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List of abbreviations

BRDU	bromodeoxyrudine
CB	calbindin
CGE	caudal ganglionic eminence
CHAT	choline acetyltransferase
CR	calretinin
GAD	glutamic acid decarboxylase
GE	ganglionic eminence
GLYR	glycine receptor
GP	globus pallidus
H	hippocampus
HPRT	hypoxanthine phosphoribosyltransferase
KCC1	potassium chloride co-transporter 1
LGE	lateral ganglionic eminence
LIZ	low intermediate zone
MGE	medial ganglionic eminence
MZ	marginal zone
NCX	neocortex
NPY	neuropeptide Y
Pax 6	Paired box 6
PBS	phosphate buffered saline
PCx	piriform cortex
PGK1	phosphoglycerate kinase 1
PHH3	Phosphohiston-H3
POA	preoptic area
PV	parvalbumin
SHH	sonic hedgehog signalling
SST	somatostatin
STR	striatum

SVZ sub ventricular zone
TBP box binding protein
VIP vasoactive intestinal peptide
VZ ventricular zone

Abstract

Glycine receptors (GlyR) are present in the developing brain before the start of neurogenesis and its functions are more than regulating neurotransmission alone. Several preliminary studies from our lab have shown a role for the Glycine Receptor alpha 2 subunit (GlyRa2) in cortical development. Experiments have shown that Glyra2 disruption causes a delay in interneuronal migration leading to a reduction in number of interneurons in the cortex at postnatal day zero (P0). It is hypothesized that GlyRa2 could influence the development of interneurons in the embryonic and early postnatal brain. Firstly, we assessed gross morphology of striatum and hippocampus in the Glycine Receptor alpha 2 subunit Knock-out model (*GlyRa2KO*) showing a significant decrease in early postnatal development, which confirmed previous data about our lab. These defects were associated with a reduction in expression of specific GABA_AR in the hippocampus at postnatal development. Furthermore, immunohistochemical stainings displayed impairments of both GABAergic and cholinergic interneuronal subpopulations in both striatum and hippocampus at early postnatal development. However, the interneuronal proliferation via the use of the mitotic marker Phosphohiston-H3 (PHH3) in the Median ganglionic eminence (MGE) did not show significant differences. Suggesting no influence of GlyRa2 in mitosis of interneuronal progenitor cells. Here, we demonstrate that the GlyRa2 subunit has an important role in development causing a quantitative defect of interneurons without affecting the mitotic phase of proliferation. These preliminary results might lead to further study on the cell cycle of proliferation and possible early differentiation in order to understand how the absence of GlyRa2 might cause these interneuronal defects

Samenvatting

Glycine receptoren (GlyR) zijn aanwezig in vroege stadia in de ontwikkeling van de hersenen. In het verleden werd hun aanwezigheid zelfs al opgemerkt voor de start van synaps vorming. Dit duidt erop dat hun functie niet enkel beperkt is tot neurotransmissie. Voorafgaande studies hebben aangetoond dat de Glycine Receptor alfa 2 sub eenheid (GlyRa2) aanwezig is in de ontwikkeling van de cortex. Bijkomend, bepaalde onderzoek data hebben aangetoond dat de uitschakeling van GlyRa2 een vertraging in interneuron migratie patronen vertoont met als gevolg een afname in het aantal interneuronen in de cortex bij de dag van geboorte (P0). Vanuit deze voorafgaande gegevens werd de hypothese gevormd dat GlyRa2 een belangrijke functie uitoefent op de ontwikkeling van interneuronen in embryonale en postnatale hersenontwikkeling. Voor het bekijken van de effecten bij de afwezigheid van GlyRa2 werden hippocampus en striatum eerste bekeken op vlak van hun oppervlakte. Gebruikte kleuringen vertoonde een afname in oppervlakte in de *GlyRa2*KO muis lijn. Deze bevindingen correleren met eerdere data waarin afname van corticale en striatale oppervlakte werd beschreven. In parallel: qPCR experimenten op twee specifieke tijdstippen in de hersenontwikkeling duidde op de verminderde expressie van bepaalde GABA_AR sub eenheden in de hippocampus. Daarop volgend werd een defect in zowel GABAergische als cholinerge interneuron subpopulaties aangetoond met behulp van immunohistochemie in zowel striatum als hippocampus (enkel GABAergische interneuron subpopulaties). Vertrekkende vanuit deze resultaten werd er gekeken naar de proliferatie van progenitors of voorloper cellen van de interneuronen in de MGE met behulp van PHH3, een merker voor mitotische lichamen. Analyse van deze proliferatie studie stelde geen significant verschil tussen beide genotypen vast. Hieruit zou blijken dat GlyRa2 geen invloed uitoefent op proliferatie van interneuronen in de MGE. Niettegenstaande deze resultaten, kan de rol van GlyRa2 in proliferatie en cel cyclus van interneuronen enkel uitgesloten worden met het uitvoeren van bijkomende BrdU experimenten op verschillende tijdstippen in de ontwikkeling. Deze verschillende experimenten op meerdere tijdstippen zou een volledig overzicht van de interneuron proliferatie in de MGE in beeld kunnen brengen. Bovendien kunnen er in de nabije toekomst andere verklaringen onderzocht worden waarop de GlyRa2 een bepalende invloed op uitoefent inzage de ontwikkeling van interneuronen in de hersenen met name het stadium van vroege differentiatie. Ondanks dat de ratio in mitotische figuren gelokaliseerd in de MGE geen onregelmatigheden vertonen kunnen we toch vaststellen dat GlyRa2 een belangrijke rol beoefent in de ontwikkeling van de interneuronen in de vroege hersen ontwikkeling.

1 Introduction

During the past years there has been a significant improvement in the understanding of the basic mechanisms and developmental stages in the mammalian brain formation. Multiple sets of studies have clarified the neurobiology of brain development including morphological, cellular and molecular organisation in the neuronal brain construction. These studies have carried out an image of brain development as a dynamic and adaptive event with a tight regulation between genetic and environmental factors (1, 2). The multitude of studies on brain development results in challenges and opportunities for researchers with different expertise to seek the fundamental principles and their impairments. This research could lead to better insights in specific developmental neuropsychiatric pathologies of the brain such as schizophrenia, autism, epilepsy, and other disorders that are a rising burden in the medical and psychological world (3). The notion in neuronal networks and either its modifications in dynamics, imbalance in excitatory and inhibitory signals, proliferation, migration, differentiation etc. are crucial for understanding a range of neurological pathologies (4, 5). Disruption of GABAergic interneurons during embryonic and early postnatal stages could be a possible cause of some of the previously mentioned neurological and behavioural impairments. Neurological disorders like epilepsy, autism, schizophrenia and other intellectual disabilities could all be related to a defect located at interneuronal development (6-8). And likewise to a deficit in inhibitory circuits which leads to an imbalance in excitatory and inhibitory signalling (9).

1.1 Interneurons

In general, the central nervous system includes two major types of neurons: inhibitory and excitatory interneurons neurons. Excitatory interneurons are mainly located in the spinal cord and only in layer IV of primary sensory areas in the mammalian brain (10, 11). These (mostly glutamatergic) interneurons function for the effective transmission of signals while inhibitory neurons control this transmission of signals in between neurons. Excitatory and inhibitory neurons are restricted to a tight regulation of an effective signalling transmission. It is the responsibility of a wide range of interneuron (mainly inhibitory) subtypes to control neuronal networks. Interneurons are confirmed to function as control mechanisms of the activity level in specific regions of the brain. Interneurons regulate the rhythmic transmission patterns between neurons, connectivity and regulate excitatory and inhibitory signalling to principal cells (12). These features result in simultaneous activation of the main cells, which will enlarge synaptic efficiency and promote enhancement of synergetic network performance(9, 13, 14).

1.1.1 Origin of the interneurons

The telencephalon in the embryonic brain development has been classified into the dorsal telencephalon (pallium) and the ventral telencephalon (subpallium). The dorsal telencephalon will develop respectively into structures as neocortex and hippocampus while the ventral telencephalon will develop into cerebral cortex and the basal ganglia (pallidus and striatum). Equivalently, the ventral telencephalon is subdivided into three progenitor domains, the lateral-, medial- and caudal-ganglionic eminences (respectively LGE, MGE and CGE). Although the LGE is the origin of striatal

projection neurons, the GE is the origin of the highly diverse interneuronal progenitor pool, observed by explants and labelling experiments. These crucial dye-labelling experiments showed that migration from the basal telencephalon expires via tangential migration of interneuron progenitor cells (15, 16). Additionally, the MGE could give shape to both striatal and hippocampal interneurons while the CGE predominantly give rise to hippocampal interneurons (17, 18).

1.1.2 Interneuron classification

The expression and distribution of different subtypes of interneuron depends on the network complexity of the specific brain region. The complexity of activity of a specific brain region is associated with the diversity level of present interneurons (19). To illustrate: Hippocampal interneurons are all GABAergic but striatal interneurons are divided into two major groups: the GABAergic as well as the cholinergic interneurons (20, 21).

The GABAergic interneuronal classification is intricate, not univocal and based on different features (Fig.1): expression of different neurochemical markers (e.g. Parvalbumin (PV), Somatostatin (SST), Calretinin (CR), Calbindin (Cb), Neuropeptide Y (NPY), reelin, vasoactive intestinal peptide (VIP) (etc.), morphological (axonal and dendritical targeting) physiological features (e.g. fast spiking, burst spiking, regular spiking etc.) and connectivity (22). However, with a combination of multiple experimental set-ups, it has become clear that distinct separate morphological interneuronal subtypes possess different firing patterns and express different neurochemical markers (23-25).

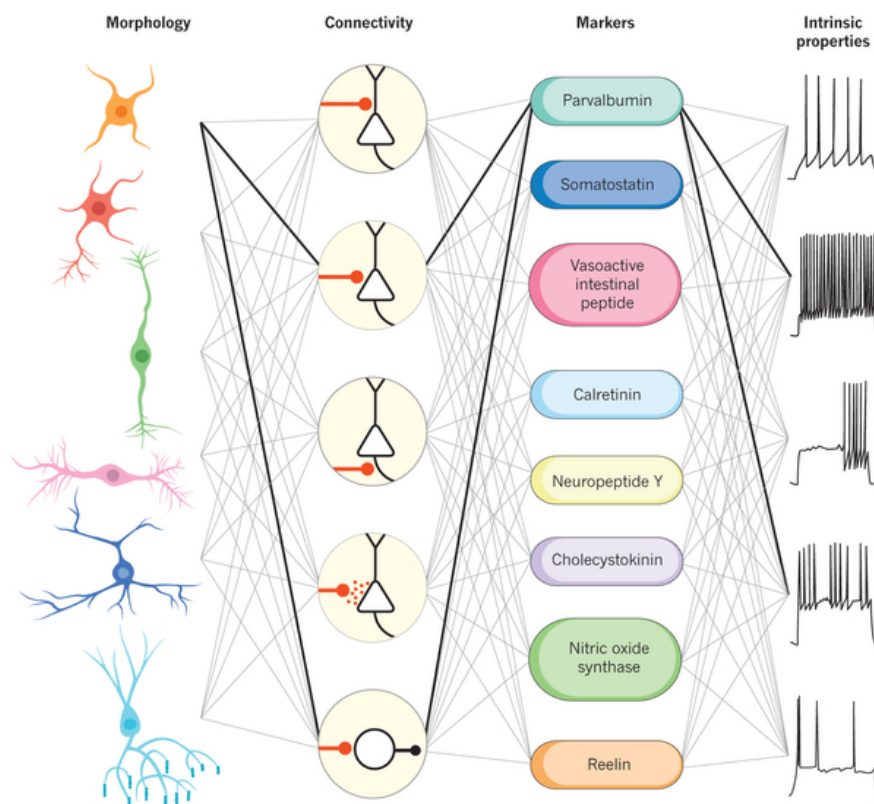


Figure 1. The classification of interneuron subtypes. Interneurons can be classified by their different features: morphological, connectivity, neurochemical markers and their physiological activity. The classification can have many overlap and is not one-sided for the different characteristics (26).

