature. Then, the ligation mixture was transformed into Top 10 chemiocompetent *E. coli* (Life Technologies) to obtain single colonies containing the desired constructs or the re-ligated empty vector. Transformation was done according to the protocol described for plasmid amplification with the addition of an extra step after the heat shock treatment. This step consisted in the addition of 800 µl of LB medium and in a 1 h agitation at 37°C to increase the efficiency of the transformation. Identification of colonies transformed with the desired construct or with the re-ligated vector was done by screening at least 10 randomly selected colonies. Isolated colonies were grown in a small volume (5ml) of LB liquid medium containing 50µg/ml of kanamycin and, after overnight incubation, DNA was isolated using the alkali and sodium dodecyl sulfate (SDS) method. Finally, after 2 hours of digestion with both BamHI and APAI at 37°C, the purified plasmids were loaded on a 1% agarose gel for electrophoresis in TAE buffer containing 0.5 µg/ml EtBr. In this way, a positive selection of bacterial cultures containing

the fluorescent Dlx-RFP reporter plasmid took place since these digested plasmids expose two bands with a band size of 0.5 kb and 4.1 kb for the insert and the vector, respectively (figure 2.2 A and B). The positive clones were subcultured overnight at 37°C to amplify the plasmid DNA as described for plasmid amplification. Further confirmation of successfully generated vectors was obtained from the electroporation of embryonic brain slices which specifically labeled migrating interneurons (figure 2.2 C).

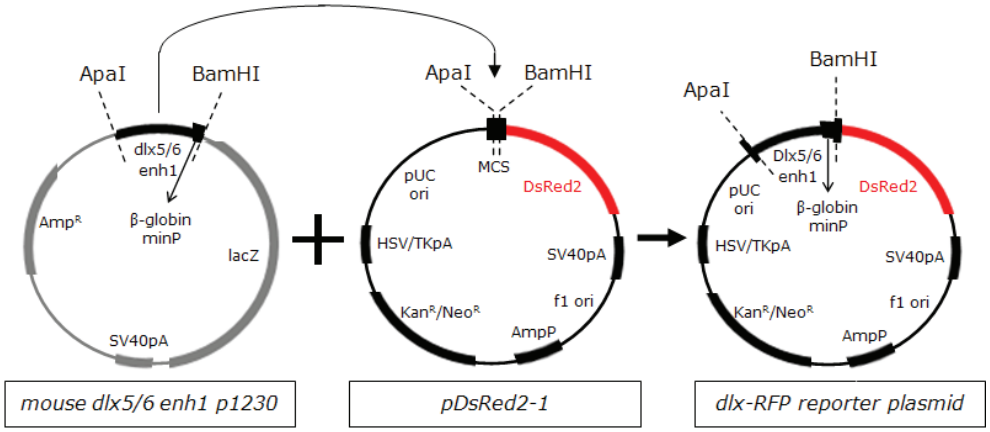

Figure 2.1. Schematic illustration of the Dlx-RFP reporter plasmid construction. Both plasmids, mouse dlx5/6 enh 1 p1230 and pDsRed 2-1, were digested using the restriction enzymes BamHI and ApaI. The purified fragment from the mouse dlx5/6 enh1 p1230 plasmid containing the dlx5/6 enhancer and the β-globin minimal promoter (black) were inserted into the digested pDsRed2-1 vector containing the coding sequence for the RFP (red). Plasmid fragments that were not used for the construction of the dlx-RFP reporter plasmid are depicted in grey. Dlx5/6 enh1, mouse dlx5/6 enhancer 1; β-globulin minP, β-globulin minimal promoter; SV40pA, SV40 early mRNA polyadenylation signal; AmpR, ampicillin resistance gene cassette; DsRed2, *Discosoma sp.* human codon-optimized Red Fluorescent Protein gene; f1 ori, f1 single-strand DNA origin; AmpP, ampicillin resistance (β-lactamase) promoter; KanR/NeoR, kanamycin/neomycin resistance gene; HSV/TKpA, herpes simplex virus thymidine kinase polyadenlylation signal; pUC ori, pUC plasmid replication origin.

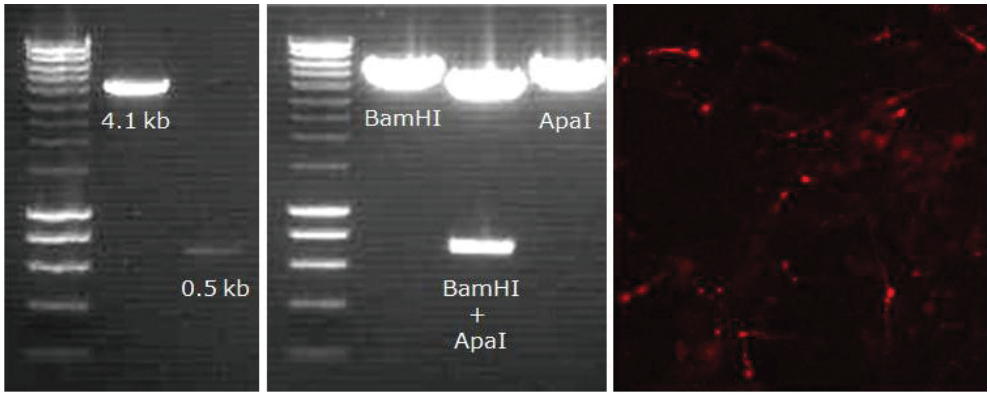

Figure 2.2. Dlx-RFP reporter plasmid construction. From left to right panels are labeled as A, B and C. (A) Agarose gel electrophoresis of isolated and purified fragments before the ligation reaction. The band of 4.1 kb represents the pDsRed2-1 fragment (75 ng/µl) and the band of 0.5 kb represents the *Dlx5/6* enhancer fragment (20 ng/µl). (B) Agarose gel electrophoresis for the analysis of the restriction pattern generated after digestion with BamH1 and ApaI of the Dlx-RFP reporter plasmid. Restriction digestion was performed with BamHI, BamHI+ApaI and ApaI respectively. (C) Confocal image showing the RFP positive cells in the MGE obtained from a coronal vibratome slice after electroporation with the generated reported plasmid.

2.10.3 MGE targeted electroporation

This method was used to deliver genes of interest or shRNA sequences into selected regions of the brain. A restricted pattern of electroporation can be achieved by the selective injection of DNA into a specific place and by the accurate positioning of the electrodes.

Gain and loss of GlyR function experiments for the assessment of interneuron migration were performed on in vitro cultured slices. Gain of GlyR function was achieved by overexpression of the wild type form of the mouse GlyR alpha 2 while loss of GlyR function was achieved by electroporation of a shRNA sequence targeted against the 3'UTR region of mouse GlyR alpha 2. Successfully electroporated interneurons were identified by co-electroporation with a Dlx-driven RFP reporter plasmid. E13 embryos were processed accordingly to generate 300 µm thick brain slices that were subsequently placed on milicell inserts (Millipore, Belgium) following the same procedure as for in vitro culture. After sectioning, the slices were transferred to an incubator (humidified environment at 37°C supplemented with CO_2 5%) until the moment of electroporation. Then, the slices mounted on the insert were transferred and placed on top of the negative electrode, which was covered by a layer of 1% agarose. Recombinant DNA (1.4 µg/µL each plasmid) was mixed with Fast Green (Life Technologies, Gent, Belgium), loaded into a micropipette and injected in the MGE by using a microinjector (Narishige, Japan). Micropipettes were made of borosilicate glass capillary tubing pulled with a P97 micropipette puller (Sutter Instruments, USA). Injection was carried out inside a flow cabinet under the visual guide through a stereomicroscope. After injection, one drop of PBS was placed on top of the slice to facilitate the positioning of the positive electrode. The positive electrode, a platinum surface attached to an agarose column, was carefully placed and hold on top of the injected region for the time length of the current delivery. The agarose column was made by punching a block of 1% agarose with a glass Pasteur pipette. After optimization, the current stimulation parameters were set to 5 pulses of 100 Volts, lasting for 10 ms, delivered every 1 second using an ECM 830 square electroporation system (Harvard Apparatus, USA). All this procedure was repeated for the contralateral MGE and every slice placed in the same insert. At the end of the electroporation procedure, the inserts and slices were washed with medium and placed back in the incubator. Three days after electroporation, slices were fixed in PFA 4% for 30 minutes, washed with PBS and imaged under confocal illumination.

2.10.4 Dorsal electroporation

The electroporation of dorsal progenitors and radially migrating cells was carried out in whole head after decapitation of E13 embryos. Plasmid mixture was injected into the left and right ventricle of the E13 brain using borosilicate glass pipettes and a pneumatic microinjector (Narishige, Japan). Plasmid mixture

included 0.05% Fast Green for visualization of the injected DNA. Micropipettes were pulled in a P1000 pipette puller (Sutter instruments, USA). After injection, the whole head was immediately electroporated using 5 mm electrodes (NEPA GENE) and a BTX ECM-830 Electro Square Porator (Harvard Apparatus). Electrodes were placed across the head in such a way that the positive pole was touching the dorso-lateral head. In this case, the current stimulation parameters were set to 5 pulses of 50 Volts, lasting for 50ms, delivered every second while using the LV mode. After this, the brains were isolated from the head in cold PBS/Glucose (25 mM) and subsequently embedded in 3% low melting point agarose dissolved in PBS. Coronal brain slices of 300 µm were obtained with a vibratome (Thermo Scientific Microm HM 650 V). Slices with a visible MGE were transferred to Millicell cell culture inserts (Millipore) in 35 mm dishes with 1 ml culture medium. This medium consisted of neurobasal medium supplemented with 1% N2, 2% B27, 2 mM L-glutamine and 1% penicillin/streptomycin (PS) solution (all from Invitrogen). Organotypic brain slices were kept in a semi-dry condition at 37°C in 5% CO2 and analyzed under the confocal microscope after two day in vitro (2 DIV). Slices were subsequently fixed for 30 min in 4% PFA and analyzed, cryoprotected in 30% sucrose and re-cut using a cryostat (Leica).

2.11 Fluorescent activated cell sorting (FACS)

In order to isolate migratory interneurons and perform subsequent western blot experiments, slice cultures, generated from the Dlx-EGFP transgenic animal, were treated for 18 h with strychnine or equivalent amount of vehicle. After this period, MGEs were dissected out by using a stereomicroscope and sharp dissection instruments (Fine science tools, Heidelberg, Germany). Tissue pieces were mechanically dissociated in cold PBS and transferred to polypropylene tubes. Then, EGFP positive cells were separated using a FACS Aria II cell sorter. Post sorting analysis showed that purity was above 95%. Immediately after sorting, cells were pelleted at 300 g for 10 minutes at 4°C and incubated in lysis buffer for 15 minutes more. After that period, small aliquots were frozen at -80 until the time of the western blot.

For FACS purification of long term interneuron migration in response to strychnine blockade, in vitro slice cultures were treated for 24 hours with 1µM strychnine or equivalent amount of DMSO. After that period, cortices were dissected out by using a stereomicroscope and sharp dissection instruments (Fine science tools, Heidelberg, Germany). Dissection of cortex was done following a horizontal line starting in the ventricular zone at the height of the cortico-striatal junction. Tissue pieces were mechanically dissociated in cold PBS and transferred to polypropylene tubes. Then, the proportion of EGFP positive cells was analyzed using a FACS Aria II cell sorter. For each condition, 50000 events were acquired at a low speed. All the parameters and selection gates were kept constant for both conditions during analysis.

2.12 Calcium imaging

Calcium imaging was performed using Fluo4 (Life Technologies, Gent, Belgium) in slices from Dlx-EGFP transgenic animals. Loading was done at 37°C for 30 minutes in agitation without holding insert. Discrimination between the EGFP signal, which identified interneurons, and Fluo4 was possible thanks to the different two-photon excitation spectra for Fluo4 and EGFP, what allowed us to selectively excite Fluo4 by using light of 820 nm and EGFP with light of 900nm. When changing from 900 nm to 820 nm, the power of the laser needed to be reduced in order to minimize photo-bleaching. Filters and dichroic were the same as for time lapse experiments. For the assessment of spontaneous calcium transients one single plane was acquired every 500 ms for 30 minutes. Glycine- or GABA-evoked responses were recorded in ACSF at 37°C. Ligand applications were achieved by whole slice perfusion. Conditioned medium was added just before imaging. Image intensity analyses were done using the freeware ImageJ software (NIH), defining regions of interest and analyzing the change of intensity in those regions in function of time. Numeric values of intensity were then converted in traces using Clampfit (Molecular Devices). The same software was used for base line correction and power spectrum calculations.

2.13 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 minutes at 90°C and used for SDS PAGE. Separated proteins were transferred to PVDV membranes that were blocked for 1 hour in PBS-Tween with 5% dry milk. Primary antibody (N18, Sata Cruz biotechnology) was used at 4µg/ml, dissolved in blocking solution and incubated for 1 hour at RT. To assess myosin phosphorylation, E13 slice cultures were allowed to recover for 6 hours and then treated with strychnine 1 µM or equivalent amount of vehicle for 18 hours. After this period, MGEs were dissected out and processed for the extraction of proteins. Ten micrograms of protein were loaded per sample line onto the SDS-PAGE gel. Phosphorylation of myosin light chain (MLC) was assessed by using anti-mouse phospho-MLC (P-MLC) antibody (Cell signalling) dissolved in PBS-T 1:1000, and anti-mouse MLC antibody (Abcam) 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 build in tool present in ImageJ (NIH) and the signal of the phosphorylated protein was normalized by the total amount of MLC.

2.14 Proliferation assay

In order to check for GlyR effect on cell proliferation, *in vitro* cultured slices obtained from E13 embryos were treated with strychnine or equivalent amount

of DMSO for 21 hours in the continuous presence of 10 μM BrdU. After that period, slices were washed with cold PBS and fixed in 4% PFA for 30 minutes. Cryoprotection was carried out in 30% sucrose overnight. After that, slices were embedded and frozen on OCT embedding medium (Tissue Tek, Zakura). Re-cutting of these sections was done using a cryostat (leica, CM3050S) set to 20 μm thick. After re-sectioning, slides were kept at -20°C until the time of BrdU immunolabeling.

2.15 Statistical analyses

For all the experiments comparing two groups, differences were calculated with a t-test or the non-parametric Mann-Whitney U test. For experiments comparing more than two groups, ANOVA or Krustal Wallis tests were used. Selected par of columns were compared using Bonferroni or Dunns post-test. Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad software Inc., CA, USA). Values are presented as mean ± SEM. Differences were considered significant at $p<0.05$.

CHAPTER 3

Glycine receptor α2 subunit activation promotes cortical interneuron migration

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Chapter based on:

Avila A., Vidal PM., Dear T. Neil, Harvey Robert J., Rigo JM and Nguyen L. Glycine receptor α2 subunit activation promotes cortical interneuron migration. Under revision at Cell Reports.

3.1 Summary

Glycine receptors (GlyR) are detected in the developing central nervous system before synaptogenesis, but their function remains elusive. This study demonstrates that functional GlyR 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 GlyR containing the α2 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 α2 activation that control cortical tangential migration during embryogenesis.

3.2 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. Projection neurons arise from progenitors located in the germinal compartment of the dorsal telencephalon and reach the cortical plate (CP) by radial migration (Noctor et al., 2001, Marin and Rubenstein, 2003, Noctor et al., 2004), while interneurons are born in the medial and caudal ganglionic eminences (MGE and CGE, respectively) (Anderson et al., 1997). Interneurons 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 (Marin and Rubenstein, 2001, Metin et al., 2006, Wonders and Anderson, 2006, Ayala et al., 2007). Interneurons account for approximately fifteen percent of cortical neurons and contribute to local networks where they fine-tune cortical neuron excitability (Seybold et al., 2012).

The molecular and cellular mechanisms that drive interneuron migration have just started to be unveiled (Bellion et al., 2005, Schaar and McConnell, 2005, Ayala et al., 2007). Tangential migration is controlled by interplay between extracellular signals and cell autonomous programs (Pla et al., 2006, Caronia-Brown and Grove, 2011). 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 migrating interneurons (Nguyen et al.,

2001, Soria and Valdeolmillos, 2002, Heng et al., 2007). Neurotransmission-independent activities of γ-aminobutyric acid (GABA) have been widely characterized in the developing cortex by several groups (Lopez-Bendito et al., 2003, Cuzon et al., 2006, Cuzon Carlson and Yeh, 2011). Activation of type A GABA receptors ($GABA_AR$) 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). In line with these findings, *in vivo* experiments have demonstrated that GABA facilitates tangential migration when Na^+-K^+-Cl^- co-transporter 1 (NKCC1) expression is moderate, converting GABA into a membrane-depolarizing signal (Inada et al., 2011). While the role of GABA and $GABA_ARs$ have been investigated in interneuron migration, only limited attention has been given to glycine. Glycine is the smallest amino acid neurotransmitter and activates glycine receptors (GlyR) - strychnine-sensitive ligand-gated ion channels (LGICs). Activation of GlyR results in chloride ion flux through the cell membrane that regulates neuronal excitability (Lynch, 2009). GlyR containing the α1 and α3 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, GlyR containing the α2 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, Kilb et al., 2008).

The present work highlights the functional expression of GlyR in cortical interneurons and demonstrates their contribution to tangential migration in the developing cerebral cortex. We showed that endogenous activation of GlyR promotes 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; 3. Actomyosin contractions that support nucleokinesis in migrating interneurons. Thus, our work brings the first *in vivo* experimental demonstration of the molecular mechanisms by which GlyR control interneuron migration in the developing cerebral cortex.

3.3 Results

3.3.1 GlyR containing the α2 subunit are functionally expressed by cortical interneurons

GlyR containing the α2 subunit are predominantly expressed in immature neurons from the developing spinal cord (Becker et al., 1988, Malosio et al., 1991, Watanabe and Akagi, 1995), as well as from various embryonic brain regions (Malosio et al., 1991). Brain sections from 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 α2 subunits in interneurons (Figures 3.1A-3.1D). The subcellular distribution of GlyR α2 subunits was assessed on cultured MGE that were microdissected from E13.5 Dlx-GFP embryos. Immunolabeling revealed a homogeneous distribution of α2 subunits over the surface of the soma and growth-cone-like structures (further termed growth cones in the text) of the leading process (Figure 3.1E). Western blot analyses performed on FACS purified GFP-positive interneurons from Dlx-GFP embryos further confirmed the specific expression of GlyR α2 subunits by cortical interneurons at different milestone stages (E13.5, E15.5, and E17.5; Figure 3.1F).

We further performed whole-cell patch-clamp recordings to test whether α2 subunits integrate into functional GlyR. 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 3.1G). A concentration-response analysis of glycine-mediated currents was best fitted by the Hill equation and yielded an EC_{50} of 69 ± 12 µM, a Hill coefficient of 1.4 ± 0.3 and an average maximal current of 270 ± 73 pA. These values were in accordance with those reported previously for α2 subunit-containing GlyR 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 µM of strychnine) with an IC_{50} value of 0.10 ± 0.02 µM (Figure 3.1H). To gain further insight into the molecular composition of the GlyR expressed by cortical interneurons, the two components of picrotoxin, picrotin (PTN) and picrotoxinin (PXN), were tested on glycine-triggered currents. While both compounds are known to equally affect α1 subunit homomers, differential-blocking abilities of these compounds have been reported towards α2 homomeric GlyR (30-50 times more sensitive to picrotoxinin) (Lynch et al., 1995, Wang et al., 2007, Yang et al., 2007). Our experiments showed that glycine currents were less inhibited by application of picrotin than picrotoxinin (Figure 3.1I). Our results also excluded the contribution of β subunits in these GlyR, as β 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 homomeric α2-subunit containing GlyR.

3.3.2 Modulation of GlyR α2 subunit expression affects interneuron migration

To understand the physiological role of α2 subunit GlyR 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 +3DIV; Figure 3.2A). Slices were co-electroporated with Dlx 5/6 enhancer element-driven RFP reporter construct (Stuhmer et al., 2002) and plasmids encoding either GlyR α2 subunit (GlyRα2; or a control, Ctr), or shRNAs that target the α2 subunit (shGlyRα2; or a control scrambled shRNA, shSCR) (Young and Cepko, 2004). Electroporation of shGlyRα2 led to complete loss of GlyR function, revealed by the absence of glycine-evoked currents in targeted interneurons (Figure 3.2B). Quantification of interneuron distribution in the dorsal (cortical wall) and ventral telencephalon suggested that acute loss of GlyR function impaired cortico-striatal boundary (CSB) crossing and interneuron entry into the cortical wall (Figures 3.2C-3.2D). By contrast, overexpression of GlyR α2 subunits promoted migration of interneurons in the cortical wall (Figures 3.2E-3.2F). Together, these results demonstrate a correlation between the level of GlyR α2 homomer expression and the migration of cortical interneurons in cultured brain slices.

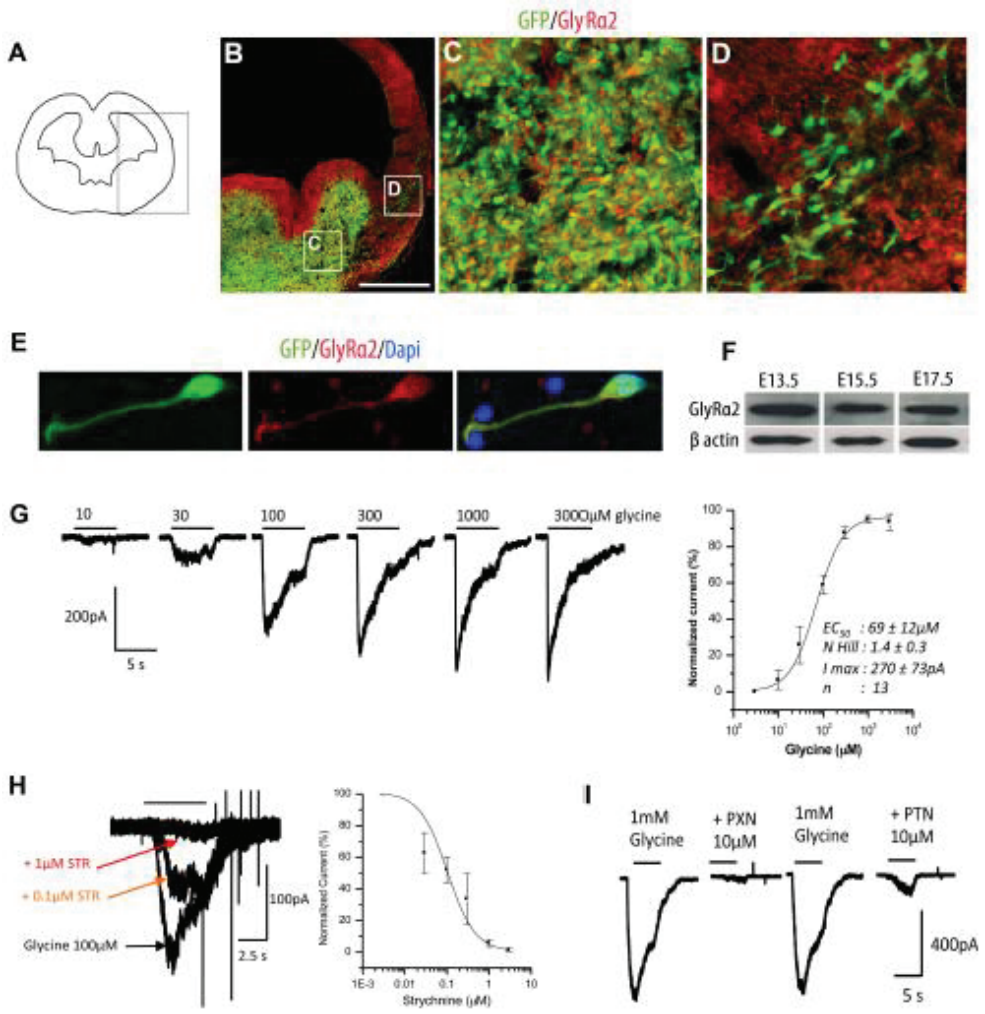


Figure 3.1. Migrating interneurons express GlyR α2 subunits during embryonic cortical development. A-D, immunolabeling performed on a brain slice from a E13.5 Dlx-GFP embryo showing partial overlap for GlyR α2 subunit (GlyRα2, red) and GFP in interneurons (green). **E**, Individual interneurons migrating out of MGE explants (GFP, green) show subcellular expression of GlyR α2 (red) at the soma and growth cone. Nuclei are in blue (Dapi). **F**, Western-blot analysis showing expression of GlyR α2 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). **H**, Representative traces of strychnine (STR) inhibition and inhibition curve (**H**) (n=5 cells). **I**, picrotoxinin (PXN) and picrotin (PTN) induce inhibition of glycine-elicited currents. Scale bar in panel B, 300µm.

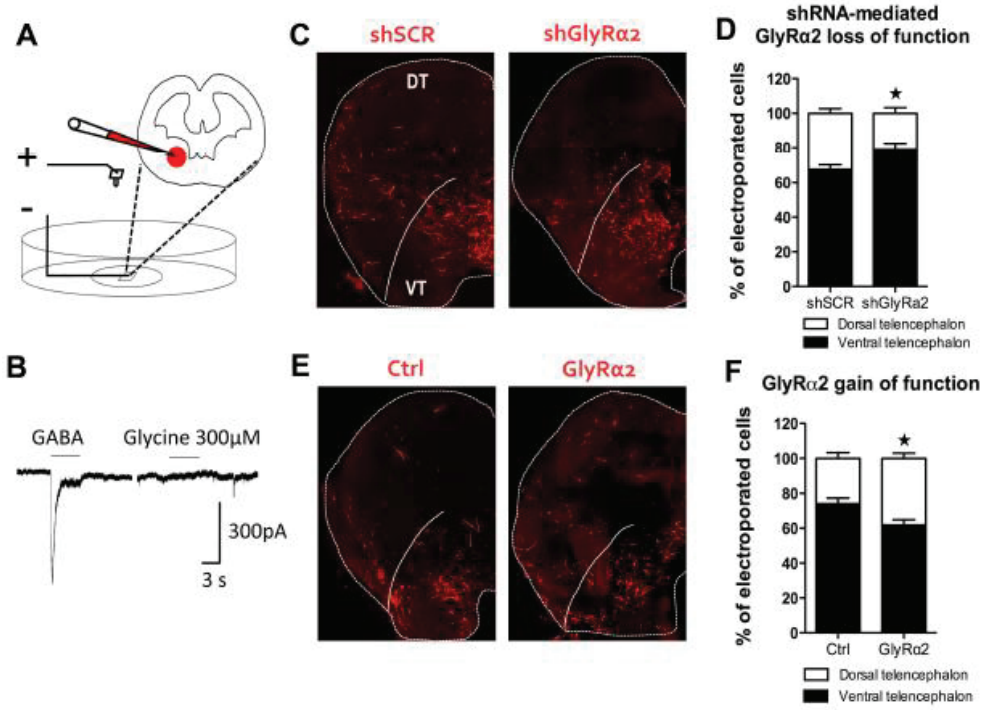


Figure 3.2. Modulation of GlyR α2 subunit expression affects interneuron migration. A, Scheme depicting focal electroporation of a plasmid solution (red) in the MGE of a cultured brain slice. **B**, lack of glycine-evoked currents in cortical interneurons after shRNA-mediated (shGlyRα2) knockdown of the GlyR α2 subunit (n=4 cells). **C-F**, GlyR α2 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 shGlyRα2 (**C**), or RFP and Ctrl or GlyR α2. Interneurons that cross the cortico-striatal boundary (solid white line) enter the cortex (**E**). Telencephalic distributions of electroporated cells with various plasmids, as indicated on histograms (**D**, **F**), * $p<0.05$, t-test; n=4-12 slices per condition. Abbreviations, dorsal telencephalon, DT; ventral telencephalon, VT.

3.3.3 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 GlyR to tangential migration (Figure 3.3A). For this purpose, brain slices from E15.5 embryos were maintained in a medium containing glycine, which tonically activates GlyR (see also Figure 3.8A). Bath application of strychnine reduced the velocity of migrating interneurons by 15%, compared to controls (Figures 3.3B-3.3C). A more pronounced effect of strychnine was observed when glycine was omitted from the culture medium (Figures 3.4A-3.4B), supporting the endogenous production and release of a GlyR agonist by cortical cells (Figures 3.4C-3.4D). It is worth noting that the 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, Metin et al., 2000, Manent et al., 2006)) over glycine via activation of their cognate receptors in cortical interneurons, or a progressive change of the chloride gradient through up-regulation of KCC2 (Bortone and Polleux, 2009) in older migrating interneurons.