GABAergic interneurons are commonly classified by their diverse expression of their neurochemical markers according to their birth origin. Specifically, interneurons originated from the MGE are mostly concerned to be CR negative, Cb positive and are, to the utmost extent, PV and SST positive (15, 27). Supplementary, interneurons derived from the CGE are in most cases CR positive (15). Important to mention, the electrophysiological features of neurons are important for network activity. GABAergic interneurons can be divided in different subtypes of interneurons with different physiological characteristics. Subdivision can be based on their difference in capacitance, resting membrane potential, input resistance, the excitability of their membrane, different specific firing patterns (silent, fast-, burst- or non burst-spiking) which could be spontaneous or evoked (19, 28).

The large cholinergic neurons, which are located in the striatum, can be identified by the presence of Choline Acetyltransferase (ChAT) (23). Cholinergic interneurons count for an approximately 1-2% of the whole neuronal cell population in the striatum (20, 29). Their acetylcholine release is essential for the proper functioning of the striatum and the connection between striatum, cortex and thalamus (30). Cholinergic interneurons regulate inhibitory signalling in medium spiny neurons (MSNs), the GABAergic projection neurons located in the striatum and other cholinergic interneurons (31).

Interneuron generation occurs approximately around E9.5 in the GE (MGE, LGE, CGE) in a well-synchronized cell fate determination (27, 32). Experimental set-ups using cell transplants and electrophysiology brought out that interneurons from the MGE and CGE preserve their capacity to develop in different subgroups even with different environment and extracellular elements. Interneurons, originated from the LGE are mainly destined to become the embryonic source of interneurons located in the Olfactory Bulb (OB) which will express CR, NPY, reelin and VIP and SST expressing interneurons destined to reside in the striatum (32-34). These results suggest that interneuronal phenotypes are determined early in interneuronal development (35).

1.1.3 Interneuronal generation involves signalling molecules and transcription factors

Interneuronal development is influenced by environmental factors. The general factor Sonic hedgehog signalling (Shh) plays an important role in interneuronal generation. Shh has a strong influence on Nkx2.1 patterning: reduction of Shh involves less cells positive for Nkx2.1. Mutants for Nkx2.1 showed a total decrease in the amount of cholinergic, CR+ and SOM/NPY/NOS+ interneurons in the striatum (17).

An important influence in the development, generation, differentiation and migration of interneurons is reserved for transcriptional signalling (fig.2). Important transcriptional factors as Mash 1, Dlx 1/2 and Nkx2.1 and their interactions are a common appearance in controlling interneuron development (36). Complementary Dlx genes, expression located at GE and embryonic septum, can act individually in the differentiation and migration on interneurons and likewise in interneuronal survival (37). Nkx2.1 accomplishes a similar role; experiments with loss of this homeobox gene function results in disturbance of temporal specification in cortical interneuron subtypes (16). MGE derived interneurons can obtain different features from the LGE and don't migrate into the cortex, different in the case of CGE-derived interneurons (38). According to these results, a subset of interneurons is completely absent in the hippocampus (18). Likewise Lhx6 is

another important transcription factor in interneuronal migration and differentiation, present in MGE and CGE but not in the LGE (39). Like in rodents, interneuronal development in humans implement Mash 1, Nkx2.1, Dlx1, Dlx2 and Lhx6 as transcription factors (40).

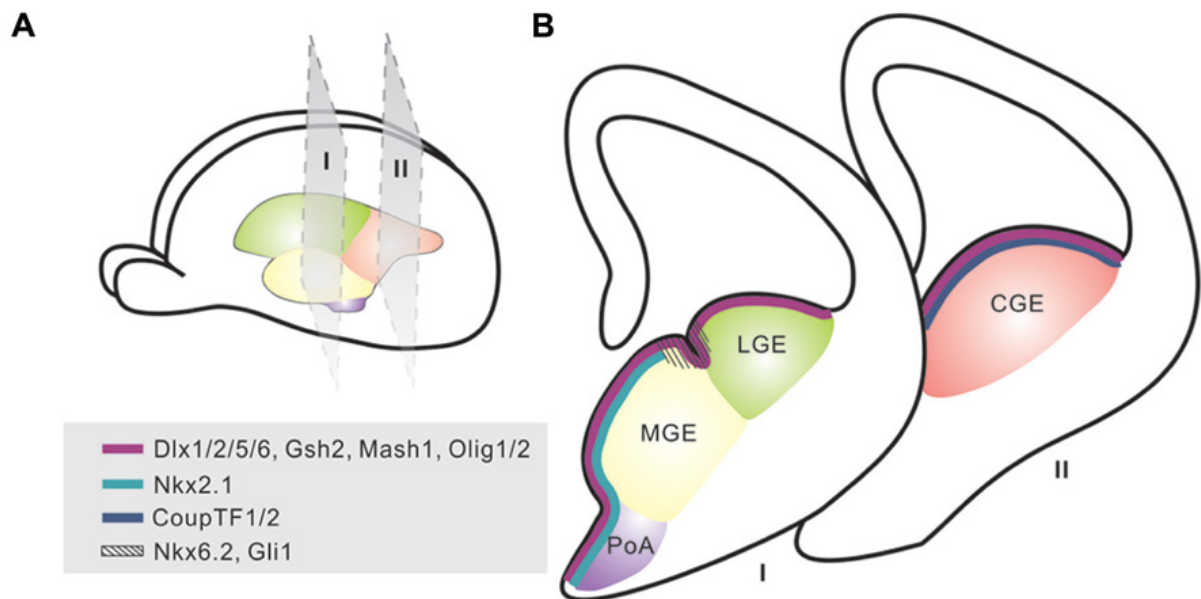


Figure 2. The expression pattern at the progenitor interneurons sites located at the ventral telencephalon. (A) Interneurons are originated from different proliferation sites located at the ventral telencephalon: the MGE, LGE, CGE and a minority in the PoA. (B) Different transcription factors are expressed in the subventricular zone throughout the whole eminence. Important factors as Dlx1/2, Mash 1 are expressed in the entire region of the GE. Nkx2.1 expression is mainly located at the MGE and the CGE. Abbreviations: PoA, Preoptic area (41).

1.1.4 Migration of interneurons

After proliferation in their proliferative zones, interneurons start to migrate to their place of destination. Tangential migration streams bring immature interneurons from their place of origin to their place of destination. This distinct migration has been based on multiple studies with the usage of different techniques like *in situ* hybridisation in slice cultures and *in vivo* transplant experiments (32, 38). Approximately, three phases of migration can be recognized which are closely related to place and time point of interneuronal production (fig.3). In the first migration stream, interneurons seem to be originated from the MGE and anterior entopeduncular area (AEP). This first migration stream appears to begin around E11.5 in the mouse. The directions are leading primary to the cortical marginal zone the sub plate and the striatum. In the secondary migration stream (at E12.5-14.5) it is the MGE who carries out the majority of the migrating cells towards the cortical plate and the developing striatum. Consequently, LGE derived interneurons in abundance to the olfactory bulb. The third and last stage of the tangential migration at E14.5-16.5 occurs from both MGE and LGE (42).

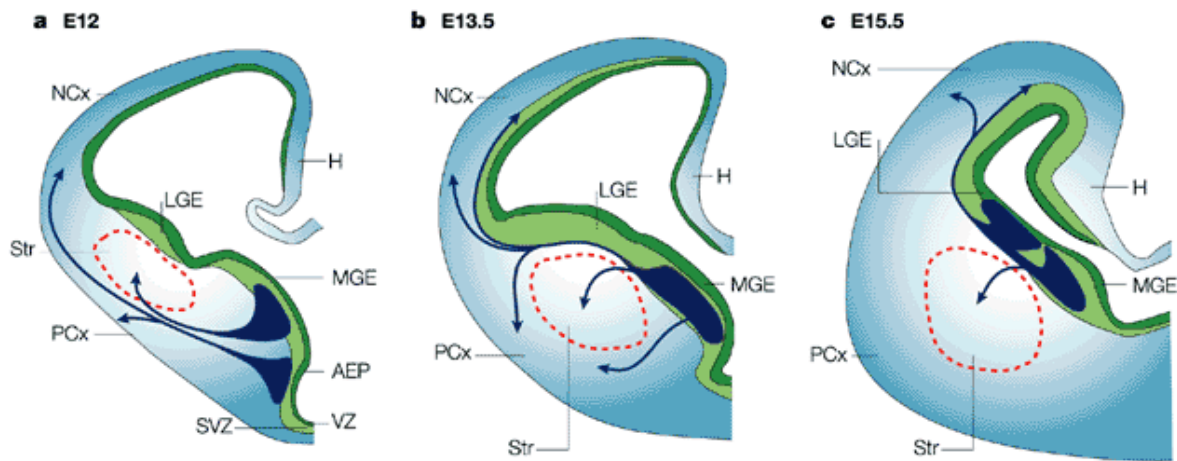


Figure 3. The tangential migration streams of immature interneurons in the developing telencephalon. Three different spatial and temporal regulated migration streams are identified in the embryonic telencephalon. (A) In early stages (E12), migrating interneurons originated primarily from the MGE and the AEP towards the Neocortex and the striatum. (B) At E13.5 a migration peak is observed. MGE-derived interneurons follow a profound rout towards the striatum and the cortex. (C) Likewise LGE-derived interneurons are migrating at E 15.5 towards the neocortex and olfactory bulb. Abbreviations; GP, globus pallidus; H, hippocampus; LIZ, lower intermediate zone; MZ, marginal zone; NCx, neocortex; PCx, piriform cortex; VZ, ventricular zone; Str, Striatum(42).

Interneuronal migration terminates with the help of intrinsic and extrinsic factors. Increase in chloride influx with a consequently cell depolarisation and modulation of the calcium influx by the activation of GABA_AR are specific elements of the intrinsic factors. Extrinsic influences are located at the extracellular environment and neurotransmitters have an important participating role (43).

1.2 The role of neurotransmitters in interneuronal development

Neurotransmitters are commonly known for their role in neurotransmission on the synaptic level but they are also present in the embryonic brain before the occurrence of synaptic genes occurs. The two most studied and well-known neurotransmitters in the act of neurologic development are GABA and glutamate. GABA is an inhibitory neurotransmitter in the adult brain but has other and different effects in the embryological and early postnatal brain. Located at the VZ, GABA stimulates proliferation of progenitor cells in contrast to the SVZ where it inhibits proliferation of these progenitor cell pools. GABA and glutamate stimulate the proliferative kinetics in the VZ but inhibit simultaneously the neurogenesis: stimulation of symmetrical division in the progenitor cells, which subsequently lead to the re-entry of the daughter cells into cell cycle. This accumulation of daughter cells causes a major increase in the progenitor population located at the VZ (44). Along brain development GABA functions as an excitatory neurotransmitter and evokes depolarisation of radial glial cells. This is caused by the Cl⁻ gradient established by the sodium-potassium-chloride cotransporter NKCC1(45, 46). Comparable with GABA, Glycine is an excitatory neurotransmitter at early stages in brain development, possible mechanisms could be similar in the context of development of the progenitor pools located in the VZ and SVZ and could give explanations in the function of Glycine in brain development. Additional, neurotransmitters like GABA, Glycine and

Glutamate play a strong contributing role in the development of interneurons. Neurotransmitter activity helps determining shape, migration and synaptogenesis of interneurons (47).

1.3 The Glycine receptor

The Glycine receptor (GlyR) is an ionotropic ligand-gated chloride channel present in the central nervous system, particularly in the spinal cord and the brain stem where the GlyR are involved in synaptic transmission, motor control and pain perception (13, 48). The receptor is a trans-membrane protein complex consisting of five types of subunits surrounding a central pore, there are possible five subunits: one to four alpha and one beta subunit (49). Its presence has been shown early during spinal cord development by affecting interneuron differentiation and synaptogenesis (50, 51) Although functioning of the GlyR was described two decades ago in the adult brain its function in other regions remains vague (48). The GlyR is considered to be involved in the extra-synaptic inhibition in the hippocampus (52-56). Furthermore its presence was identified in the brain cortex, the inhibitory and excitatory hippocampal neurons, thalamic and brain stem nuclei and the cerebellum. Likewise, activity disturbance of the GlyR can provoke different brain pathologies. For example: impairments in receptor functioning of $\alpha 1$ or β subunits can lead to hyperekplexia phenotypes in humans, mice and cattle's (57, 58). Furthermore, mutations in genes responsible for encoding GlyR alpha 2 have been found in autistic patients (57, 59, 60).

GlyRs is a trans-membrane protein complex formed by an assembling of five subunits, which are symmetrically arranged around a central pore (61). These five subunits include: four exceedingly homologous ligand-binding subunits ($\alpha 1 \rightarrow \alpha 4$) and one β subunit. The pharmacological and kinetic properties of the receptor change according to the subunit composition (62) like the chloride conductance and the affinity for other ligands like strychnine, taurine and alanine (63-65). The receptors, which contain the $\alpha 1$ and $\alpha 3$ subunits, are acknowledged for their synaptic role in the spinal cord and brain stem in the adult brain (66, 67). By contrast to the GlyR containing the $\alpha 2$ subunits, characterized by slower desensitisation and display slower kinetics, which are widely divided in the embryonic brain (48, 51). Important to mention that presence of the subunit alpha 4 had not yet been investigated at embryonic brain development (57). Conversely to the inhibitory functions of glycine in the CNS, the GlyR activation in the developing neocortex is excitatory which lead to an increase in intracellular concentration of calcium and results in a membrane depolarisation in neuronal progenitor cells (68). This discrepancy arises due to the inverted gradient of chloride in the embryonic and early postnatal stages (64, 68, 69).

1.3.1 Glycine receptors in embryonic and postnatal brain development

As previously mentioned, neurotransmitters and their receptors are crucial in the developmental process of the CNS. As they are part of the extracellular environment which gives external cues to developing neurons(47, 70). Next to GABA and glutamate receptors, the primary inhibitory and excitatory acting receptors in the adult brain (71) Glycine receptors have been detected before synaptogenesis in the embryonic brain (51). The first approach to study the expression of the GlyR expression in the CNS has carried out by the use of radioactive labelled strychnine. Nevertheless, this technique only indicated receptors with a high affinity for the ligand, which is mainly non-

existent in the embryonic brain (48, 72). Regarding the functional expression of GlyR in the developing brain, *GlyRa2*KO animals showed in specific studies no morphological defects in the cortex at P0 (58). Nevertheless, recent studies have observed the opposite: a decrease in cortical thickness and striatal area at birth (73). These incompatible findings have carried out questions about the presence of a moderate microcephaly in the brain, the expression of the GlyRa2 and other subunits of inhibitory receptors like Glycine and GABA_A. Possible explanations for the moderate microcephaly could state in the fact that the GlyRa2 is important in the proliferation of interneurons as well as projection neurons. Immunohistochemical labelling with the use of proliferation marker KI67 in embryonic brains carried out a decrease in cycling progenitor cells localised in the SVZ and the VZ in the *GlyRa2*KO in comparison with the WT (73). A possible suggestion for the moderate decrease could be in the presence of compensational mechanisms dependent of the other inhibitory receptor subunits of GlyR and GABA_AR that try to overcome the absence of GlyRa2.

During the first postnatal weeks GlyR alpha 2 subunits and beta mRNA expression are dramatically changed(48). In the second postnatal week, alpha 2 expression in the early post-natal brain diminishes while the alpha-1 beta heteromer expression increases and becomes detectable (58, 74). Synaptic heteromeric GlyRa2 has been detected in different areas in the adult CNS including spinal cord, brain stem, olfactory bulb and retina(75). In early development the homo-oligomeric GlyRa2 is found extra synaptic with a role in non-synaptic tonic transmission of signals from nearby nerve terminals in a non-vesicle glycine release mode(71).Consequently at P15, alpha 2 and beta expression reaches a stabile distribution in the cortex, which will remain the same throughout adulthood (48).

The overall focus in this project is the role of the glycine receptor during late embryonic and early postnatal development of the interneurons specifically at two important brain structures: the hippocampus and the striatum. Interneurons in the hippocampus are studied extensively in the context of their different features and their role in network oscillations and represent a key in the understanding of network activity in the different important regions of the hippocampus (76, 77). Striatal interneurons mainly originate from the MGE (a minority from the LGE) are subdivided into four categories namely the cholinergic interneurons (1), GABAergic interneurons containing PV (2), SST (3), CR (3) NPY and NOS (4) (20). Preliminary data shows that cholinergic interneurons originate from the MGE whereas SST positive interneurons generally derive from the LGE (34). Recent studies about migration of MGE derived neurons to the LGE and the expression of Nkx2.1 has given insights considering the development of striatal interneurons (78).

Experiments with the use of an animal model: a constitutive knock-out (full knock-out) for GlyRa2, which is a mice line with a deleted Exon 7, have resulted in the disruption of cortical progenitor homeostasis and cortical interneuron migration (51, 73). Using this as preliminary data, interneuronal subpopulations located at the hippocampus and striatum are under evaluation to observe the role of the GlyRa2 in other structures besides the cortex. Suggesting that GlyRa2 has an effect on the development of the different types of interneurons located at the hippocampus and the striatum should be an evidence of the important role of this receptor in brain development. The aim of the study is to observe the function of the GlyRa2 in the proliferation and migration of

the hippocampal and striatal interneurons. First measurement of the global areas of both hippocampus and striatum are performed to observe any morphological defect. Followed by a qPCR experiment for both structures to detect a possible compensational mechanism in the expression of inhibitory receptors (GABAA R α 1- α 5 and GlyRa1- α 4 and β) at P7 and adulthood. Together an immunolabeling of GAD65 was done in Dlx5/6GFP+ interneurons located in the hippocampus at P14. Additionally, a sequence of immunohistochemistry techniques has been carried out to observe a possible impairment in the number of various subtypes of interneurons (PV, CHAT, SST) with first a global view on the hippocampal interneuronal by the use of a Dlx5/6-GFP transgenic mice strain. To observe possible defects at the level of the global proliferation in the proliferation pool located at the MGE, mitotic marker PHH3 was achieved at the specific time point of E12; closely to the proliferation peak of interneurons located at the MGE. All these experiments are carried out to achieve a better understanding in the role of GlyRa2 in the early stages in brain development.

2 Materials and methods

2.1 Animals

Animal experiments carried out in this project were performed according to the guidelines of the local ethical committee of Hasselt University. MF1 and C57BL/6 strains were used. MF1 animals were used to maintain the transgene *Dlx5*, 6:Cre-IRES-GFP enhanced green fluorescent protein (GFP), controlled by *Dlx5*, 6 as enhancer element (*Dlx*-GFP) to target specific population of interneurons (79). Both mice strains contained deletion of exon 7 of *Gla2* as used in previous articles and experiments (51, 80). Mice were genotyped by the KAPA Hotstart Mouse Genotyping Kit (KAPABiosystems) to distinguish WT and *GlyRa2*KO and *Dlx*-GFP negative or positive animals.

2.2 Histology and immunolabelings

2.2.1 Tissue processing

Pups at postnatal day 7, 14 and 30 (P7, P14 and P30) were perfused transcardially with 4% PFA diluted in 1x phosphate buffered saline (PBS) (81). Brains were post fixed in 4% PFA for one day at 4°C and transferred for overnight in 30% sucrose solution (hypertonic medium) to remove all the water at 4°C. For cryosection: brains were placed in crymol vinyl molds (Tissue-Tek) with O.C.T. compound and placed on dry ice to freeze. Brains were cut in sections at 20 µm in the cryostat (CM-3050-S, Leica) and left to dry before they were stored at -20°C before the eventual immunostaining. For embryonic experiment, brains were dissected from the skull, rinsed in PBS and transferred to 4% PFA for 1 hour incubation followed by post fixation in 30% sucrose solution overnight at 4°C. In the case of coronal free floating sections: brains were embedded in 3% Low melting point Agarose (Fisher Scientific) and fixed to the vibratome holder (Microm, Termo Scientific) by usage of cyanoacrylate glue (Ted Pella). Sections were cut at a thickness of 50 µm. After receiving the coronal slices, sections were placed in a 24 well plate containing 1x PBS and kept on 4°C.

2.2.1 Nissl staining

Nissl stainings were performed on both P7 and P14 coronal sections. 50 µm sections were first placed on slides and air dried before placement into 1:1 alcohol/chloroform overnight to de-fat the tissue and limits background fat staining. Subsequently a rehydration through 100%, 95%, 80%, 75% to distilled water was carried out, each step taking 2 minutes. A staining step with 0,2% Cresyl Violet solution for 5 minutes was followed by a quick rinse in distilled water (± 30 sec.) with a differentiation step in 95% ethyl alcohol for 2 minutes and checked under a light microscope to obtain the best result. Differentiation step was adjusted in time according to the sections. Dehydration in 100% alcohol and a clearance with Xylene was performed for both 2 times 5 minutes. Finishing with mounting the slices with a permanent mounting medium.

2.2.2 Immunohistochemistry

For immunohistochemical staining, frozen sections (20µm) and free-floating (50µm) were used for the different immunohistochemical stainings.

Phosphohiston-H3 (PHH3) labelling was performed as thus: frozen sections were washed three times with TBS-Tween for rehydration of the tissue. DNA denaturation and antigen retrieval was carried out by the incubation in citrate buffer for an estimated time of 20 min. at 95°C. After antigen retrieval, slices were washed three times in TBS-Tween for 5 min each. To prevent non-specific binding, a blocking buffer containing 10% Normal Donkey Serum (NDS, Trecucula) and 0,2% Triton X-100 (Sigma) diluted in Tris buffered Saline (TBS) was applied. Slices were incubated over night at 4°C with 1: 200 diluted primary rabbit anti-PHH3 antibody (06-570, Millipore) in 50% of the blocking solution. Secondary antibody donkey anti-abbit (A555, life technologies) used in a concentration of 1:500 with an incubation time of 1 hr. at room temperature. Slides were washed in TBS-Tween followed by counterstaining with DAPI-containing mounting medium (Vector Laboratories) for counterstaining, slices were covered with a cover glass and observed under the microscope. The labelling of +GAD65 as well as GFP labelling was carried out without the antigen retrieval step with citrate. Other steps were performed equally with a blocking solution consisted of 10% NGS 0.2% Triton and incubation with primary antibodies GFP 1:100 (ab6556, Abcam) +GAD65 5:100 (Developmental studies hybridoma bank) diluted in 2.5% NGS and 0.2% Triton as well as secondary antibodies: 1:500 Goat anti mousse (A555, life technologies) and goat anti rabbit (A488, life technologies).