Taurine is also present in the developing cerebral cortex (Flint et al., 1998, Benitez-Diaz et al., 2003) and taurine deficiency is associated with cortical development defects (Sturman, 1988). Due to the high levels of taurine in the developing brain, this transmitter has been proposed to act as an endogenous GlyR agonist in the developing cortex (Flint et al., 1998, Yoshida et al., 2004). However, glycine has also been detected in the developing brain (Benitez-Diaz et al., 2003) and our immunolabeling experiments suggest that cortical plate neurons represent a potential source of endogenous glycine (Figures 3.4C). We conducted time lapse experiments performed in the presence of taurine, and in combination with strychnine, to test the ability of this transmitter 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 3.4A-3.4B). This result is in agreement with a recent study that suggested that taurine may act via a different target, the K^+-Cl^- 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 towards the centrosome, a process termed nucleokinesis (Bellion et al., 2005, Schaar and McConnell, 2005). To examine the involvement of GlyRs on this process, we set up an *in vitro* assay in which MGE explants from E13.5 Dlx-GFP embryos were grown one day on homochronic cortical mixed feeder (Figures 3.5A-3.5B) (Bellion et al., 2005,

Godin et al., 2012). Strychnine application reduced migration speed in this assay (Figure 3.5C). Under control conditions, nuclear translocation frequency was 1.37 ± 0.07 events / hour and was constant during the whole experimental time period (Figures 3.5E-3.5F). Exposure to strychnine reduced this activity to 1.11 ± 0.06 events / hour (Figure 3.5D). 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.

3.3.4 Genetic disruption of the GlyR α2 subunit impairs interneuron migration *in vivo*

To further assess the contribution of GlyR containing the α2 subunit to cortical interneuron migration *in vivo*, we engineered a novel *Glra2* knockout mouse line (Figure 3.6) where exon 7 - containing the membrane spanning domains M1-M3 was deleted. Cortical interneurons that lack GlyR α2 subunit homomers were assessed in embryos arising from Dlx-GFP/*Glra2* knockout mouse line (Figures 3.7A-3.7B). 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 travelling in the deep SVZ migration stream were affected (Figures 3.7C-3.7E). Time-lapse recordings confirmed a reduction of both velocity of migration and frequency of nuclear translocation for interneurons traveling in the SVZ stream. It is worth noting that the amplitude of nucleokinesis remained unaffected in all migration corridors. Together, these results suggest that the functional expression of GlyR α2 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.

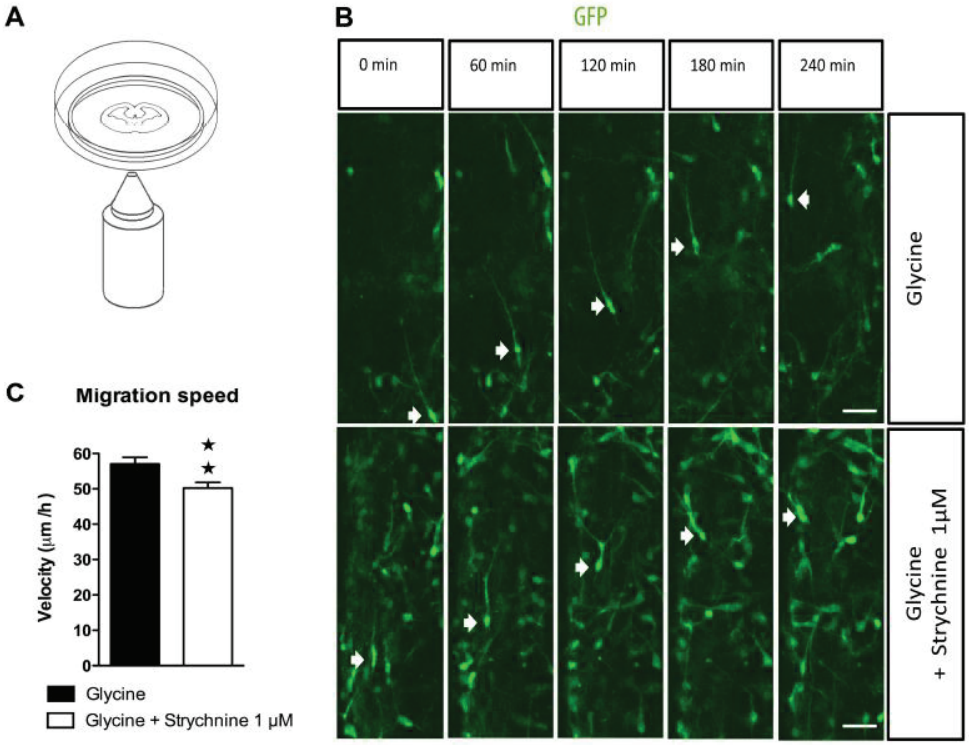


Figure 3.3. GlyR blockade inhibits interneuron migration in brain slices. **A**, Scheme illustrating a brain slice processed by confocal microscopy for real-time imaging. **B**, Time-lapse sequence of GFP-expressing interneurons migrating in E15.5 Dlx-GFP brain slices incubated in medium containing glycine 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 6 different brains. (See also Figure 3.S1).

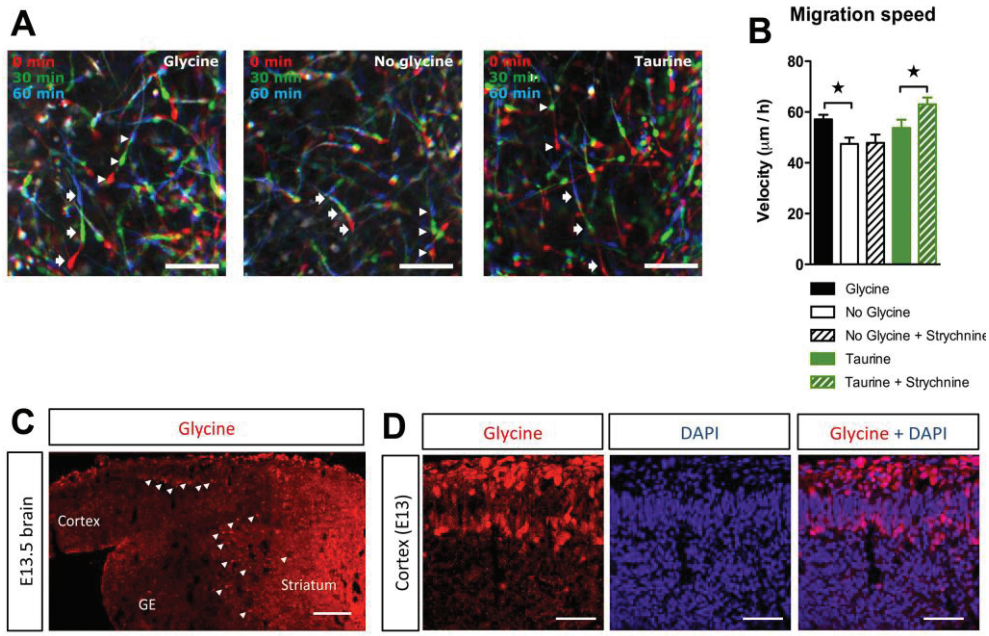


Figure 3.4. Glycine is the main endogenous GlyR alpha 2 agonist that promotes interneuron migration in the developing cortex. A-B, time-lapse recordings of interneurons migrating in E15.5 Dlx-GFP slices incubated in medium containing specific drugs, as illustrated. Color-coded pictures show different 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 (**B**), ANOVA-1, Dunnet's multiple comparison post-test *p<0.05. **C-D**, glycine immunoreactivity (red) in E13 mouse telencephalon. White arrows point to strongly immunoreactive glycine containing cells (**C**). Glycine accumulates within the cortical. Nuclei are in blue (Dapi) (**D**). Scale barsare : 50 µm (**A**), 200 µm (**C**) and 100 µm (**B**).

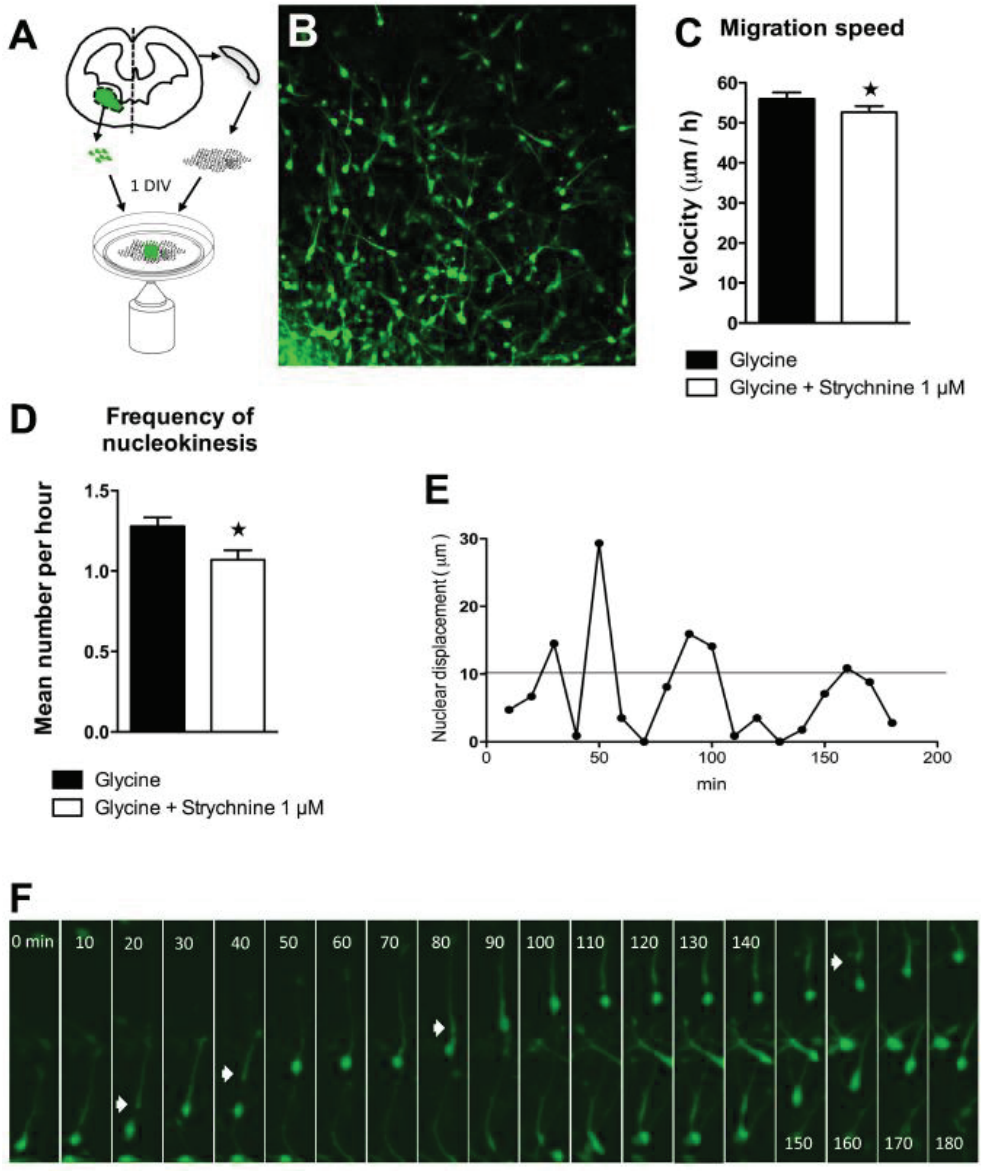
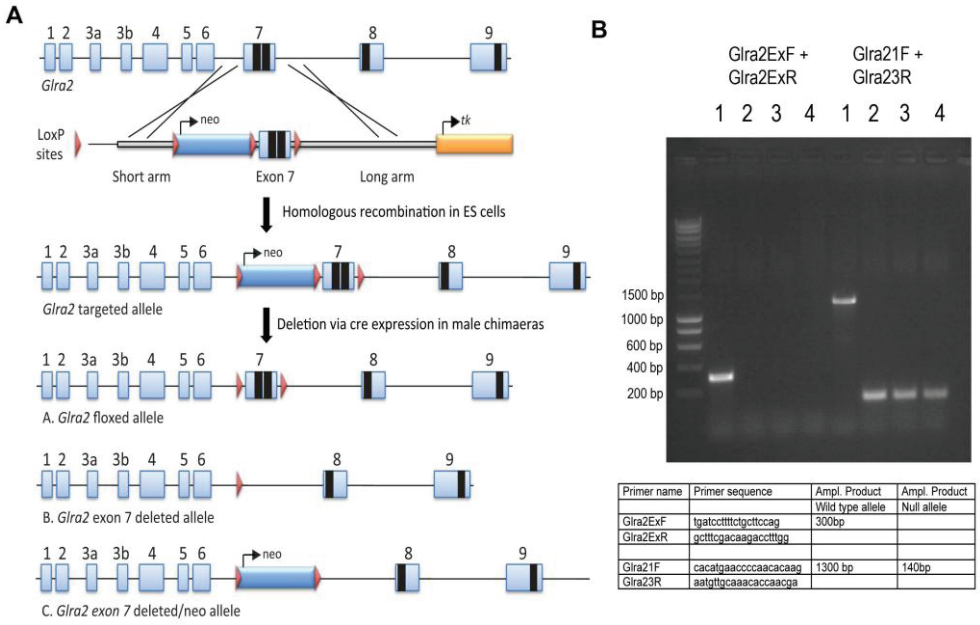

A
1 DIV
B
C
Migration speed
Velocity (μm / h)
60
50
40
30
20
10
0
Glycine
Glycine + Strychnine 1 μM
D
Frequency of nucleokinesis
Mean number per hour
1.5
1.0
0.5
0.0
Glycine
Glycine + Strychnine 1 μM
E
Nuclear displacement (μm)
30
20
10
0
0
50
100
150
200
min
F
0 min
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180

Figure 3.5. Nucleokinesis in migrating interneurons after GlyR blockade. **A-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=182-312 cells from 7 explants) (**C**) and nucleokinesis frequency (n=104-112 cells from 7 explants) (**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 μm. (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.



Primer name	Primer sequence	Ampl. Product Wild type allele	Ampl. Product Null allele
Glra2ExF	tgatccttttctgcttccag	300bp	
Glra2ExR	gctttcgacaagacctttgg		
Glra21F	cacatgaaccccaacacaag	1300 bp	140bp
Glra23R	aatgttgcaaacaccaacga		

Figure 3.6. Generation of the Glra2 knockout mouse line. A, the mouse GlyR α2 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 chimaeras 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. **B**, Genotype was carried out using two sets of primers targeted against exon 7. Numbered lines in the gels are different animals. Animal 1 is wild-type while animals 2 to 4 are KO. The table under the gel list the primers using for genotyping.