ChAT staining labelling was performed on free-floating sections (P14 and P30). Slices were washed 3 times 5 minutes with Phosphate-buffered Saline (PBS). To prevent non-specific binding, blocking solution was applied containing 10% NDS, 0,2 % Triton X-100 diluted in PBS at RT. The primary antibody ChAT was diluted at 1:1000 (Millipore AB144) in 5% NDS and 0,1 % Triton X-100 dissolved in PBS and kept overnight at 4° C covered on a shaker. After incubation, slices were washed for 3 times 5 minutes in PBS following a 1-hour incubation with the secondary antibody diluted at 1:200 in 5 % NDS plus 0,1 % Triton X-100 dissolved in PBS. Finally slices were washed 3 times 5 minutes, placed on glass slides and mounted using Vectashield hard set mounting medium with DAPI (Vector laboratories) and a cover slip. SST staining was performed equally in free floating sections but optimized with an extra rinsing with 0,1% Triton X-100 diluted in PBS 3 x 5 minutes to have a good permeabilization of the cell membranes. The blocking solution consisted of 5% NDS; 0,2% cold water fish skin gelatin (Sigma, G7765) and 0,25% Triton diluted in PBS. Sections were incubated with primary antibody anti-SST (Invitrogen) for 36 hours at 4°C diluted in blocking solution and slides were incubated with the appropriate secondary antibody, diluted in blocking solution, between 3-5 hours respectively.

2.3 Quantification and image acquisition

2.3.1 Fluorescence microscope

Images of Nissl and SST labelling were performed by using a Nikon NIS-Eclipse 80i microscope equipped by a Digital sight DS-2MBWc fluorescent camera. Pictures were obliged by the Nikon NIS-Elements BR 3.10 software. Camera settings (gain and exposure) were set according to the experiment and ROI. Brightfield, DAPI, FITC and TRITC channels were used to visualize the immunoabelings. Nissl stainings were obtained by using a Nikon plan fluor 4x (numerical aperture (NA) 0,1), 10x (NA 0,25) and 20x (NA 0,5) objectives. Images for SST+ cells in the hippocampus

and striatum cells were taken by the use of 4x (NA 0,1) and 40x (NA 0,75) objectives. Images of Dlx-GFP + interneurons located at the hippocampus were taken with a 10x objective for construction of a mosaic.

2.3.2 Confocal microscope

To visualize the immunolabelled brain sections of PV and ChAT, images were taken by a Carl Zeiss Axiovert 200M motorized microscope equipped with a LSM 510 META confocal laser scanner system (Carl Zeiss). For optimal quantification purposes, images and Z-stacks of 10 μm were obtained by using a Plan-Neofluar 40x/1.3 Oil DIC objective. The A555 fluorophore was visualized using a 543 nm spectral line of the He-Ne laser. DAPI excitation was obtained by the use of a two-photon excitation at 780 nm with the light emitted by a mode locked MaiTai laser (Spectra Physics). Excitation of GFP, Alexa555 and DAPI were obtained in the different immunolabellings. Image acquisitions were obtained by the use of the Zeiss laser-scanning microscope LSM510 software (version 4.2 SP1). For the hippocampus, the ROI, based on the brightness, were performed on the specific areas of the hippocampus namely the CA1, CA2, CA3 ad DG. Simultaneous for the striatum, pictures were taken for the ROI with the rate of 6-pictures/ hemisphere.

2.4 RNA isolation and quantitative PCR

For this experiment both adult mice and mice age P7 were sacrificed and dissected. Hippocampus as well as striatum (P7 only) were isolated and stored at -80°C . RNA isolation was performed by the use of Rneasy Mini Kit (Qiagen) and quantified with the use of a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific). Subsequently 2 μl of total RNA per 20 μL of was transferred into cDNA in a 20 μl reaction making sufficiently use of the high Capacity cDNA reverse Transcription Kit (Applied biosystems) according to manufacturer's instructions and stored at -20°C . Subsequently cDNA was diluted in 180 μl Nuclease free H_2O to dilute for a final concentration of 10 ng/ μl in a final volume of 200 μl before the use in downstream qPCR. Quantitative PCR (qPCR) were performed in duplicate for each cDNA sample for each in a 10 μl containing reaction mix which contained 5 μl Fast SYBR Green (applied Biosystems); Nuclease free water 1,9 μl ; 0,3 for each Forward and Reverse Primer (10 μM) and finally 7,5 μl of cDNA. The gene-specific primer pairs of Glycine receptor $\alpha 1$ - $\alpha 4$ (Idt) (table 1) were previously used in the cell physiology lab and Glycine receptor β (Idt) was used from published data (82). Primers for GABA $\alpha 1$ - $\alpha 5$ (Idt) were ordered based on existing publications (83). By the use of GeNorm, the appropriate reference genes were selected from 9 well-known housekeeping genes (Eurogentec company). Determination for the optimal number of control genes together with their minimal variety confirmed that TATA Box Binding Protein (tbp) and Hypoxanthine-guanine phosphoribosyltransferase (hppt) were the most suitable for P7, tbp and Phosphoglycerate Kinase 1 (PGK1) for adult (table 2). Relative quantification of gene expression was calculated using the comparative Ct method ($2^{-\Delta\Delta\text{Ct}}$) Data were normalized by the use of the selected Housekeeping genes following statistical analysis to compare WT and KO values with a two tailed corrected Mann-Whitney test.

Table 1: Primers for GlyR and GABAAR for qPCR for P7 hippocampus and striatum and adult hippocampus

Gene	Primer sequence	
	Forward (5'-3')	Reverse (3'-5')
Glycine α1	GGA AGA GGC GAC ATC ACA A	TGG ACA TCC TCT CTC CGG AC
Glycine α2	CAC TGG CAA GTT TAC CTG CAT	AAG CAG GCT CGG GAG ATG GTG TC
Glycine α3	GCA CTG GAG AAG TTT TAC CG	AAG CAG GCT CGG GAG ATG GTG TC
Glycine α4	CAG CAT CAG ATT GAC CCT CA	GCA GGA GCA TCT TCT AGC CA

Table 2: Housekeeping genes for the normalization of the qPCR experiment in P7 hippocampus and striatum samples and hippocampus in adult

Gene	Primer sequence (5'-3')	
	Forward	Reverse
Tbp	ATG GTG TGC ACA GGA GCC AAG	TCA TAG CTA CTG AAC TGC TG
HMBS	GAT GGG CAA CTG TAC CTG ACT G	CTG GGC TCC TCT TGG AAT G
HPRT	CTC ATG GAC TGA TTA TGG ACA GGA C	GCA GGT CAG CAA AGA ACT TAT AG CC
Pgk1	CTC ATG GAC TGA TTA TGG ACA GGA C	GCA GGT CAG CAA AGA ACT TAT AG CC

2.5 Image analysis

All analysis was performed by usage of Fiji (just ImageJ) (NIH) freeware. To simplify the analysis of the confocal data, from the Z-stacks who were spanning for 10 μ m, the picture with the brightest field was selected and quantification was based on this single frame. Counting of the cells was performed with the use of the cell-counter plug-in in ImageJ. For the Dlx-GFP assay MosaicJ plugin in Fiji ImageJ was used to construct a mosaic to analyse and quantify Dlx+ interneurons located at the hippocampus of pictures with a 10x objective.

2.6 Statistical analysis

Statistical analysis was carried out by the use of Graphpad Prism 5.0 software (Graphpad software Inc.). WT and *GlyRa2*KO mice were always compared between each other. Differences were calculated with a t-test or the non-parametric Mann-Whitney test. By exception, the use of an unpaired t test with Welch's correction provided the statistical analysis for the Dlx-GFP positive interneurons in the hippocampus at P14 with the 2-way ANOVA test to study the distribution and how these means could differ a post hoc test was used to test if groups presented significant differences. All values are presented as mean \pm standard error mean. Statistical tests with a p-

value $< 0,05$ were considered as significant. By using asterisks, the level of significance was being displayed: * $p < 0,05$; ** $p < 0,01$ and *** $p < 0,001$.

3 Results

3.1 The effect on morphology in the GlyRa2 knock-out model

Previous studies have been carried out and observed contradictory effects in the context of the GlyRa2 role in brain morphology. Studies observed no change in morphology in cortex (P0), cerebellum (P0) and retina (adult) (58). But recently experiments have shown the appearance of a moderate microcephaly in the *GlyRa2*KO model, specifically a decrease in cortical thickness and striatal area (73). To obtain an answer about this observations, experiments by using DAPI-staining (striatum) and Nissl staining (hippocampus) are carried out.

3.1.1 Genetic inactivation of GlyRa2 leads to a reduction in Striatal area

Analysis of the striatal area in both WT and *GlyRa2*KO at the age of P14 and P30 revealed a significant reduction in the total area of this brain structure (fig. 4).

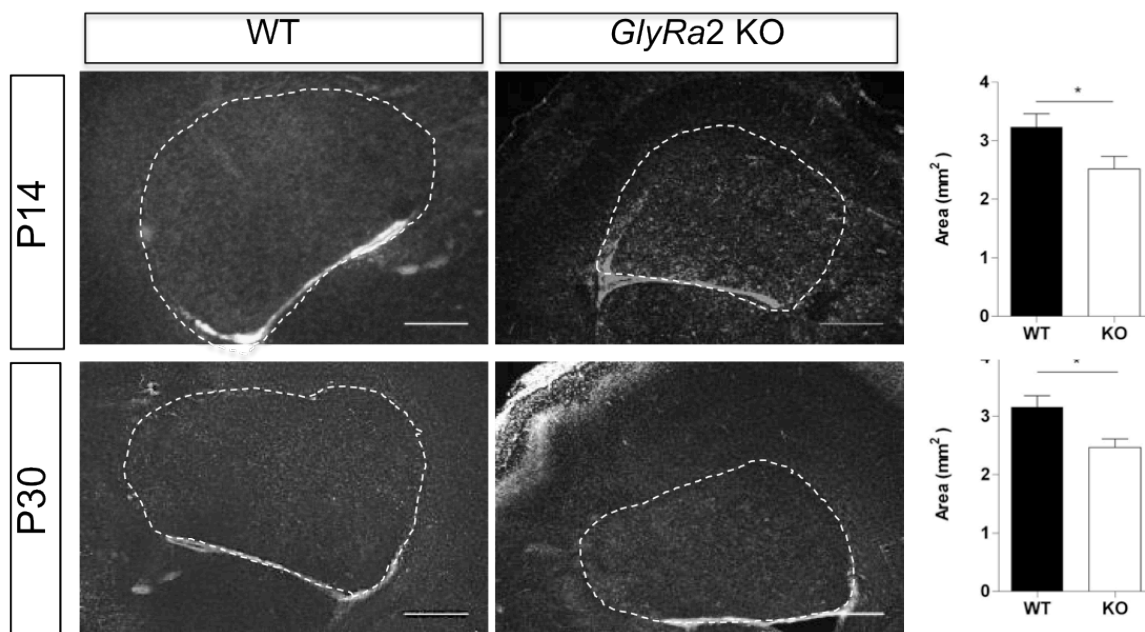


Figure 4: The effect of the GlyRa2 full knock out on striatal area: early *Glyra2*KO postnatal mice show a reduced size of the striatum at P14 and P30. Coronal brain slices were stained with DAPI to recognize the striatal area (solid white line). The presented values are expressed as mean \pm SEM, for P14 n= 7 and P30 n= 11, for each genotype 4 brains were used; * $p < 0,05$, Mann Whitney test.