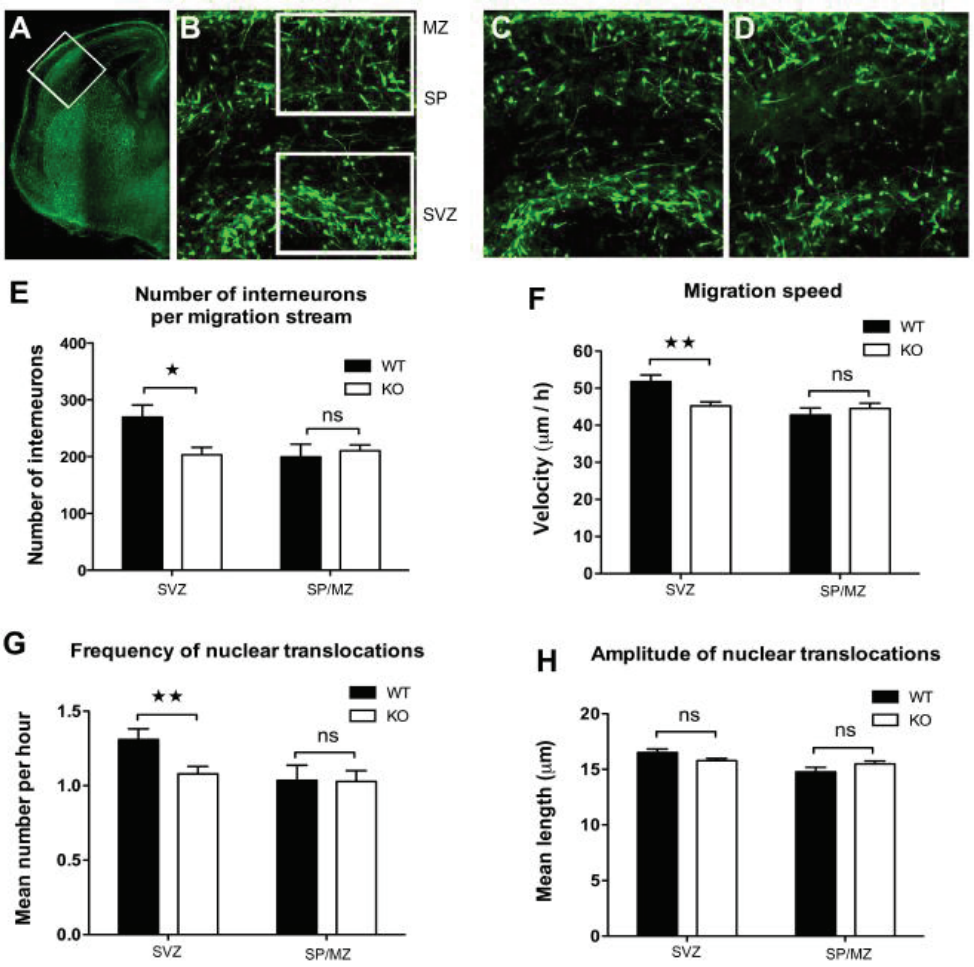


Figure 3.7. Genetic targeting of Glra2 impairs interneuron migration in the SVZ. **A-D**, *in vivo* analyses of interneuron migration in the *Glra2* knockout line. Two-photon imaging performed on E15.5 *Glra2*;Dlx-GFP shows interneurons migrating in the subventricular zone (SVZ), subplate (SP) and marginal zone (MZ) streams. Neurons migrating in the SP and MZ were analyzed together (common boxed area). **C-D**, magnified area of the migratory corridors boxed in **B**. **E-H**, histograms showing absolute numbers (**E**), velocity (SVZ, n=178-344 cells and SP/MZ, n=68-161 cells from 4 brains per genotype) (**F**), frequency of nucleokinesis (SVZ, n=97-195 cells and SP/MZ, n=39-86 cells from 4 brains per genotype) (**G**), and amplitude of nuclear translocation (SVZ, n=107-198 cells and SP/MZ, n=35-94 cells from 4 brains per genotype) (**H**) of interneurons (GFP, green) navigating in SVZ or SP/MZ. Abbreviations, WT, wild-type embryo littermates from *Glra2*;Dlx-GFP mouse line; KO, knockout embryos from *Glra2*;Dlx-GFP mouse line (See also Figure 3.S2).

3.3.5 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 3.8A). In addition, spontaneous calcium oscillations were recorded in interneurons (Figure 3.8B) that were modulated by strychnine (Figures 3.8C-3.8E). 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 3.8F-3.8G). It is also noteworthy that co-application of gabazine, a specific $GABA_AR$ 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 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 Polleux, 2009) (Figure 3.8H). Bath co-application of these blockers with strychnine showed that omega-conotoxin had an additive effect on cell migration inhibition. This result suggests that 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).

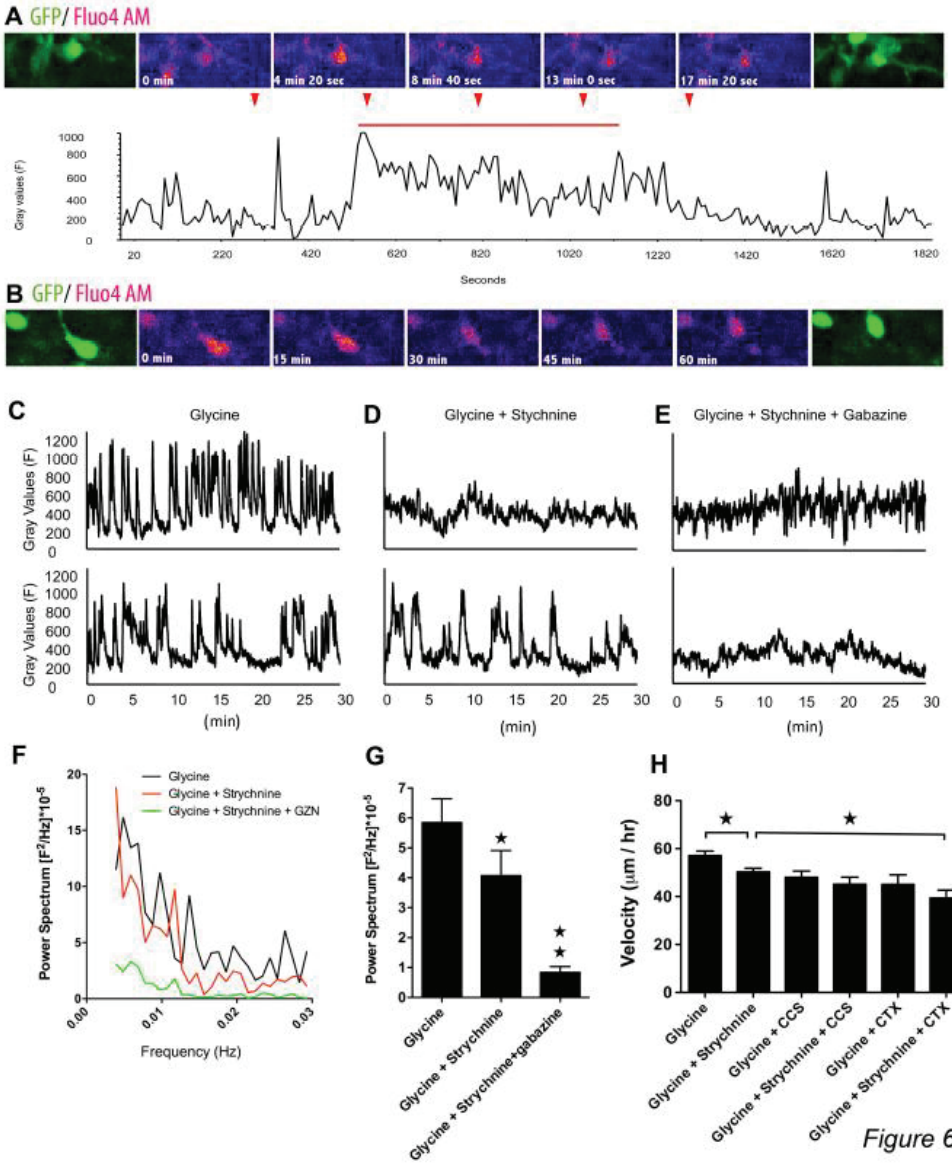

A GFP/ Fluo4 AM
0 min
4 min 20 sec
8 min 40 sec
13 min 0 sec
17 min 20 sec
Gray values (F)
Seconds
B GFP/ Fluo4 AM
0 min
15 min
30 min
45 min
60 min
C
Glycine
D
Glycine + Stychnine
E
Glycine + Stychnine + Gabazine
Gray Values (F)
(min)
F
Glycine
Glycine + Strychnine
Glycine + Strychnine + GZN
Power Spectrum [F²/Hz]*10⁻⁵
Frequency (Hz)
G
Power Spectrum [F²/Hz]*10⁻⁵
Glycine
Glycine + Strychnine
Glycine + Strychnine+gabazine
H
Velocity (µm / hr)
Glycine
Glycine + Strychnine
Glycine + CCS
Glycine + Strychnine + CCS
Glycine + CTX
Glycine + Strychnine + CTX

Figure 6

Figure 3.8. GlyR activation controls intracellular calcium dynamics. A-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. **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 μM strychnine (STR), 10 μM calciseptine (CCS) - a L-type calcium channel blocker, and 1 μM omega-conotoxin (CTX) - a 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). * $P<0.05$, Dunn's Multiple Comparison Test.

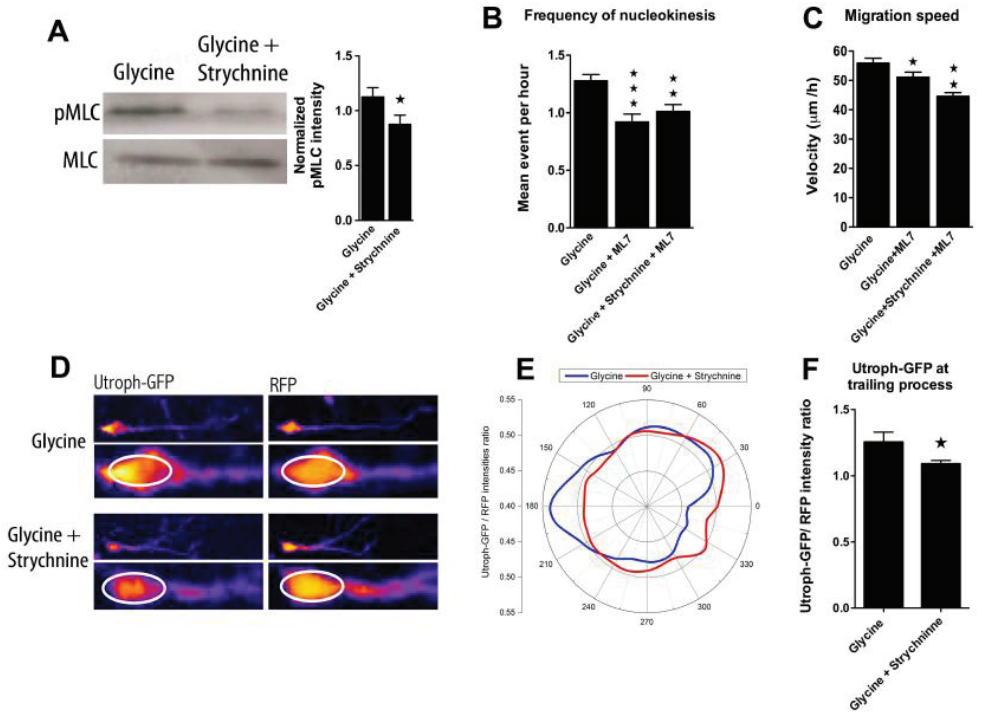


Figure 3.9. GlyR activation controls actomyosin contractility during nucleokinesis. A, Western blot analysis showing phosphorylation levels of myosin light chain (MLC) in MGE dissected from slices cultured in glycine-containing medium with or without strychnine (n=4 different brains). **B-C**, histograms of nucleokinesis frequency (n=77-115 cells form 7 explants) (**B**) and migration speed (n=182-200 cells from 7 explants) (**C**) of E15.5 Dlx-GFP cortical interneurons migrating out to MGE explants after treatment with various drugs, as indicated. **D-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). *p<0.05 , t-test.

3.3.6 Myosin II phosphorylation acts downstream of GlyR 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, Schaar and McConnell, 2005, Martini and Valdeolmillos, 2010). 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 pathway (Emmert et al., 2004). Interestingly, nucleokinesis correlates with calcium oscillations (Martini and Valdeolmillos, 2010). Therefore, we decided to test whether changes in the calcium transients after GlyR activation would regulate 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 3.9A). Treatment with ML-7 (a specific MLCK blocker), which reduces pMLC levels (Godin et al., 2012), mimicked strychnine application (Figures 3.5C-3.5D), and affected both migration velocity and nuclear translocation (Figures 3.9B-3.9C). Similar results were obtained with application of blebbistatin, a drug that prevents ATP loading on myosin II heavy chains (data not shown). Co-application 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 3.9B-3.9C). 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). This probe is based on the calponin homology domain of utrophin that binds F-actin without stabilizing it (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 result in modification of Utroph-GFP signals (Godin et al., 2012). MGE-isolated interneurons were co-transfected with RFP to compensate for the differences in electroporation efficiencies and changes in cell shape during the analysis (Figure 3.9D). Under control conditions, Utroph-GFP signal was detected as a non-homogeneous 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 3.9E-3.9F). 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.

3.4 Discussion

Neurotransmitters have roles beyond neurotransmission - particularly during the development of the central nervous system (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 GlyR has remained elusive. Our results demonstrate that functional GlyR are expressed by cortical interneurons in the developing forebrain. These GlyR are homomers, composed of α2 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.

3.4.1 GlyR are expressed by cortical interneurons during early stages of corticogenesis

Previous studies support the expression of α2 subunit GlyR in immature cells during cortical development (Malosio et al., 1991). However, to date, functional cortical GlyR 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 GlyR at several developmental milestones (E13.5, E15.5, and E17.5). GlyR immunoreactivity was not restricted to the cell body, but 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 α2 homomers. The EC_{50} value for glycine in cortical interneurons was 69 μM, in close agreement with previous studies (Flint et al., 1998, Kilb et al., 2002, Okabe et al., 2004). The sensitivity of these GlyR to picrotoxin suggests that the β subunit does not contribute to the formation of these GlyR (Pribilla et al., 1992). While acute knockdown of GlyR α2 subunits prevented glycine-elicited currents in cultured brain slices, we cannot completely rule out the presence of other GlyR subunits (e.g. α3 or α4) *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 α2 subunit loss of function experiments, suggesting that activation of GlyR containing the α2 subunit are central to cell migration.

In addition, our study support 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 GlyR are different. A small concentration of glycine is more effective than a high concentration of taurine on GlyR containing the α2 subunit. Indeed, the reported differences in EC_{50} are up to hundred times higher for taurine as compared to glycine for the same neurons (e.g. EC_{50} of 406 μM for taurine and 32 μM for glycine), and cortical neurons GlyR sensitivity to taurine is very low after birth (EC_{50} of 7.7 mM) (Schmieden et al., 1992, Hussy et al., 1997, Yoshida et al., 2004).

3.4.2 GlyR activation controls tangential migration of cortical interneurons

Developmental functions of GlyR 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 GlyR contribute to radial migration during late embryonic development. However, this effect was only seen *in vitro* after drastic pharmacological treatment of cultured slices with an excess of glycine and in the presence of two blockers of glycine transporters (Nimmervoll et al., 2011).

Our work unveils a novel function for GlyR activation in the tangential migration of cortical interneurons. Real-time imaging demonstrated that blockade of GlyR 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 *Glra2* 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 *Glra2* exon 7 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 suffer from lack of GlyR α2 subunit expression. However, since the molecular composition of those migratory streams are 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 α2 subunit homomers. This could be explained by the existence of compensatory mechanisms such as: 1. The expression of different GlyR that do not incorporate α2 subunits in interneurons navigating the MZ and SP streams; 2. A lack of functional GlyR 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 suggests that the delays in interneuron migration observed in *Glra2* knockout E15.5 embryos correlates 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 $GABA_A$Rs, which controls cell migration termination (Bortone and Polleux, 2009). It has been reported that GABA does not influence the speed of migration of cortical interneurons, but only the pausing time (Bortone and Polleux, 2009). Thus, GlyR and $GABA_A$R 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.

3.4.3 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 voltage-gated calcium channels 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 $GABA_A$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 observed 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(Pleasure et al., 2000). While GlyR α2 subunits were detected in both the soma and growth cone of cortical interneurons (Figure 3.1E), our data suggest that regulation of actomyosin contractility is dependent on GlyR activation at the rear of the nucleus rather than the growth cone, where other mechanisms may promote the phosphorylation of MLC. Real-time imaging of Utroph-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 GlyR containing the α2 subunit control cortical interneuron migration, thus expanding the known physiological functions of glycine and GlyR in cerebral cortex development.

CHAPTER 4

Dorsal progenitors and radially migrating neurons express functional glycine receptors that control cell proliferation

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Chapter based on:

Avila A., Vidal PM., Nguyen L. and Rigo JM. Dorsal progenitors and radially migrating neurons express functional glycine receptors that control cell proliferation. In preparation.

4.1 Summary

Early studies dealing with the role of neurotransmitters during brain development have suggested that the strychnine-sensitive glycine receptor (GlyR) could play a role during brain development. However, the physiological consequences of GlyR activation during early stages of cortex development in projection neurons have only been partially explored. The present work reveals that GlyRs are present in the proliferative regions of the cortex where they differentially control the rate of cell proliferation in the ventricular and sub-ventricular zone. Glycine-elicited currents in E13 radial progenitor cells depended on GlyR activation. Interestingly, strychnine blockade or specific silencing of GlyR increased cell proliferation in the sub ventricular zone, whereas it decreased proliferation in the ventricular zone.

4.2 Introduction

The development of the brain is a highly regulated process that requires the integration of the intrinsic genetic program with different signals present in the dynamic biochemical environment (Casanova and Trippe, 2006). Despite the high complexity of the adult brain, the cerebral cortex emerges from a small proliferative region located in the ventricular and sub-ventricular zones (VZ and SVZ) of the telencephalic vesicles. Dorsal progenitors located in these regions coordinate self-renewal with differentiation to subsequently give rise to excitatory projection neurons and glial cells (Noctor et al., 2001). The dorsal telencephalon begins as a thin layer of neuro-epithelial cells that gives rise to the first neurons of the mantle zone and to radial glial cells, a restricted population of cortical progenitor cells. Radial glial cells locate their cell bodies in the VZ and extend an apical process that contact the pial surface and works as a scaffold for newly generated neurons that migrate radially interacting with these structures (Rakic, 2003). Radial glial cells also give origin to projection neurons, mainly during early stages of cortex development, through symmetric or asymmetric divisions that can also give origin to more restricted populations of progenitor cells such as the intermediate progenitors (Malatesta et al., 2003, Rakic, 2003, Anthony et al., 2004, Mo et al., 2007). Intermediate progenitors are situated in the SVZ and differ from radial glial cells in the expression of specific markers, and the lack of an apical process. Intermediate progenitors are believed to amplify the neurogenic process and to be responsible for the extension of the cortex surface present in mammals (Takahashi et al., 1995b, Kakita and Goldman, 1999).

Accumulating studies have highlighted the general mechanisms of action through which the main inhibitory and excitatory neurotransmitters GABA and glutamate lead to changes in the rate of cell proliferation, cell migration, or changes in cell morphology (Cancedda et al., 2007). However, to date many

questions remain unsolved (Wang and Kriegstein, 2009). An important discovery is the excitatory effect exerted by GABA during development. Due to the developmental regulation of the intracellular levels of chloride, the effect of this classic inhibitory neurotransmitter leads to membrane depolarization and thus activation of voltage gated calcium channels that, in turn, induce changes in the rate of proliferation at the VZ and SVZ of the immature brain cortex. Strikingly, knockout animals for GAD67, the enzyme that produces GABA, do not show any apparent defect in cortex development. The putative compensatory effects exerted by activation of other neurotransmitter receptors, such as GlyR, may clarify this apparent discrepancy. However in the literature there is not yet any evidence to support this hypothesis.

The GlyR are pentameric assembling of subunits that behaves as an ion channel when expressed and activated in the cell membrane. This channel belongs to the ligand gated ion channels family, and selectively controls the flux of chloride ions between the cytoplasm and the extracellular environment (Lynch, 2009). This anionic channel is gated by glycine, but also by other amino-acids like taurine or β-alanine. So far, glycinergic neurotransmission has been found to play an important role in the synaptic transmission of sensory stimuli and in the generation of motor outputs in the spinal cord (Moss and Smart, 2001), but this receptor is also present in some regions of the brain like the hippocampus and the cortex. Importantly, extrasynaptic GlyR are present in the embryonic (Malosio et al., 1991, Platel et al., 2005) and postnatal brain. After birth, functional GlyRs have been found in cortical (Kilb et al., 2008, Kunz et al., 2012) and sub-plate neurons (Flint et al., 1998), and Cajal Retzius cells (Okabe et al., 2004), where they could play a developmental role. Moreover, this putative developmental role is supported by findings in the retina, where GlyRs exert a control on the generation of photoreceptors (Young and Cepko, 2004, Young-Pearse et al., 2006), and in the spinal cord, where it influences the generation of synapses (Scain et al., 2010). In this work, we performed whole cell recording in embryonic brain slices, slice cultures and shRNA interference to assess the presence and the role of GlyR in the early development of the brain cortex with a focus on dorsal progenitors and the control of cell proliferation.

4.3 Results

4.3.1 GlyR expression starts early during brain development

Previous works have described the presence of GlyR mRNAs in the embryonic rat brain (Malosio et al., 1991). However, there is no available data that describe the actual distribution of GlyR proteins in the dorsal cortex. We thus performed immunohistological analysis against GlyR alpha 2 subunits in embryonic mouse brain sections. We detected a widespread expression of GlyR subunits at embryonic day 13 (E13) (Figure 4.1). At this age, GlyR expression was observed throughout the thickness of the developing cortex, with a predominant VZ

location. Similar results were obtained using the 4A GlyR antibody, which recognizes all GlyR subunits (figure 4.2); suggesting that alpha 2 subunits might be the dominant GlyR subunit present at early stages of cortical development. Interestingly, immunolabeling for GlyR alpha 2 was restricted to both progenitors and postmitotic neurons at E15, and to cortical projection neurons settled in the cortical plate, at E17. At birth, GlyR alpha 2 subunits were only detected in some layers of the cortex as previously shown in rat (LoTurco et al., 1995, Flint et al., 1998).

In order to assess the functional expression of GlyR, we performed whole-cell patch clamp recording in slice culture. For this purpose, we infected E13 brain slices and recorded GlyR elicited currents on EGFP expressing cells after one or two days post-infection (figure 4.3A). In these conditions, the application of glycine triggered fast activating currents that showed a slow desensitization in the presence of the ligand (figure 4.3B). The maximal average generated current was 324 ± 140 pA. Successive applications of glycine were used to build a concentration response curve characterized by an EC50 of 72 ± 10 µM and a Hill coefficient of 1.7 ± 0.2 (figure 4.3C). All glycine-elicited currents were blocked by strychnine at 1 µM (figure 4.3D).

4.3.2 GlyR activation induces an increase in intracellular calcium in progenitor cells

The cellular effects of the activation of glycine and $GABA_A$ receptors have invariably and extensively been linked to membrane depolarization during early development. Moreover, the depolarization induced by neurotransmitters generally triggers an increase in intracellular calcium that likely plays a role during development. To test whether GlyR activation is able to modulate the intracellular calcium concentration in progenitors from the VZ, we loaded embryonic slices with Fluo4 and recorded its signal upon glycine application. Applications of glycine (300 µM) induced reversible increases in intracellular calcium in cells that were mainly located in the VZ (figure 4.3E). Glycine-elicited and spontaneous increases in calcium were also seen to travel along radial fibers. As expected, a similar effect was elicited by the application of GABA (100 µM, data not shown).

Taken together, these results demonstrated the functional expression of GlyR in the developing cortex at early stages of development. Moreover, GlyR activation promotes changes in intracellular calcium concentration. this suggests that GlyR might contribute to calcium-dependent regulation of specific events in cortical development.

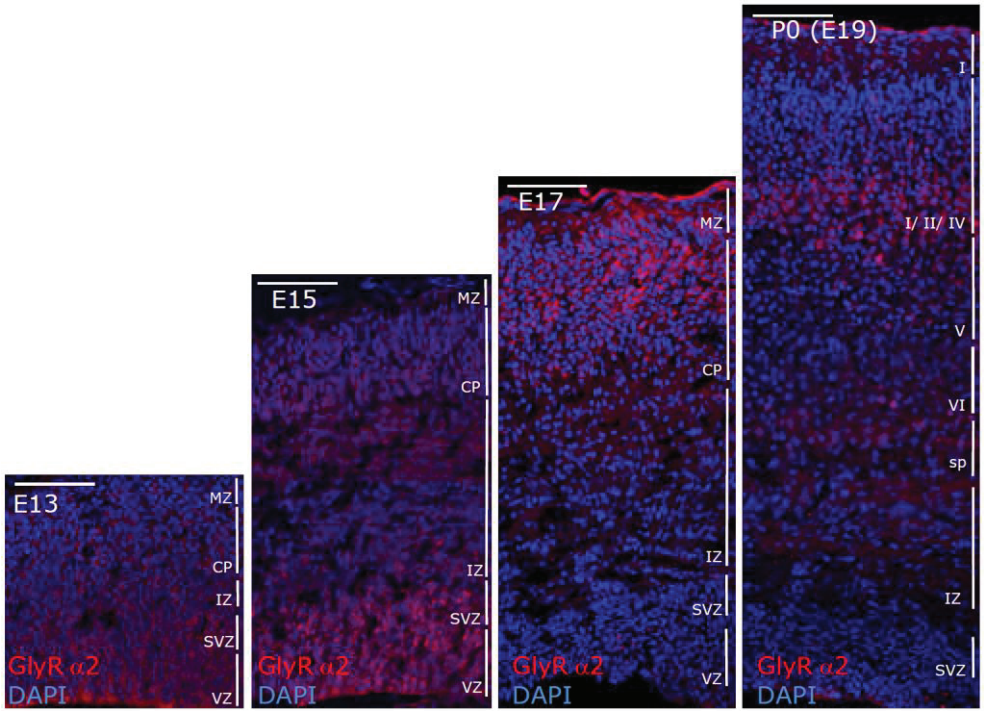

Figure 4.1. Glycine receptor expression during embryonic cortex development shows dynamic changes. Immunolabelings directed against GlyR alpha 2 subunits were performed at different stages during cortex development. Initially, GlyR is diffusely expressed through the whole cortex at E13. Then, it changes to a more localized pattern of expression at E15 that is restricted to the VZ. At E17, GlyR expression is no longer detected in the VZ, but it appears in the superficial layers of the cortex. Finally, by P0 the expression changes again and it becomes restricted to the cortical plate consistently with what was reported by others. GlyR labeling is presented in read and DAPI in blue. Scale bar is 75 μm.

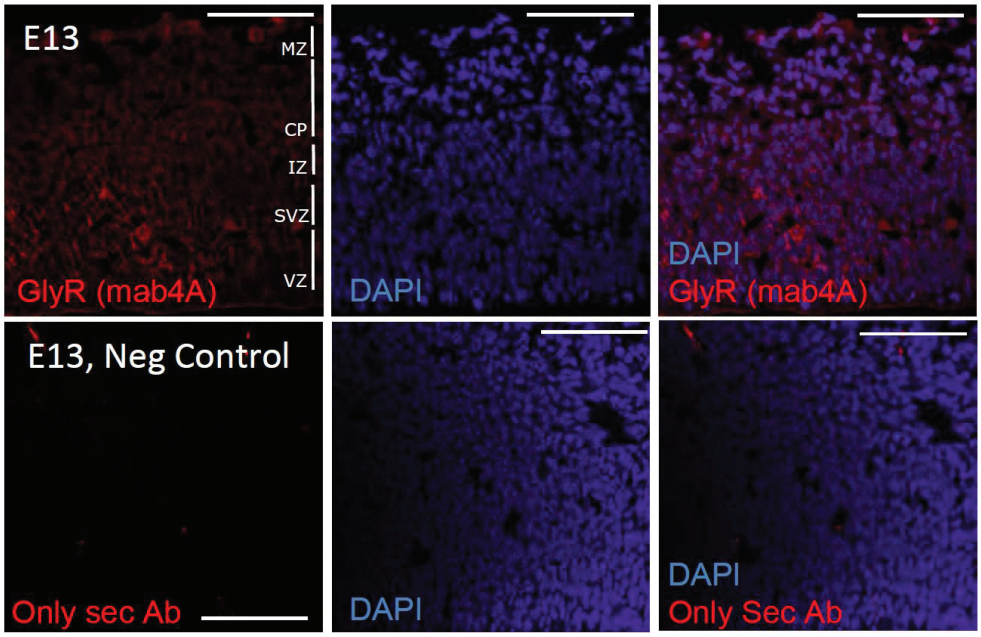

Figure 4.2. Expression pattern detected with a general GlyR antibody is similar to the pattern obtained with the alpha 2 specific antibody at E13. The general GlyR antibody 4A displays a diffuse labeling of the E13 embryonic cortex in a similar way as the alpha 2 subunit specific antibody (N18). Inferior panel shows the negative control for the immunolabeling. Scale bar is 75 µm.