3.1.2 The effect of genetic silencing of GlyRa2 in Hippocampal formation

To look further at other specific regions, which also can be affected by the genetic disruption of the GlyRa2, Nissl stainings were performed to study the morphological features of both WT and KO. For all the coronal slices in the context for hippocampal information, sections were selected according to the same location of the hippocampal brain structure using specific Atlas based on the age. Nissl staining is a well-known technique to investigate the morphology of specific structures commonly

used to see morphological impairments. Free-floating coronal slices (50 μ m) of ages P7 and P30 were stained and imaged. Analysis of the total area showed a significant reduction in hippocampal size in both ages (P7 and P14) (fig.5A). To obtain further information about the effect of the *GlyRa2KO*, the specific regions of the hippocampus are studied (fig 5B). Analysis of the thickness of the CA1, CA2, CA3 and DG (DGEC= ectal limb; DGen= endal limb) was measured at a single image with specific location and measured in the right angle, specific for each slice and region of interest. Slides were taken all equal for the same region of the hippocampus with the typical hippocampal shape and the barrel cortex as a reference. Quantification of the different measurement showed a significant decrease in thickness of the *GlyRa2KO* animals at both ages in every specific sub region of the hippocampus.

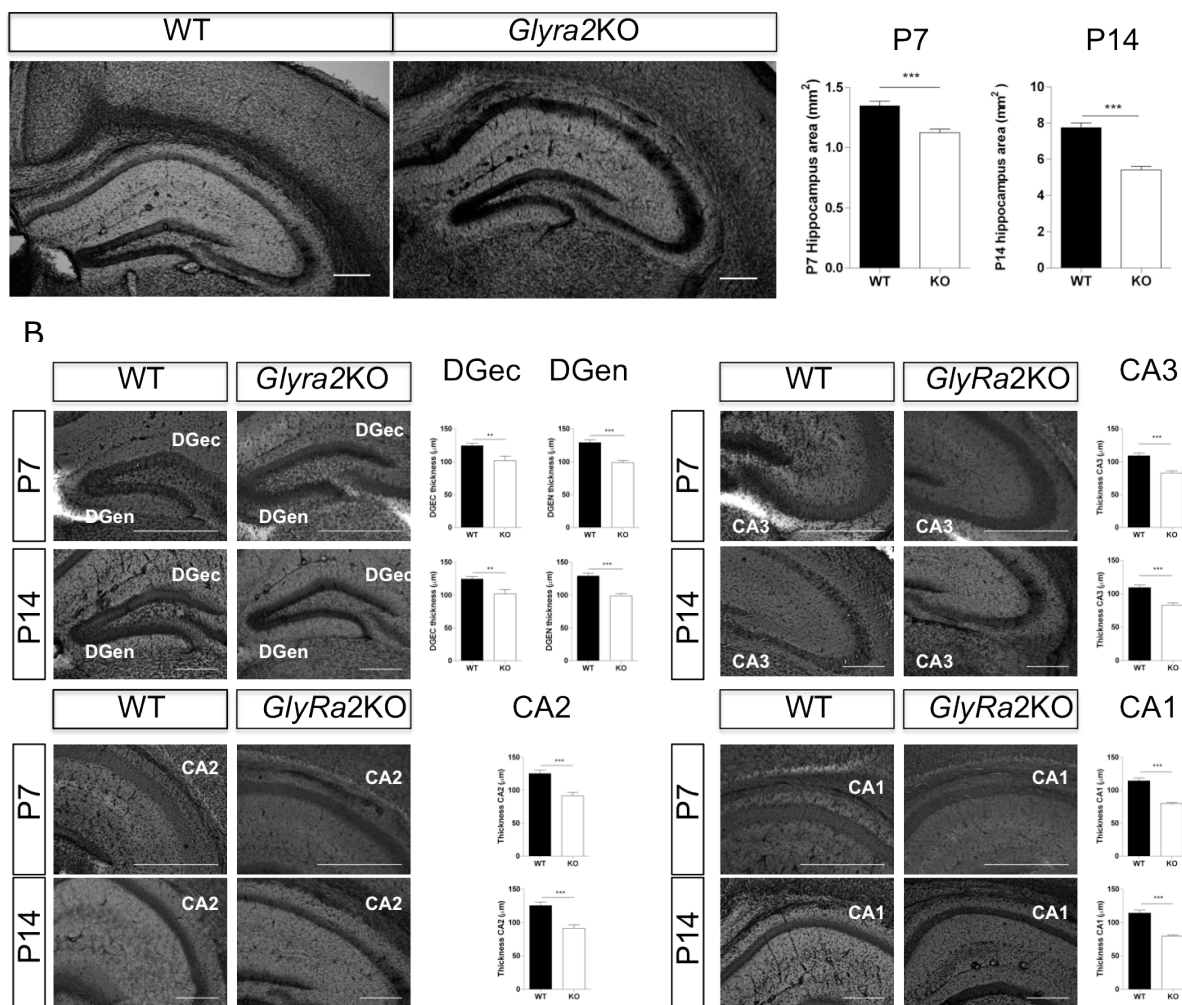


Figure 5: The effect of *GlyRa2KO* on hippocampal formation: early *GlyRa2KO* postnatal mice (P7 and P14) show a reduced size of the hippocampal area. Free floating coronal slices are stained with cresyl violet to obtain good representation of the hippocampal morphology. **A**, The global area of the hippocampus is measured at P7 and P14. **B**, The specific regions of the hippocampal formation are considered in both ages based on a single image of the ROI. The presented values are expressed as mean \pm SEM, for P7 n= 3 and P14 N=6. Per genotype. For each brain 4-6 brain sections were used; * p<0,05; ** p<0,01; ***p<0.005, Mann Whitney test. Scale bares: 500 μ m (**A**) and 50 μ m (**B**). Abbreviations; DGEC, ectal limb, DGen, endal limb.

Together these results show that there are morphological changes in the *GlyRa2*KO mice. Striatal and hippocampal areas are decreased at different time points in early ages. The role of GlyRa2 in the development of these structures should be very clarified to distinguish if it is a proliferation defect or a migration/patterning effect or both.

3.2 Cholinergic interneuronal population in the striatum is affected in the *GlyRa2*KO mouse line.

Preliminary data in the *GlyRa2*KO model has carried out a reduction in cortical projection neurons and a decrease in interneuronal migration patterns towards the cortex. Accordingly, a reduction in striatal and cortical size was presented in the *GlyRa2*KO mice (73). It could be suggested that the GlyRa2 has a similar role in both brain structures. Parallel effects on the populations of interneurons would be a possible consequence.

For the striatum, two major groups of interneurons are present: the cholinergic and the GABAergic interneurons. Cholinergic interneurons are triangle shaped and are the greatest in size compared to all the other interneurons in the striatal area(84) . The ChAT-expression of these interneurons is observed in free-floating coronal slices of WT and mutant animals. To obtain a reliable result 6 pictures for each hemisphere = ROI, which resembles the region of interest. Although there is a reduction in size, there is an accumulation of cholinergic interneurons in the striatum at the both specific ages (P14 and P30) in the GlyRa2 mutant mice (fig.6). Statistical analysis showed a significant increase of cholinergic interneurons in the striatal area in the animal knockout model compared with the WT. Together these results demonstrate that the reduction in striatal area is not in correlation with the amount of cholinergic interneurons that are residing in the striatum. Interestingly, the genetic knock out of the GlyRa2 leads to an increase in cholinergic interneurons.

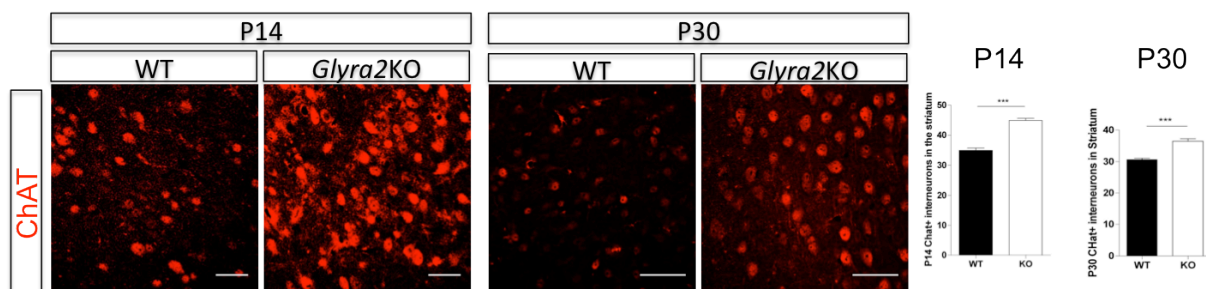


Figure 6: The concentration of cholinergic interneurons in the striatum: immunohistochemical pictures of ChAT expression of WT and *GlyRa2* KO mice. Representative confocal images (brightest field of z-series projections) are obtained by free-floating coronal slices (50µm) for ChAT (red) in the striatum in both ages of P14 and P30. The total amount of cholinergic interneurons appears to be significantly higher in the GlyRa2 mutant mice. 6 images for each hemisphere are taken to resemble the ROI. ***p< 0,005, t-test; n= 4 animals for each condition. Scale bare: 50 µm.

3.3 The effect of the GlyRa2 genetic inhibition on GABAergic interneuron populations in the striatum

Besides the group of cholinergic interneurons, GABAergic interneurons are the second major group of interneurons that are localised in the striatum. As like the cholinergic interneurons, GABAergic interneurons have an important role in the signal transmission in the striatum and between striatum and other parts of the brain (20, 30). Two important subtypes of GABAergic interneurons: PV and SST are selected to illustrate the possible effects when the GlyRa2 is genetically disrupted.

To determine the number of PV+ and SST+ expressing interneurons, immunolabelings in free floating sections (SST) and cryosections (PV) were carried out to visualize a possible difference in this subtype of interneurons located in the striatum. To observe a possible effect of the GlyRa2 WT and *GlyRa2*KO sections were stained and analysed by the use of a fluorescent microscope for SST and PV (fig 7). Statistical analyses determined a significant difference between the WT and the GlyRa2 mutant: *GlyRa2*KO mice had a significant decrease in the number of both PV+ and SST+ neurons in the striatum. This result suggests that the presence of PV+ and SST+ interneuronal populations are affected by the use of the *GlyRa2*KO mice model. GlyRa2 could have an effect on the proliferation of this interneuronal sub types considering the significant decrease in number when the GlyRa2 is absent.