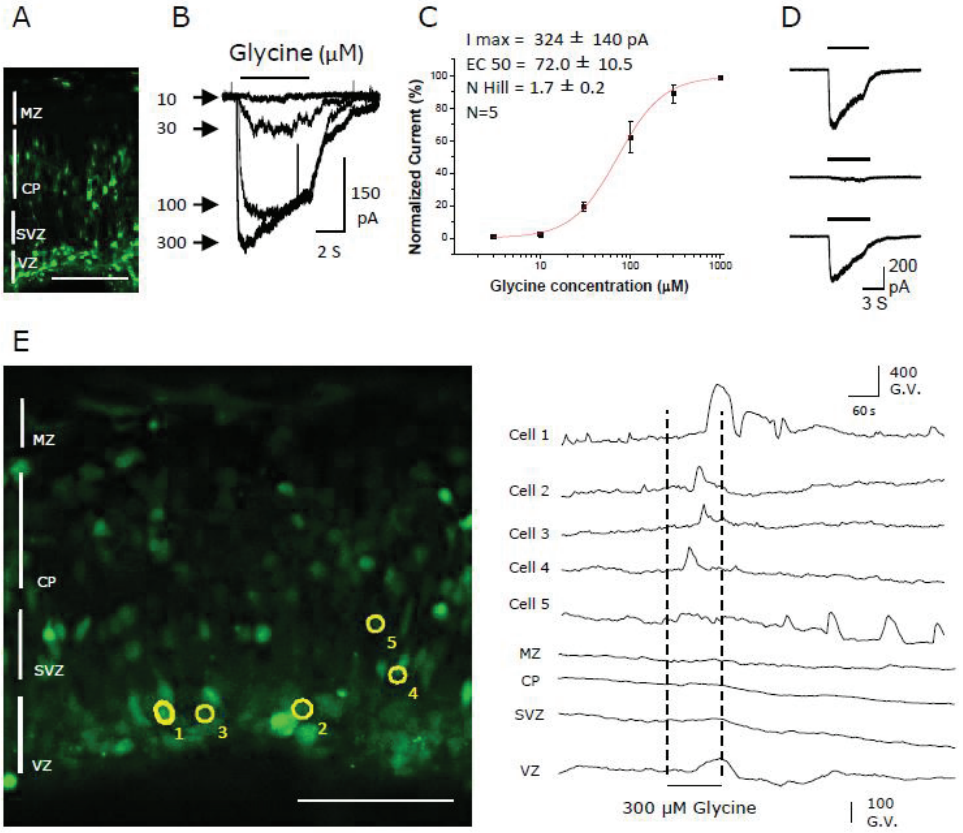

Figure 4.3. GlyR are functionally expressed in dorsal radially migrating neurons and can increase intracellular calcium. A, EGFP retrovirus infected E13 slices after 2 days *in vitro* (2DIV). Infected cells are located in the ventricular and intermediate zone. The majority of infected cells are radially oriented. **B,** glycine elicited currents on radially migrating neurons located in the CP and SVZ of infected slices after 2DIV. **C,** Concentration response curve built from the amplitudes of glycine elicited currents presented on B. **D,** Glycine elicited currents (300 µM) are reversibly and fully blocked by 1 µM of strychnine. Horizontal bar shows the duration of the application of glycine (top trace), glycine plus strychnine (middle trace) and glycine after wash out of strychnine (bottom trace). **E,** E13 cortical neurons from slices loaded with Fluo4 respond to glycine with an increase in intracellular calcium. Scale bar is 150 µm. Yellow circles identify the cells presented in the traces at the right side. Traces on the right lower corner show the average responses of the different cortical layers. Glycine mainly triggers calcium increase at the VZ.

4.3.3 Acute knockdown of GlyR decreases proliferation in the VZ

We next investigated the functional consequence of GlyR activation in cortical progenitor cells. We first assessed cell proliferation by performing a loss of function experiment. We performed acute depletion of GlyR alpha 2 subunit by *ex vivo* shRNA electroporation in the dorsal telencephalon. ShRNA-mediated silencing was achieved by targeting the 3'UTR region of GlyR alpha 2 subunit (Young and Cepko, 2004). After electroporation the brain was sectioned in thick slices that were subsequently kept in culture for 48 hrs. The slices were stained for KI67 in order to visualize proliferating cells. In these conditions there was no difference in the total number of electroporated cells between the control and the shRNA condition and, as expected, most of the KI67 staining was near the ventricular surface where some mitotic figures could be seen (figure 4.4A). We observed a significant decrease in proliferation upon GlyR alpha 2 subunit down regulation (17.9 ± 1.5 % of the electroporated cells were proliferating; figure 4.4B) as compared to control (28.0 ± 2.0 % of cells were Ki67 positive; figure 4.4B). A few double positive cells for GFP and KI67 were also observed in the cortical plate, but not significant differences were observed upon GlyR silencing in this region.

We further hypothesized that GlyR activation could promote differential regulation of cell proliferation in the VZ and SVZ. Hence, we performed an accumulative bromodeoxyuridine (BrdU) assay (Takahashi et al., 1995a). For this purpose, slices were treated with strychnine (1 or 5 μM) or vehicle, for 21 hours in the continuous presence of BrdU. After that, slices were re-sectioned, stained and evaluated for BrdU positive cells (figure 4.4C). Interestingly, there was a significant increase in the number of BrdU positive cells upon strychnine application (figure 4.4C). This increase was dose-dependent, and it was mainly localized in the upper part of the VZ or SVZ, demonstrating the dual function of GlyR in the control of cortical cells proliferation.

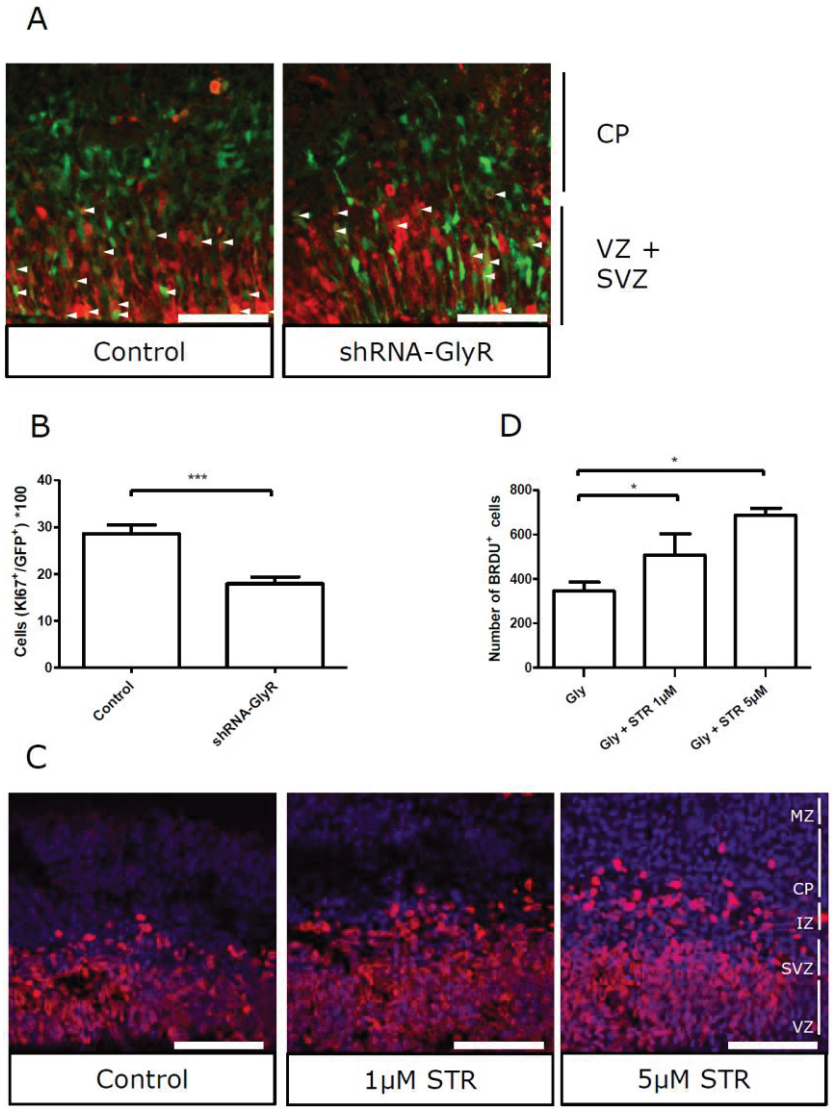

Figure 4.4. GlyR differentially affects cell proliferation in the cortex. A, Representative pictures of control and shRNA electroporated cortex at E13. Scale bar 75µm. White arrows point double EGFP (green) and KI67 (red) positive cells in the VZ and SVZ. **B,** Quantification of double EGFP and KI67 positive cells in the VZ and SVZ of EGFP and EGFP + shRNA-GlyR electroporated embryos. *** $p<0.001$. **C,** BRDU staining (red) for strychnine treated slices after 21 hours of *in vitro* culture in the continuous presence of BRDU and strychnine. Scale bar 75µm. **D,** Quantification of BRDU positive cells present in the whole field presented in C. *$P<0.05$.

4.4 Discussion

Here we demonstrated that GlyR are functionally expressed during early embryonic cortical development. Moreover, we showed that GlyR dynamically control the proliferation of dorsal progenitors.

4.4.1 Early expression of GlyR in the embryonic brain

Our data clearly shows that dorsal cortical progenitors express fully functional GlyR in progenitors of the VZ and in migrating neurons in the SVZ at E13. These findings are in agreement with other results from our lab that demonstrate the early expression of GlyR in ventral progenitors and migratory interneurons (see chapter three). Our results contrast with previous experiments that reported no glycine-elicited membrane currents in the VZ of developing rat embryos (LoTurco et al., 1995, Flint et al., 1998). This might be due to the fact that progenitor cells do not continuously express glycine receptor during development and this expression decreases near birth, as suggested by our immunolabelings. Another possibility is the differences between species that were used on these studies. Our data corroborate early studies that described the mRNA expression of GlyR subunits (Malosio et al., 1991) in the embryonic cortex (Malosio et al., 1991) and complement them with a pharmacological and functional description of GlyR- evoked currents. Previous electrophysiological experiments, performed in dissociated postnatal striatal progenitors (Nguyen et al., 2002) are in agreement with our data and suggest that dorsal cortical progenitors express non-synaptic homomeric alpha two containing GlyR, but the full pharmacological description of GlyR related currents in the embryonic cortex remains to be carry out.

4.4.2 Glycinergic control of cell proliferation during corticogenesis

The lack of phenotype in knockout mice with impaired production of GABA (Asada et al., 1997, Kash et al., 1997) has suggested the presence of compensatory mechanisms exerted by other neurotransmitters, such as glycine, during cortex development. Our data support for the first time a role for GlyR activation in cortical progenitors. GlyR activation seems to differentially affect cell cycle regulation in VZ and SVZ. These results completely mirror the effect of GABA in the germinal zone of the developing cortex (Haydar et al., 2000) and confirm the hypothesis that the control exerted by the classical inhibitory neurotransmitters, on cell proliferation, is differential for apical and basal progenitors. A study using specific progenitors markers such as Pax6 and Tbr2 would confirm this hypothesis. The reported differences in the magnitude of the effect could be understood based on the differential expression of glycine receptor alpha 2 and some of the GABA subunits, which are down regulated in the ventricular zone of mice and humans (Fietz et al., 2012). However, the mechanism behind the two opposite effects detected in the VZ and SVZ is still

not clear. Since GlyR effect is dependent on chloride gradient, it is tempting to speculate that chloride gradients might be different between in the two regions.

Previous analyses of GABA effect on cell proliferation have shown that $GABA_AR$ activation effectively shortens the G1 phase of the cell cycle (Haydar et al., 2000). In addition to that, changes in the length of the G1 phase have been associated with a preference for differentiative over proliferative divisions (Lukaszewicz et al., 2002, Dehay and Kennedy, 2007). Specifically, it has been demonstrated that NT3 lengthens the G1 phase and promotes differentiative divisions, while bFGF shortens the G1 phase promoting proliferative divisions (Lukaszewicz et al., 2002). Consequently, changes in G1 length might have a consequence on the neurogenic (Dehay et al., 2001, Calegari and Huttner, 2003, Calegari et al., 2005, Lange et al., 2009) and gliogenic processes by affecting the number of progenitor cells involved in differentiative divisions (Hodge et al., 2004, Pilaz et al., 2009) or directly affecting cell fate determination (Calegari and Huttner, 2003, Calegari et al., 2005). Our results suggest that GlyR activation mostly controls progenitors cells located in the SVZ, which mainly give birth to upper layer neurons and astrocytes during later stages of cortex development (Takahashi et al., 1995b, Kakita and Goldman, 1999). In this context, GlyR might have a similar effect than EGFR in the generation of astrocytes derived from the SVZ *in vivo* at later stages (Burrows et al., 1997) and, more interestingly, might directly influence neurogenesis.

In conclusion, our work contributes to a better understanding of the role of GlyR during brain development and their involvement in the control of cell proliferation.

CHAPTER 5

General discussion and conclusions

Neurotransmitter-induced oscillations in the membrane resting potential of the cell, together with electrical activity involving voltage gated ion channels and direct cell electrical coupling, can heavily impact on brain development. In general, the flux of ions across the cell membrane can have a multitude of effects during brain development, influencing different processes such as proliferation, differentiation, cell migration, axon path finding, and dendrite outgrowth (Spitzer, 2006). Analysis of each of these cellular processes is beyond the scope of our study, but some conclusions and assumptions, based on our study, can be made regarding proliferation, migration and differentiation.

5.1 Glycine receptors and cell proliferation

GABA and glutamate have been found to act before cell cycle exit during cortex development and to control cell proliferation. These neurotransmitters promote membrane depolarization and trigger efflux of chloride in cells located in the ventricular zone of the dorsal cortex. This effect goes along with a decrease in DNA synthesis and cell proliferation (LoTurco et al., 1995). The regulation of cell proliferation exerted by GABA and glutamate likely participates in a feedback loop that communicates a stop signal to proliferating cells once the cortex has reached its maturity, and GABA and glutamate become more abundant. It has been reported that cerebellar neuroblasts may also be affected by GABA; but in this case, $GABA_AR$ activation has a positive effect on proliferation (Fiszman et al., 1999). In addition, GABA signaling also modulates cell cycle progression of glial cells. It has been found that tonic activation of $GABA_AR$ decreases GFAP positive cell proliferation in the postnatal SVZ (Liu et al., 2005). In our study, we have described for the first time the presence of functional GlyRs in ventricular zone progenitors of the developing dorsal telencephalon, and we have also shown that these receptors might differentially change the rate of cell proliferation in the ventricular and sub ventricular zone of the dorsal telencephalon.

Here, we have focused on the study of GlyR in dorsal progenitor cell proliferation as we have not found defects in ventral neurogenesis. PH3 staining for mitosis was unchanged in GlyR KO compared to wild-type littermates (data not shown). Despite of that, we also found GlyR expression in the proliferative regions of the ventral telencephalon. Additional functions of GlyR may be related to cell fate determination in these regions, but this remains to be examined. The effect of GlyR activation on cell proliferation can have long-term consequences on the generation of excitatory neurons. Interestingly, the differential effect mediated by GlyR regarding the control of proliferation at the VZ and SVZ is similar to the effect reported for $GABA_AR$ (Haydar et al., 2000). By means of cumulative BrdU assays it has been shown that GABA decreases the overall generation of cortical neurons. This effect can be understood considering that the promotion of cell proliferation in the VZ leads to a sequestering of cells that do not differentiate to generate neurons or intermediate progenitors. Following the same logic, in our

case, where we have used strychnine to block GlyR, it is expected that this should induce an increased neurogenesis by the induction of cell cycle exit in VZ progenitor cells. However, this is not clear and the opposite effect could also be observed depending on the contribution of the VZ and SVZ to the newly generated neurons and on the magnitude of GlyR effect on these two populations of progenitors. Nevertheless, accumulative BrdU labeling do not clearly define the origin of labeled cells. Cell autonomous defects could also be involved especially since GlyR have been described to be present in other cell types such as Cajal Retzius cells and immature CP neurons at this age (Platel et al., 2005). It has been demonstrated that CP neurons can release glutamate in response to GlyR activation and indirectly signal to the proliferative region. Additionally, our methods do not allow us to conclude about the types of division that are affected by GlyR activation. This subject is important in order to fully understand GlyR effect on dorsal neurongesis. Differentiative divisions would make more difficult to assess the origin of the BrdU positive cells in the SVZ.

5.2 Glycine receptors and cell migration

In the present study we demonstrated a new role for GlyR in the control of the speed of interneurons migration. Similar effects have been reported for other neurotransmitters and ion channels that can also influence the resting membrane potential of the cells. $GABA_A$ receptors are present since early stages of central nervous system development and the study of their effects have generated abundant data during the last years. It has been shown that $GABA_AR$ activation can influence the migration of certain interneuron cell types directed to populate the olfactory bulb (Bolteus and Bordey, 2004). This effect was found to be similar in magnitude, but opposite in direction compared to our study. In addition, it has been shown that GABA may play a role in the termination of interneuron migration by increasing interneuron latency at later stages during cortex development. Moreover, it is believed that this may influence the final positioning of interneurons in the cortex (Bortone and Polleux, 2009). Thus, GlyR appear to have an overlapping role in the control of cell migration as they were found to be co-expressed on $GABA_AR$ expressing interneurons. This could be particularly determinant in understanding the compensatory mechanisms that appear to be acting in GABA deficient mice that do not show gross cortical abnormalities. Recently, similar effects on the regulation of cell migration have been found by the manipulation of potassium channels and glutamatergic neurotransmission in interneurons. Specifically, the over expression of potassium channels or the blockade of glutamatergic receptors, which decrease neuronal excitability and hyperpolarize the cellular membrane potential, have been shown to interfere with the proper lamination of CGE-derived interneurons (De Marco Garcia et al., 2011). In summary, the role of chloride currents and the subsequent depolarization may also have a long lasting effect and influence interneuron positioning. In addition, chloride currents may have an advantage

compared to sodium currents during this process. Since chloride currents depend on a smaller electrochemical gradient, they have a small impact on the resting membrane potential of the cell compared to sodium currents, which in turn can depolarize the cell to values that are dangerously high and can produce excitotoxicity. It has been suggested that sodium currents might be more important at later stages of brain development, once GABA have turned into an inhibitory signal and can control the excessive excitation provided by these currents (Ben-Ari, 2001). Nevertheless, since interneurons are a small population of neurons, compared to cortical projection neurons, a moderate disruption is likely to be unnoticed by using traditional histological methods. However, minor disturbances in the balance of excitation and inhibition can have profound consequences on the proper function of the affected cortical circuits (Penagarikano et al., 2011). Future experiments will need to address this hypothesis in the context of GlyR actions on cell migration. Currently, our data only support GlyR function on early phases of interneuron tangential migration.

5.3 Molecular mechanisms downstream GlyR activation

5.3.1 Molecular control of cell proliferation

The cellular mechanism that ultimately controls proliferation seems to be related to depolarization and calcium influx. Coordinated calcium waves are a hallmark of proliferative radial glia in the VZ. They appear to be mediated by extracellular ATP and are mainly dependent on intracellular release of calcium and the presence of connexin channels. Blockade of these spontaneous oscillations by metabotropic P2Y1 ATP receptor blockers decreases proliferation (Weissman et al., 2004). Thus, neurotransmitters such as GABA and glutamate exert a modulatory effect on these oscillations by promoting calcium influx from the extracellular compartment. Additionally, intracellular calcium stores are known to be functional in the developing cortex from E13 onwards, and they can contribute to the amplification of the signal by means of calcium induced calcium release (Faure et al., 2001, Platel et al., 2005). In our study, we have described the presence of GlyR in the ventricular zone of the developing dorsal telencephalon and have shown that these receptors can trigger calcium influx. Based on previous findings, it is possible that the changes on the spontaneous oscillations induced by GlyR activation are responsible for the changes on cell proliferation. If this is the case, it would be interesting to know the mechanisms behind this process that are sensitive to the frequency or the amplitude of calcium oscillations and can influence the cell cycle progression. Interestingly, myosin II is required for interkinetic nuclear migration of neuronal progenitors and it contributes to the delamination of intermediate progenitors (Schenk et al., 2009). Thus, in a similar way to what has been described for interneurons, MLCK could be one of the possible targets activated downstream GlyR.

5.3.2 Molecular control of interneuron migration

In the present study we have proposed a mechanism whereby GlyR activation in interneurons induces a depolarization of the cell membrane and this subsequently triggers an influx of calcium that affect myosin function by the activation of myosin light chain kinase, which finally leads to changes in the speed of interneuron migration. Intracellular calcium plays a central role in cell biology. It is believed that cells initially developed mechanisms to get rid of the toxic effects of high calcium concentrations using pumps that extruded or captured calcium ions. However, the extrusion of calcium or its capture in intracellular stores generated a concentration gradient that was conveniently exploited as a way of signaling mechanism. Today, the intracellular calcium and its dynamic changes, by way of calcium oscillations, are understood as a fundamental part of signal transduction networks. $GABA_AR$ and GlyR have a depolarizing action during development that subsequently triggers the opening of VGCC and the influx of calcium. Complementarily, NMDA receptors are permeable to sodium and calcium and can directly lead to depolarization and influx of calcium ions. Moreover, activation of L-type calcium channels by GABA induced-depolarization has a central role during the termination of interneuron cell migration (Bortone and Polleux, 2009). In our study, we provided correlative evidence supporting a L-type calcium channel dependent effect of glycine on cell migration. We show that blockade of GlyR by strychnine affect cell migration in the same direction as the blockade of VGCC. Moreover, when we treated interneurons with both blockers for GlyR and for L-type VGCC the effect on cell migration was not additive. It is interesting that although interneurons express other VGCCs (Bortone and Polleux, 2009), the effect of GABA and glycine is restricted only to the L-type VGCC. At the moment, there is no explanation for this, but a similar mechanism has been found to work on migrating fibroblasts. In those cells, calcium influx through L-type calcium channels changes the level of activation of MLCK and the phosphorylation of MLC at the trailing end of the cell (Yang and Huang, 2005). In the same way, the regulation of nuclear translocation in migratory interneurons is also dependent on MLCK activity, MLC phosphorylation and calcium oscillations (Martini and Valdeolmillos, 2010). In interneurons, myosin II phosphorylation promotes actomyosin contraction and pushes the nucleus toward the centrosome in an intermittent process. In our study we have provided evidence to support the involvement of GlyR in the calcium-mediated changes on myosin function during interneuron migration. In the same way as the proposed mechanism in fibroblasts, we proposed that GlyR-induced depolarization changes the phosphorylation of MLC acting through openings of L-type VGCC. We have observed that blockade of L-type calcium channels and GlyR equally affect nuclear translocations in a non-additive way, suggesting that both channels belong to the same pathway. Consequently, our results also demonstrated that GlyR blockade by strychnine decreases myosin phosphorylation.

5.3.3 Additional effects downstream GlyR activation

Our experiments do not exclude the possibility that the GlyR-mediated calcium increases can have other effects in addition to the modulation of myosin II activity. Recently, it has been shown that depolarization can impact on the cell autonomous machinery and change the activation of key transcription factors. Specifically, the effect of potassium channels on cell migration depends on the down regulation of *Dlx1* and one of its targets, Elmo1 (De Marco Garcia et al., 2011). Moreover, it is unlikely that the same mechanism that regulates interneurons migration would be involved in the control of cell proliferation on dorsal progenitors upon GlyR activation. Thus, there must be other targets sensitive to calcium operating downstream GlyR activation in the developing brain. For this purpose, we tested the effect of strychnine on the phosphorylation of the cAMP-responsive-element binding protein (CREB). Interestingly, our experiments show that strychnine blockade decreases the phosphorylation of CREB on E13 MGEs providing preliminary information (data not shown) that encourages further studies involving GlyR modulation of gene expression. Although CREB was originally found to be located downstream cAMP signaling, today it is one of the most characterized stimulus inducible transcription factors. There are several pathways that converge on CREB activation and the most important are the PI3K/Akt pathway, the MAPK pathway, the cAMP/PKA and the Ca^{2+}/CaMK pathway (Shaywitz and Greenberg, 1999). Several CREB target genes have been described in the nervous system and some of them are known to play an active role in neurogenesis. A few examples are: BDNF, Prolactin, bcl-2, PSA-NCAM, NGF, Cyclin D2, Mastermind-like 1 (Maml1), Notch 1, Hes 1, CaMK, Ng2 and SST (Conkright et al., 2003). Interestingly, other transcription factors, such as c-fos, can also be influenced by CREB activation (Ahn et al., 1998). These observations, together with the variety of transduction cascades that converge on CREB activation, make CREB one of the most important players of stimulus-induced gene transcription. CREB activation is largely recognized as an important event during brain development in the regulation of cell proliferation (Dworkin et al., 2007), cell survival (Bonni et al., 1999), neurodegeneration (Mantamadiotis et al., 2002) and differentiation of SVZ olfactory bulb neuroblasts (Giachino et al., 2005). Recently, the function of CREB has been studied in the control of cell migration. Using knock-out animals, it has been shown that CREB and CREM dysfunction leads to anatomical brain abnormalities related to interference with the proper cell migration of progenitor cells and immature neurons (Diaz-Ruiz et al., 2008). Moreover, migratory interneurons of the olfactory bulb show a transient increase in CREB phosphorylation during migration and differentiation (Giachino et al., 2005). Among the extracellular signals that are known to exert an effect on CREB activation, depolarization and its subsequent effect on intracellular calcium is one of the most studied. This depolarization and subsequent calcium influx effectively change the activation of CREB in response to GABA during adult neurogenesis (Merz et al., 2011). In consequence, changes on CREB activation

have an effect on cell differentiation and maturation of newly generated hippocampal neurons (Jagasia et al., 2009).

5.4 Physiopathological consequence of GlyR activation during development

Defects in cell proliferation and cell migration can lead to different neurodevelopmental associated pathologies such as epilepsy and autism (Penagarikano et al., 2011). However, at this stage we do not know if the migration delay leads to permanent interneuron positioning defects that would result in disruption of their network integration. On the other hand, mutations of the GlyR are mostly found in the GlyR alpha 1 gene, which is the adult form, and is almost completely absent during development. However, there is one mutation of the GlyR alpha 2 gene which has been linked to the development of autism (Piton et al., 2011). This is a missense mutation (R350L) where an arginine residue is replaced by a leucine in the intracellular loop. Although this mutation was found in a female patient, where there are two copies of the gene, one on each X chromosome, the change on the receptor might have a consequence over particular aspects of her disorder. In the case of autism and schizophrenia, another disorder with a developmental component, it has been suggested that the interaction between multiple gene variants rather than one individual mutation is the more likely scenario that originates the disease. Moreover, it has recently been found that GlyR alpha 2 gene is enriched in serotoninergic neurons, which are known to be involved in autism and related disorders (Dougherty et al., 2013). Thus, GlyR alpha 2 gene has been proposed as a strong candidate for further screening in autistic patients (Piton et al., 2011). Interestingly, the fact that mutant glycine receptors are observed in patients with developmental associated diseases such autism suggests that GlyR could be a target to develop therapeutical strategies. However, currently this is unlikely due to the lack of GlyR alpha 2 selective agonist or antagonists. Until now, cyclothiazide, the only molecule that has shown to selectively affect alpha 2 containing GlyR, lacks specificity because it affects AMPA and GABA receptors as well (Zhang et al., 2008).

CHAPTER 6

Perspectives

The study of GlyR and its effects on the proliferation of dorsal progenitors and tangential migration of interneurons can be expanded in an array of directions. Future research could move forward independently in each of these two subjects, or combine approaches to analyze the final effect of GlyR activation in the generation of cortical circuits. In the same way, further studies could also focus on specific aspects of GlyR effects at different levels of complexity. While there is a lot to learn about the molecular and cellular mechanisms downstream GlyR activation, there are even more questions about the possible effects of GlyR activation at the tissue level and on the functional organization of the brain cortex.

6.1 Perspectives on the study of cell proliferation

The results of our study present correlative information about the molecular mechanisms involved in the control of cell proliferation by GlyR. They suggest that GlyR-mediated effects on calcium signaling could have a direct consequence on cell proliferation. A more direct approach could consider the manipulation of the calcium buffering capabilities of the cell which should modify the effects of GlyR activation. Complementary, spontaneous calcium oscillations in radial glia cells are controlled by intracellular mechanisms and the contribution of VGCC to their generation have not been thoroughly considered. Here, we expect that chloride efflux will induce sufficient depolarization to activate VGCC, which are supposed to be the mediators of the increase in intracellular calcium. To understand this, it is firstly necessary to study VGCC expression and functionality in relation to GlyR activation on cortical progenitors, and migratory neurons. Additionally, calcium sensitive downstream targets involved on the control of the cell cycle should be studied as well.