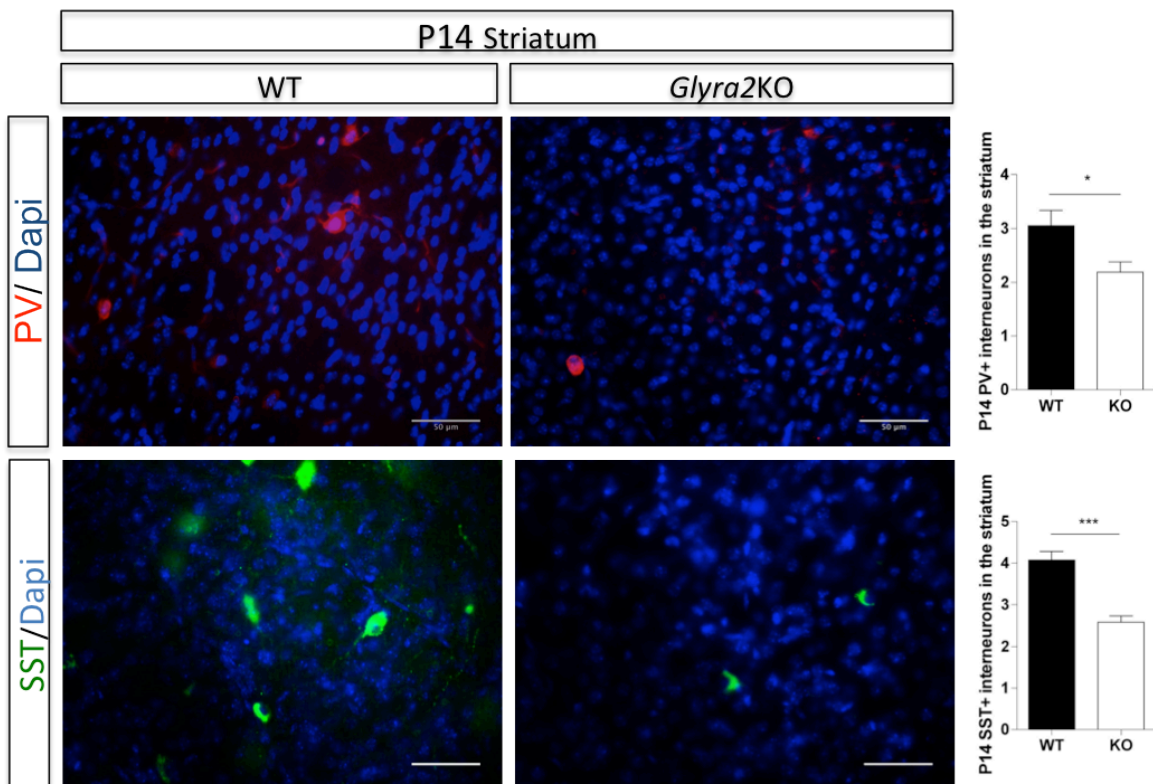


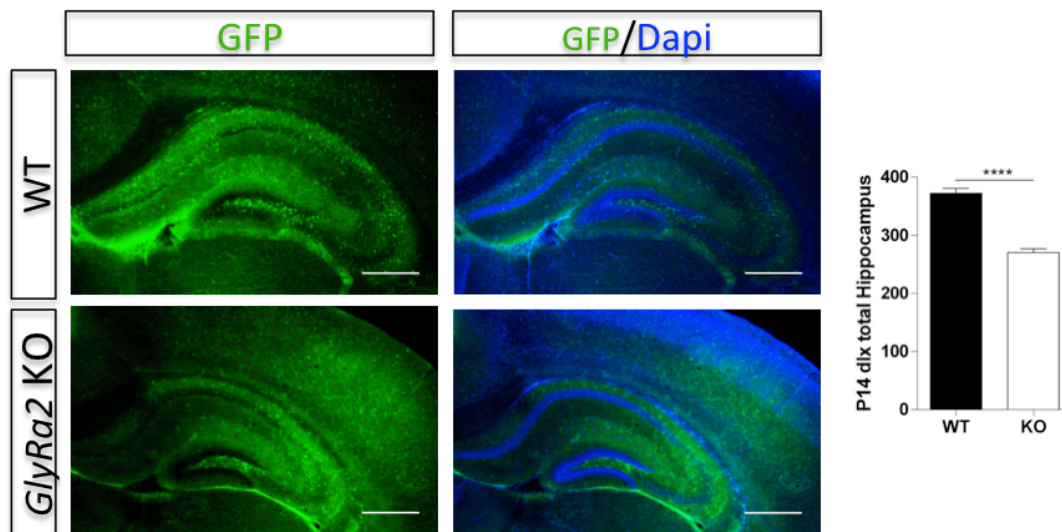
Figure 7: The population of PV+ and SST+ interneurons located in the striatum: Fluorescent images of immunohistochemical labelling of PV + and SST+ interneurons in the striatum at P14 Frozen cryosection (20 µm) presenting immunolabeling against PV (red, upper panel) and Dapi (blue) together with free floating coronal sections (50µm) which illustrate SST+ interneurons (green, lower panel) in coronal slices of WT and the GlyRa2 mutant mice. The numbers of PV+ and SST+ interneurons counted in the ROI were both significantly

decreased in the GlyRa2 mutant mice. 6 images for each hemisphere are taken to resemble the ROI. * $p < 0,05$ *** $p < 0,001$, t-test; $n = 3$ animals for each condition. Scale bare: 50 μm .

3.4 The number of dlx 5/6 positive neurons in the Hippocampus in *GlyRa2*KO

To search the possible explanation for the reduction in hippocampal area, first the Dlx 5,6: Cre-IRES-GFP (Dlx-GFP) mice line was used to see a difference in the Dlx 5/6 expression. The Dlx5/6 transcription factors are expressed in the LGE and the MGE sub ventricular zones and visualize the GABAergic interneurons, which are destined to migrate to the cortex and hippocampus. Hippocampal interneurons are mainly generated in the GE and migrate tangentially towards the developing neocortex. The majority of these GABAergic interneurons express PV or SST (85, 86). Free-floating sections (50 μm) of Dlx-GFP mouse brains were stained to enhance the GFP signal (fig 8A). Histochemical analyses performed on P14 old mice-sections showed an overall reduction in Dlx-GFP expressing interneurons in the Dlx-GFP/*GLra2* knockout mouse line. Statistical analysis confirmed a significant reduction in the GlyRa2 mutant mice compared to the *GlyRa2*WT mice but the distribution of the interneurons localised in the different regions remained unchanged (fig. 8B). All together, these results propose a possible defect in number of GABAergic interneurons located in the hippocampus when the GlyRa2 receptor is genetically silenced.

A



B

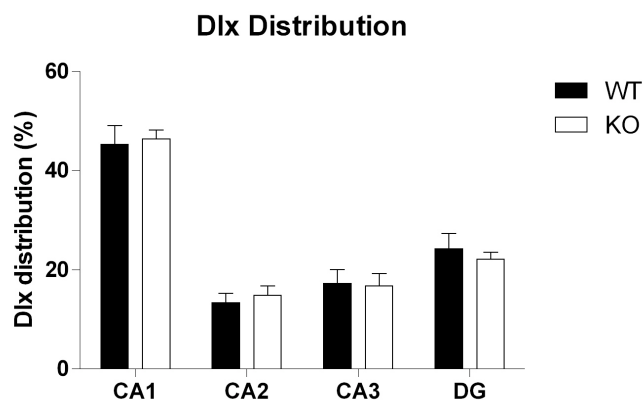


Figure 8: Dlx5,6 expressing interneurons in the hippocampus: A, Representative fluorescent images of Dlx-eGFP at the age of P14 are presented. Free floating sections of both GlyRa2 Wt and KO samples are compared labelled with Dlx-GFP (green) and Dapi (blue). The amount of DLX-GFP expressing interneurons were counted and resulted in a significant decrease in the *GlyRa2* KO. **** $p < 0,0001$, t test with Welch's correction, $n = 4$ animals for each condition. Scale bare: 500 μm . **B,** The distribution of the Dlx-GFP interneurons along the specific regions of the hippocampus. No significant difference was observed between the WT and the KO group, 2way ANOVA, $n = 9$ brain slices for each animal with 4 animals per genotype. Abbreviations; DG, dentate gyrus.

3.5 GABAergic interneuron subpopulations in the hippocampus

Previous experiment showed a reduction of hippocampal interneurons with a Dlx-GFP expression. Interneurons with a Dlx-GFP expression, localized in the hippocampus, are GABAergic and consist with the majority of PV+ and SST+ expressing interneurons. We used immunolabelings for both neurochemical factors to observe if both groups are affected by the absence of the GlyRa2 in the knockout mice line.

3.5.1 The population of SST+ interneurons in the hippocampus

We performed a histochemical analysis for SST+ interneurons located in the hippocampus for the investigation if the decrease of Dlx-GFP positive interneurons is caused by a possible decrease in the number of SST+ neurons. Free floating coronal sections of both GlyRa2 and KO mice were labelled and fluorescent pictures were taken of the whole area as well as the specific regions of interest which resemble the 4 specific area's (CA1, CA2, CA3 and DG) of the hippocampus. Statistical analysis of the number of counted SST+ cells has concluded that the two groups, WT and KO, both have an equal concentration of SST+ interneurons in the whole area of the hippocampus and that the distribution of the SST+ interneurons was not affected in the genetic silencing of the GlyRa2. These results conclude that GlyRa2 absence does not affect the SST+ interneurons in the hippocampus, not by number and not by the distribution in this brain region.

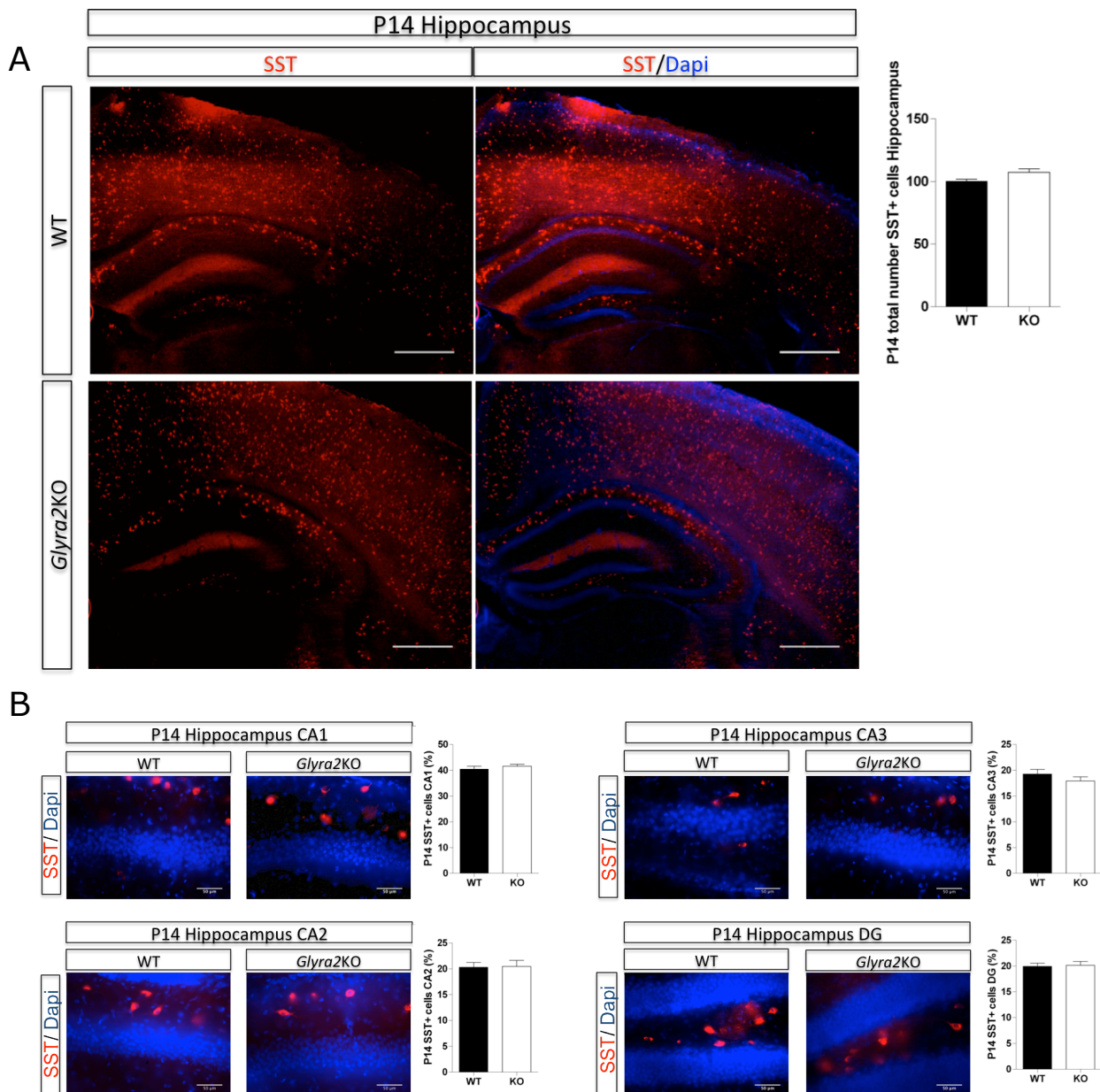
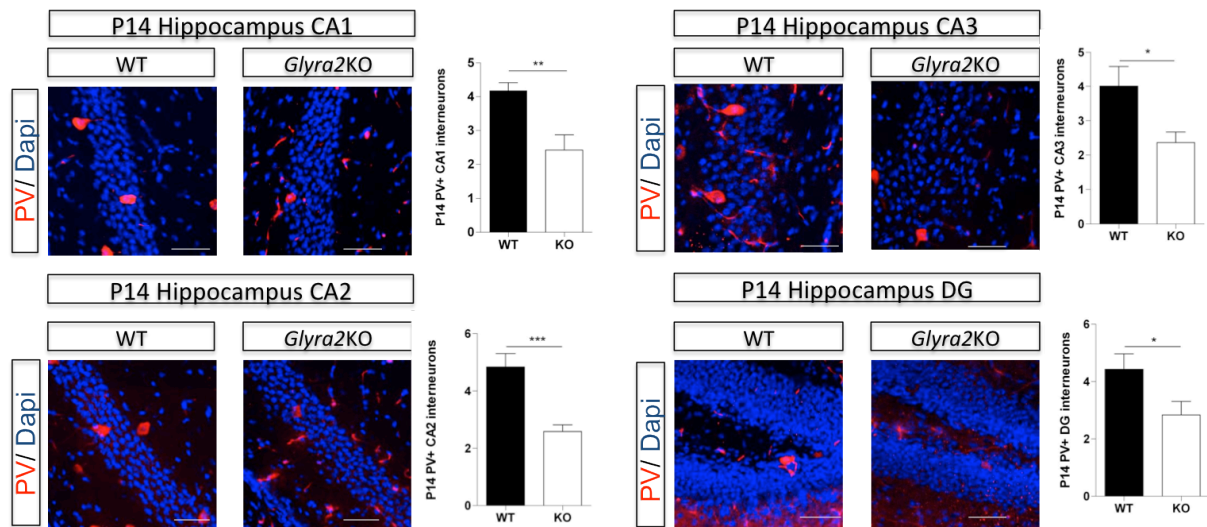


Figure 9: The population of SST+ interneurons located in the Hippocampus: Fluorescent images of immunohistochemical labelling of SST+ interneurons in the striatum at P14. Early *Glyra2KO* postnatal mice (P14) show no change in SST+ interneurons in the hippocampus. Free-floating coronal slices were labelled with SST+ (red) and Dapi (blue). **A**) The total number of SST+ in the hippocampus at P14. **B**) The number of SST+ interneurons located in the specific regions of the hippocampus represented as the distribution (%) compared to the total number of hippocampal SST+ interneurons. One single image, taken on the same location, was considered as the ROI. The presented values are expressed as mean \pm SEM, $n=3$. For each brain 3 brain sections were used, Mann Whitney test. Scale bars: 500 μ m (**A**) and 50 μ m (**B**). Abbreviation; DG, dentate gyrus.

3.5.2 The number of PV+ interneurons located at the hippocampus

The other major group of GABAergic interneurons, that express the *Dlx-GFP* enhancer, are the Ca+ buffer expressing PV positive interneurons. These interneurons count for an approximately 20% of the total GABAergic cell concentration and are well categorized by their fast spiking performance upon cell depolarisation (87). Frozen cryosection (10 μ m) with immunohistochemical labelling

against PV were imaged using confocal microscope to obtain a clear view of this subtype in GABAergic interneurons on the diverse areas in the hippocampus. It appears that WT has a more number of cells in the different hippocampal areas (CA1, CA2, CA3 and DG) (fig. 10). Statistical analyses confirmed a significant decrease in number of interneurons expressing PV in all the different regions of the hippocampus. Regarding this result and attachment of previous results of the striatum (fig 7), suggestions about the existence role of GlyRa2 in the development of PV+ interneurons seems to be a more relative concept.



3.6 Proliferation assay for observation of the interneuronal proliferation pool

We performed an immunolabeling at E12 to observe if the GlyRa2 plays an important role in the proliferation of interneurons. At E9 proliferation of the majority of hippocampal and striatal interneurons starts in the MGE with a peak at E13,5 (88). To study proliferation of the interneuronal progenitor cells in the MGE, the mitotic marker Phosphohiston-H3 (PHH3) was used at the age of E12 (Fig.11). PHH3 detects the core event during interphase: the phosphorylation of histon H3, which also exclude apoptotic cells (89). Altogether, PHH3 is a reliable and effective mitotic marker. Statistical analysis demonstrated no significant change in the two genotypes. Preliminary data of our lab with PHH3 staining done at E15 (data not shown) confirmed these results. All together these findings demonstrate that GlyRa2 has possible no essential role in the mitosis of the interneurons located at the MGE. But to be correct, different labelling BrdU-experiments within different time points (after 1 day, 12h, 1h) could give a global view on the proliferation events located in the MGE.

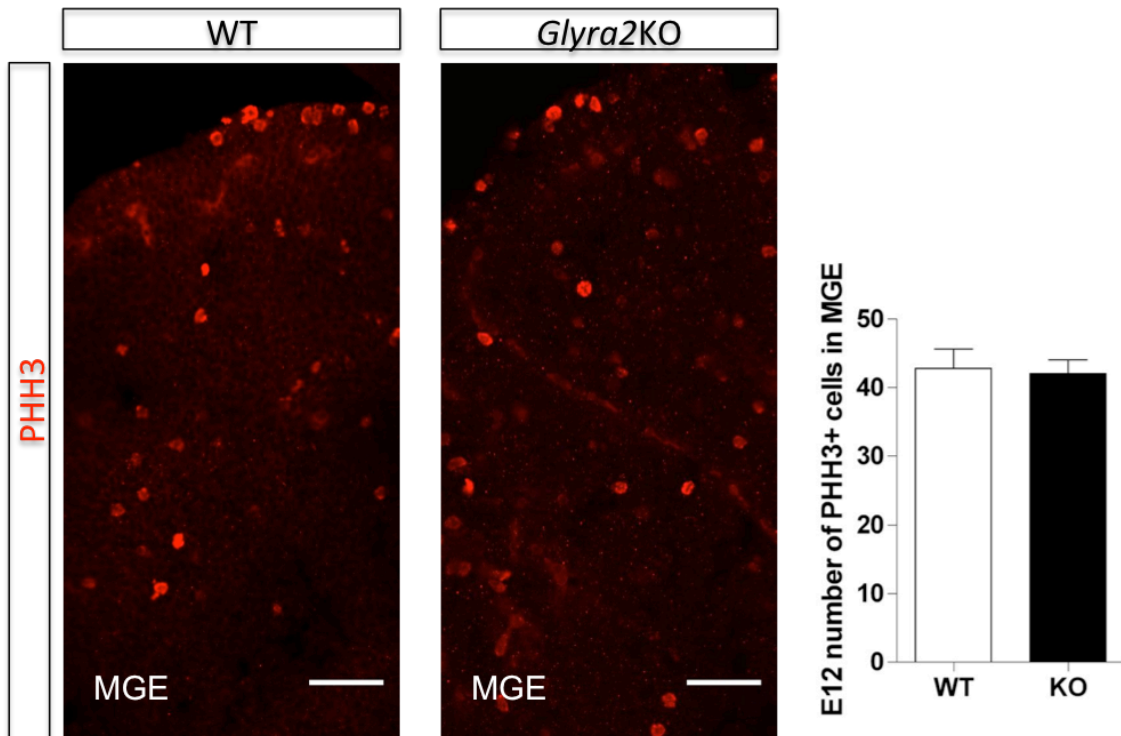


Figure 11: Proliferation at E12 in the MGE: Confocal images of immunohistochemical labelling of proliferating interneuronal progenitors located at the MGE. Histological images of frozen E12 cryosections (20 μ m) of WT and *GlyRa2KO* mice pictures are presented for PHH3 (red) and Dapi (blue). The numbers of PHH3+ cells were not altered between genotypes. Two pictures for each hemisphere were taken to get an appropriate ROI. Values are expressed with the mean \pm SEM, t-test; n= 3 animals for each condition. Scale bare: 50 μ m. Abbreviation; MGE, Medial ganglionic eminence.

3.7 The change of mRNA expression pattern in the hippocampus and striatum of inhibitory receptors in the presence of *GlyRa2KO*

A qPCR study was carried out to obtain information about the mRNA expression of different inhibitory receptors. The 5 Glycine alpha (α 1-5) subunits and 5 GABA subunits (α 1-4 and β subunits) were compared at two specific developmental time points: post-natal day 7 and adult. Comparison of the mRNA levels of the inhibitory receptors was compared between the two groups (WT and KO) for P7 and adult in hippocampal tissue and P7 for striatal tissue. For the normalization, housekeeping genes (table 2) were selected for the proper analysis of the mRNA samples.

Interestingly isolation of hippocampal RNA samples from WT and *GlyRa2KO* showed that only mRNA expression of GABAA a4 was significantly reduced in the KO samples of hippocampus at P7 (fig. 11). In addition, GABAA a1 mRNA expression was significantly different in adult samples (fig.12). Other mRNA levels did not significantly differ from each other between the groups. For the striatal tissue, no significant difference in the expression of mRNA between the two littermates was observed. Taken together: *GlyRa2KO* leads to a decrease in hippocampal GABAA a4 mRNA expression at the early postnatal age of P7 and affects only hippocampal GABAA a1 expression level in adult mice.