The effect of GlyR activation on cell proliferation can have a long term consequence in the generation of neurons. Interestingly, the differential effect of GlyR in the control of proliferation at the VZ and SVZ is similar to the effect reported for $GABA_AR$ (Haydar et al., 2000). There, it has been shown that GABA decreases the overall generation of cortical neurons. The study of this possibility constitutes one of the main perspectives of our studies since an influence on the generation of excitatory neurons could have a direct impact on the functional properties of the cortex. There is considerable evidence suggesting that changes in the rate of cell proliferation of cortical progenitors and the generation of intermediate progenitors are responsible for the extensive expansion of the brain cortex in humans compared with other mammals. Although it is difficult to prove, the fact that GlyR affect the rate of proliferation in cortical progenitor support the idea that GlyR could be a factor responsible in part for the cortex expansion present in more evolved mammals.

6.2 Perspectives on the study of cell migration

Since GlyR effect is ultimately dependent on the chloride gradient that directly depends on the expression of NKCC1/KCC2, it would be important to analyze how the dynamic changes in the expression of NKCC1/KCC2 in migratory interneurons affect GlyR-mediated effects. It has been shown that GABA mediated effect differs in magnitude depending on the expression level of NKCC1/KCC2 during interneuron migration. This could explain more in detail how GlyR differentially controls interneuron migration at different ages, and at the level of the different migratory streams. At the cellular level, there is increasing evidence about the morphological changes experienced by migrating interneurons and the molecular mechanisms that control nucleokinesis and leading process branching dynamic are well understood. However, little is known about the coordination of leading process remodeling and nuclear translocation. Our studies about GlyR have not included the analysis of the leading process remodeling, but a preliminary analysis could be performed on the existing data. Perhaps, this analysis could indicate if neurotransmitters are involved or not in the coordination of the nuclear translocation and leading process remodeling during migration.

Our present results do not provide evidence related to long term effect of the GlyR-mediated control of cell migration. It remains an open question whether GlyR activation is involved or not in the final positioning of interneurons in the mature cortex. It would be interesting to check the distribution of different interneuron subtypes in the mature cortex of GlyR KO animals or in other models where GlyR are knockdown in specific interneuron subtypes. Interestingly, positioning defects may have functional consequences on the assembling and functioning of cortical circuits. The same models could be used in electrophysiological experiments to measure synaptic activity and evidence the presence of possible connectivity defects. Nevertheless, there are several examples of molecules that in addition to a role in the control of cell migration can independently influence other developmental processes such as synaptogenesis (Fazzari et al., 2010). This may be the case for GlyR, which can potentially influence several molecular processes by affecting the endogenous calcium dynamic and, by extension, other developmental processes.

Interneurons are a highly heterogeneous population of neurons. Here, we have focused our study on MGE derived interneurons that invade the cortex at E15. At this age we found that GlyR activation may contribute to their migration. However, when we investigated other ages such as E17, we found that GlyR activation may have an opposite effect. Thus, GlyR activation differentially affects different subpopulations of interneurons depending on their age. This may be explained based on the developmental regulation of the chloride gradient, which is responsible for GlyR induced- depolarization. Nevertheless,

these studies could be refined and expanded to explore GlyR effect in more restricted interneuron cell populations in the cortex or others structures such as the rostral migratory stream that provides neurons to the olfactory bulb. Certainly, staining using different interneuron markers such as CR, CB, VIP, SST and PV, in the mature cortex of GlyRKO animals, would serve as an indication for interneurons subtype that might be affected by GlyR activation. Additionally, a different transgenic mouse line could be used to selectively analyze specific interneuron populations (Taniguchi et al., 2011). These analyses could further expand our understanding on interneurons diversity and provide additional information to support a classification based on their early response to neurotransmitters.

6.3 Perspective on the study of cell differentiation

Our study has focused on the early stages of embryonic brain development and leaves unexplored the possible consequences on the morphological features acquired by neurons once they have found their final location in the cortex. However, some predictions can be made based on previous findings related to the effects of GABA. The over expression of KCC2 by in utero electroporation of dorsal progenitors, which prematurely transform excitatory GABA actions into inhibitory, although it does not have any impact on cell migration, has a long lasting effect that changes the morphology of neurons at later stages. KCC2 electroporated cells show an impaired development of branches, which are shorter and fewer (Cancedda et al., 2007). More generally, the effects of KCC2 are associated to its chloride transporting property, but the induction of morphological changes have also been associated with the direct interaction of KCC2 with the cytoskeleton (Li et al., 2007). Consistently with the trophic effect of depolarization at early stages, the over expression of potassium channels, which decrease neuronal excitability by hyperpolarizing the membrane potential, has similar effects to KCC2 manipulations and interferes with the proper morphological development of projection neurons (Cancedda et al., 2007). The overexpression of potassium channels during development also lead to morphological abnormities in cortical interneurons. This effect can be induced after P3 in CGE-derived interneurons and is independent on the effect that potassium channels have on interneuron lamination (De Marco Garcia et al., 2011). The blockade of NMDA and AMPA/Kainate receptors induces morphological defects reminiscent of potassium channel over expression in interneurons (De Marco Garcia et al., 2011). In this work the authors provide correlative evidence that suggest that the electrical activity changes the levels of *Dlx1* and one of its targets, Elmo1. This decrease in Elmo1 leads to morphological defects induced by potassium channel over expression and glutamatergic blockade. Thus, early electrical activity can change the expression of transcription factors and influence the morphological development. In the present work we have observed dynamic morphological changes on migratory

internneurons, but we were not able to confirm whether these changes will be still present after the end of cell migration. Since morphological features appear to be defined after P3 for projection neurons and interneurons, a future pharmacological approach to block GlyR should take this in consideration.

6.4 Perspectives on the study of cortical circuits

Besides the embryonic role of GlyR, their role in postnatal brain maturation remains unexplored. It has been demonstrated that GlyR are functionally present on the cortical plate, Cajal Retzius cells, and subplate neurons during postnatal cortex development, where they have a depolarizing action that can trigger the influx of calcium. Moreover, functional experiments have demonstrated that GlyR activation can modify spontaneous synaptic activity and the firing of action potentials. However, the functional role of GlyR has been left to speculation. Probably it has an influence in the generation of the early patterns of activity or it influences morphological maturation of neurons, but this remains an untested hypothesis. Giant depolarizing potentials are a hallmark of developing networks (Ben-Ari, 2001). Interestingly, GABA has an important role in their generation and maintenance. Thus, it would be interesting to also test the effect of glycine and its receptor in the immature cortical networks. Indeed, a role of glycine in this type of early activity is supported by the fact that mice which are devoid of GABA present no clear developmental dysfunction due to the supposed compensation exerted by other related neurotransmitters such as glycine. Moreover, there are a few articles that have documented the expression of GlyRs in different cell types (Kilb et al., 2002, Okabe et al., 2004, Kilb et al., 2008) of the postnatal cortex. Future experiments should clarify the role of these receptors.

Finally, although the experimental paradigms and reported conclusions are in agreement with the current theories and observations regarding brain development, and the excitatory effect of neurotransmitters, conclusions should be taken with caution aware of the possible artifact that might be introduced by our manipulations interfering with both $GABA_AR$ and GlyR function (Bregestovski and Bernard, 2012). In this context, less invasive techniques and *in vivo* measurements should confirm the validity and extend our conclusions in the future.

Publications and presentations

Publications related to the thesis

Avila A., Vidal PM., Dear T. Neil, Harvey Robert J., Rigo JM. and Nguyen L. Glycine receptor α2 subunit activation promotes cortical interneuron migration. 2013 Jan 23. Under revision at Cell Reports.

Avila A., Vidal PM., Nguyen L. and Rigo JM. Dorsal progenitors and radially migrating neurons express functional glycine receptors that control cell proliferation. In preparation.

Other publications of the author

Vidal P.M., Lemmens E., **Avila A.,** Vangansewinkel T., Chalaris A., Rose-John S., Hendrix S. ADAM17 is a survival factor of microglial cells in vitro and in vivo after spinal cord injury in mice. Submitted to Clinical Pharmacology & Therapeutics.

Swijsen A., **Avila A.**, Brône B., Janssen D., Hoogland G., Rigo JM. Experimental early-life febrile seizures induce changes in GABA(A)R-mediated neurotransmission in the dentate gyrus. Epilepsia. 2012 Oct 2. doi: 10.1111/j.1528-1167.2012.03694.x.

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Avila A., Nguyen L. and Rigo JM. Glycine receptor activation influences early cortical development. Acta Physiologica. 2010 Volume 200, Supplement 678 Part II :P-06

Selected poster presentations

Vidal P.M., Lemmens E., **Avila A**., Vangansewinkel T., Hendrix S. Inhibition of ADAM17 increases microglial death after spinal cord injury. 5th Annual meeting of the Society for Neuroscience. New Orleans, USA. October 13-17, 2012.

Avila A., Vidal P., Nguyen L. and Rigo JM. The Glycine Receptor is Expressed and Functionally Active in Migratory Interneurons and Influences Early Cortical Development. 41th Annual Meeting of the Society for Neuroscience. Washington DC, USA. November 12-16, 2011.

Swinnen N., Smolders S., **Avila A**., Legendre P. and Rigo JM. Complex invasion pattern of the cerebral cortex by microglial cells at the onset of neuronal cell migration in the mouse embryo. 41th Annual Meeting of the Society for Neuroscience. Washington DC, USA. November 12-16, 2011.

Avila A., Vidal P., Nguyen L. and Rigo JM. Glycine Receptor Activation Influences Early Cortical Development. IBRO meeting. Firenze, Italy. July 14-18, 2011.

Avila A., Nguyen L., Rigo JM. Migrating GABAergic interneurons respond to glycine with a glycine receptor mediated current. FENS school of neuroscience. Multiple facets of GABA in Brain Development. Obergurgl, Austria. January 10-17, 2010.

Oral Presentations

Migratory Interneurons Express Functional Glycine Receptors During Early Development of the Cerebral Cortex. Euron and Theme joint PhD meeting. University of Bonn. Bonn, Germany. September 22-23, 2011. This presentation was selected as the best by the principal investigators attending to the meeting.

Glycine Receptor Activation Promotes Cortical Interneuron Migration by Regulating Nucleokinesis. Inter-university Attraction Poles (IAP) 7 ELECXITE Annual meeting. Brussels, Belgium. April 26, 2013.

Glycine Receptor Activation Promotes Cortical Interneuron Migration. Belgian Society for Neuroscience. Brussels, Belgium. May 30, 2013.

Nederlandse samenvatting

GlyR zijn transmembranaire eiwitcomplexen gevormd door de symmetrische assemblage van vijf subeenheden rond een centrale porie. Er bestaan vijf types subeenheden, met name vier verschillende alfa-subeenheden en één beta-subeenheid. De GlyR kan gevormd worden hetzij als een alfa-homomeer, of als een heteromeer waarin de beta-subeenheid is opgenomen. Niet enkel glycine, maar ook andere liganden zoals taurine en alanine kunnen de GlyR activeren. Elk van deze moleculen kan binden aan het extracellulair domein van de receptor en vervolgens de opening van de centrale porie bevorderen, waardoor een selectieve flux ontstaat van chloor- en bicarbonaationen. Net zoals voor $GABA_AR$, is de GlyR-gemedieerde ionflux enkel afhankelijk van de elektrochemische gradient voor deze ionen die ontstaat over de celmembraan. GlyR komen algemeen voor in het ruggenmerg waar ze bijdragen aan synaptische transmissie en een belangrijke rol spelen in motoriek en pijnperceptie. Ze verschijnen al vroeg tijdens de ontwikkeling van het ruggenmerg en beïnvloeden het ontwikkelingsproces. Hoewel het voorkomen van functionele GlyR in de volwassen hersenen al twee decennia geleden werd beschreven, is de functie die ze uitoefenen in deze regio niet goed gedocumenteerd.

De ontwikkeling van de hersenen start vroeg tijdens de embryogenese en voltooiing van dit proces bij de mens kan jaren duren. De eerste stappen omvatten nauwkeurige coördinatie van celproliferatie en migratie. Algemeen worden hersencircuits gevormd door exciterende en inhiberende neuronen. Deze neuronen ontstaan uit verschillende voorlopercellen, gelokaliseerd in de ontwikkelende hersenen. Na differentiatie migreren neuronen uit de proliferatieve zone en dringen vervolgens de cortex binnen. Deze processen staan onder strikte controle van de intrinsieke genetische machinerie en de extracellulaire signalen aanwezig in de biochemische omgeving. Neurotransmitters en hun receptoren behoren tot deze biochemische omgeving en oefenen invloeden uit vanaf het begin van de neurogenese. In de muis komt de GlyR vroeg tijdens de embryogenese tot expressie, waarbij het mRNA van de alfa2-subeenheid het meest voorkomt. De informatie over de functionaliteit van deze receptoren is echter beperkt. Om deze reden hebben we in dit werk de rol van GlyR tijdens vroege stadia van de embryonale hersenontwikkeling onderzocht en daarbij gefocusseerd op celmigratie en -proliferatie.

De eerste hoofdstukken van dit proefschrift zijn gewijd aan de inleiding van het onderzoeksproject en het beschrijven van de materialen en methoden. De volgende hoofdstukken, drie en vier, beschrijven de experimentele resultaten. In het eerste deel van dit proefschrift beoogden we de rol van GlyR tijdens de ontwikkeling van interneuronen op te helderen. Deze studie toont aan dat functionele GlyR tot expressie worden gebracht door embryonale corticale interneuronen *in vivo*. Verder observeerden we dat genetische verstoring van deze receptoren tot defecten leidt in interneuron migratie. Daarnaast ontdekten we dat extrasynaptische activering van GlyR, die de alfa2-subeenheid bevatten,

door endogeen glycine, voltagegevoelige calcium kanalen activeert en calciuminflux bevordert in corticale interneuronen. Deze calciuminflux moduleert verder de actomyosine contractiliteit om zo tijdens migratie de nucleaire translocatie te fine-tunen. Alles tesamen belichten onze gegevens de moleculaire gebeurtenissen die corticale tangentiële migratie sturen tijdens embryogenese.

In het tweede deel van de thesis bestudeerden we GlyR in de context van de dorsale neurogenese en het genereren van projectie neuronen. Deze studie onthult de aanwezigheid van GlyR in de proliferatieve gebieden van de cortex, met name de ventriculaire en sub-ventriculaire zone, waar deze receptoren de snelheid van celproliferatie differentieel reguleren. Glycine-opgewekte stromen in radiale E13 progenitorcellen waren afhankelijk van GlyR activatie. Opmerkelijk was het effect van strychnine blokkade of van specifieke GlyR silencing: celproliferatie in de sub-ventriculaire zone nam toe, terwijl in de ventriculaire zone dit proces afnam.

Tot slot beschrijven de twee laatste hoofdstukken de conclusies en perspectieven voor toekomstig onderzoek.

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