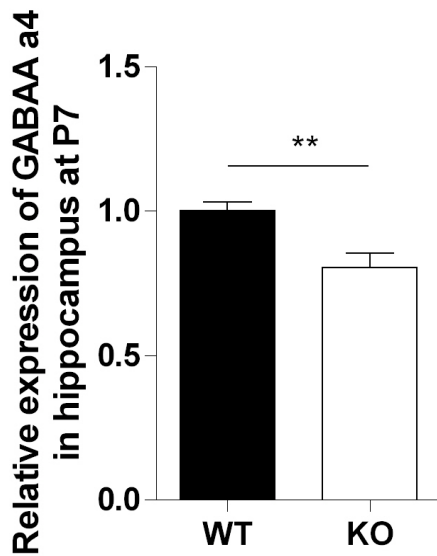


Fig 12: The expression of GABAA α4 subunit mRNA from P7 mice in the hippocampus: Expression of the 5 GlyR subunits (α1-5) and 5 GABAA-receptor (α1-4, β) channel subunits were examined using RT-qPCR in hippocampal tissue. Only the relative expression of GABAA α 4 mRNA differed significantly between WT and *GlyRa2*KO. The mRNA level of each of the GlyR and GABA A subunits used were normalized to the reference genes and presented as mean with ± SEM. The reference genes used were hypoxanthine phosphoribosyltransferase (*Hprt*) and Box Binding Protein (*tbp*) for P7. **p<0,01, Mann Whitney test; n=6 for each group.

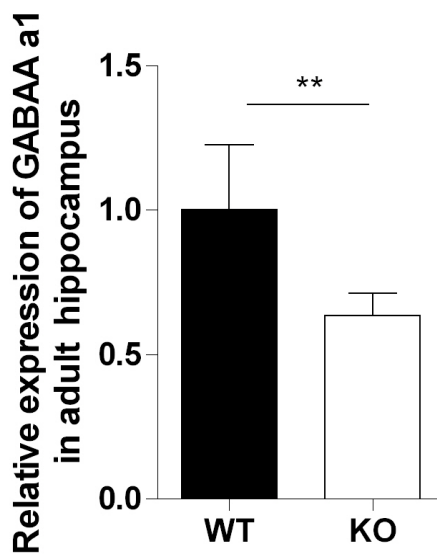


Fig 13: The expression level of GABAA α1 mRNA from hippocampal tissue in adult mice: Expression of the 5 GlyR subunits (α1-5) and 5 GABAA-receptor (α1-4, β) channel subunits were examined using RT-qPCR in hippocampal tissue. Only mRNA expression of GABAA α 1 was significantly different between WT and *GlyRa2* KO animals. All samples (GlyR and GABAAR subunits) were normalized using housekeeping genes and presented as mean with SEM. As reference genes Box Binding Protein (*tbp*) and Phosphoglycerate Kinase 1 (*PGK1*) were used. Values are presented as mean with ± SEM. **p<0,01, Mann Whitney test; n=6 for each group.

3.8 the expression of GABAA at the level of hippocampal interneurons when Glyra2 is knocked out

qPCR results carried out the finding that specific GABA_AR subunits are affected at different time point in the hippocampus. GABA_AR expression was observed in cryosections of P14 old mice to discover if there is a visible effect at the level of the interneurons. To detect expression of GABA_AR in hippocampal Dlx-GFP interneurons, double immunohistochemical staining was performed at cryosection (20 μm) on P14 old slices with glutamic acid decarboxylase 65 (GAD 65), the GABA synthesizing enzyme. GAD65 in the GFP positive interneurons are clearly stained over the whole soma while the expression of GAD65 is concentrated in the perisomatic structures (the perisomatic boutons). These boutons were counted for each Dlx-positive cell in the different WT and *GlyRa2*KO animals and gave an indication about the synaptic events. Histochemical stainings illustrated the decrease of GAD65 expression at the perisomatic areas of the interneurons by the fact that the GAD65 signal (red) was almost absent (fig. 12). Statistical analysis gave a high significant decrease in the number of perisomatic boutons at the Dlx-GFP interneurons in the *GlyRa2*KO animal. This result complements the reduction of the specific GABA_AR expression in the hippocampal development.

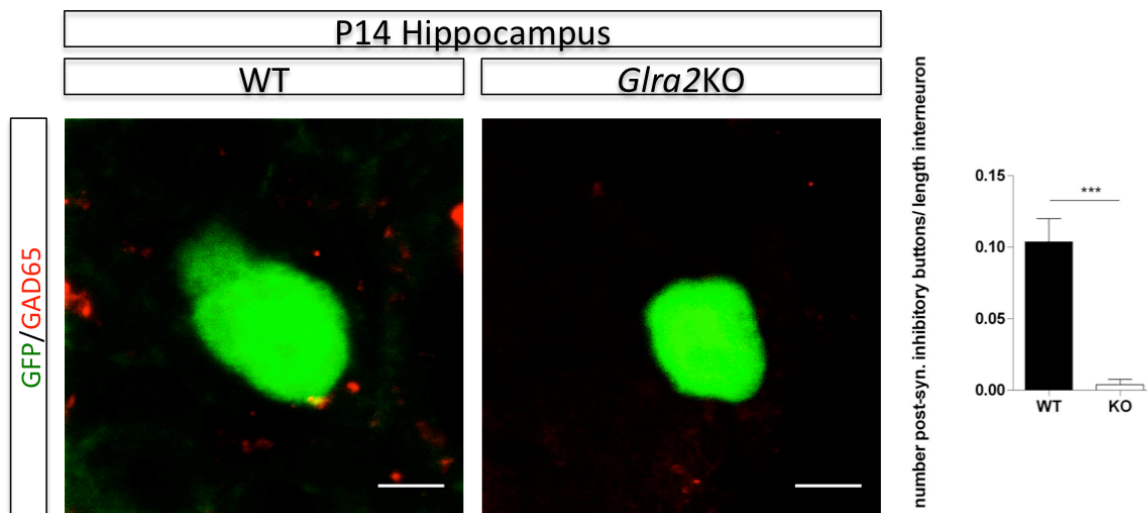


Figure 14: The expression and distribution of GAD65 in the Hippocampus: confocal images of double fluorescence immunohistochemical staining with GAD65 and GFP illustrates the Dlx-GFP interneurons (green) in the hippocampus and the expression of GAD65 (red) in WT and *GlyRa2*KO mice. Dlx-GFP expression appears bright in the soma while GAD65 is visualised by dotted profiles (perisomatic boutons). Values are presented as mean with \pm SEM *** $p < 0,001$; t-test; $n = 3$ animals for each condition. Scale bare: 50 μm.

4 Discussion

The role of neurotransmitters in the adult brain is not only restricted to the contribution of signal transmission in the adult brain alone. Neurotransmitters are also present before the start of synaptogenesis. In addition, neurotransmitters have an important function in development of the brain and spinal cord (47). In the past, only the role of Glutamate and GABA was well documented and studied throughout embryonic and early post-natal brain development (90). Our lab has recent published data about the importance of the presence of the GlyRa2 in cortical and striatal development (73). The absence of GlyRa2 leads to a morphological decrease in both cortex and striatum at postnatal day 0 (73). Moreover *GlyRa2*KO brains display a reduction in proliferation of cortical projection neurons and interneuronal migration (73). Here, we wanted to further investigate two other brain structures (hippocampus and striatum) with the focus in the development in specific cell populations: striatal and hippocampal interneurons in the absence of GlyRa2 to see if they correlate to the cortical interneurons. First, global morphology was studied in both structures with subsequently comparison of the most prominent interneuron subgroups (GABAergic: PV and SST in hippocampus and striatum plus striatal cholinergic interneurons). Second, proliferation rate of interneurons was observed to see if *GlyRa2*KO plays an important function in this phase of interneuronal development. We also observed the mRNA expression of the inhibitory receptors GABA_A and Glycine to observe possible compensational mechanisms in the absence of GlyRa2.

4.1 Genetic disruption of the GlyRa2 subunit affects brain morphology

Currently, the influence of GlyRa2 in the developing brain is not commonly studied and only a few studies have been carried out. The only published data of GlyRa2 in brain development, which only roughly studied the influence of GlyRa2 with the use of a different KO model, has resulted in no changes in cortical morphology(58). The performed experiments from our lab have been focusing on cortical development, resulting in cortical impairments in the absence of GlyRa2 (51, 73). Reasons about these findings could be explained by the different use in KO models and the difference in observation: our lab actually measured cortical and striatal area at P0 while the other published data did an approaching study of the different areas at P0 (58, 73).

To state the previous results, Dapi labelling (striatum) and Nissl staining (hippocampus) were performed on P7 and P14 slices for hippocampal area and P30 animals for the striatal area in order to observe the effect of *GlyRa2*KO and thereby state the importance of GlyRa2 in brain development. Our present study demonstrated that *GlyRa2*KO mice expose a significant reduction in both striatal and hippocampal area as a significant decrease in thickness of all hippocampal regions (CA1, CA2, CA3 and DG). These reductions in striatal and hippocampal area might be caused by several reasons such as reduction in neuronal cell numbers, spine density decrease and shortening of axons. In the past, the reduction of neuronal cells was observed in the cortex at P0. Specifically, a decrease of projection layers in the upper and lower layers of the cortex was detected, caused by depletion of basal progenitor cells and the favouring of direct neurogenesis (73).

4.2 GlyRa2 has a role in the amount of different types of interneurons located in striatum and hippocampus.

Previous studies support the importance of GlyRa2 in interneuronal development (48). In particular, experiments using time-lapse imaging showed that *in vitro* GlyR blocking, with the use of strychnine, impaired nucleokinesis and speed of migration of interneurons in the cortex (51). In addition, with the use of a transgene animal model: *GlyRa2*KO mice, experiments have shown an interneuronal migration defect in the cortex of *GlyRa2*KO animals (51). This interneuronal migration delay in the absence of GlyRa2, which was observed at E15.5, is combined with a decrease in number of cortical projection neurons at P0 (51, 73).

To declare and confirm previous results, diverse subtypes of interneurons located at other brain structures (striatum and hippocampus) were taken into consideration at different stages of early-postnatal development. Like the cortex, striatal and hippocampal interneurons are originated from mainly the same progenitor pools located in the subpallium (43). It would be convincing to believe that cortical interneuron impairments, seen in the *GlyRa2*KO, should be consistent in other interneuronal populations distributed in other brain regions. Moreover, striatum and cortical interneurons require repulsive signals to reach their final place of destination (43, 91). After migration, interneurons arrive at their place of destination where they differentiate in different subtypes and distribute according to the specificity of the brain region(43). To clarify if *GlyRa2*KO has an important role in interneuronal development, first the major groups of the interneuronal subtypes in both hippocampus and striatum were studied.

Immunohistochemical stainings were carried out to observe a possible defect in the different interneuronal subtypes in these different brain regions. Results demonstrate a rise in ChAT positive interneurons in the striatum at both postnatal phases (P14 and P30). According to these results the second major interneuronal group, the GABAergic interneurons were taken into consideration. These interneurons are the largest source of inhibition and are important in network regulation (87). Considering this heterogeneous group of interneurons, results demonstrated a reduction of both subtypes (PV+ and SST+) in the absence of GlyRa2 in the striatum. Loss of GABAergic interneurons could lead to an imbalance in excitatory-inhibitory signalling (92, 93). In addition, an increase in cholinergic interneurons could implement impairment in the regulation of inhibitory responses in the MSNs, a disruption of the striatal output through GABAergic inhibition as also possible effect in the neuromodulatory control in the context of dopamine and acetylcholine signalling (31).

Taken together, these findings are leading to the investigation of the proliferation of GABAergic and cholinergic interneurons. Proliferation assays could give insights in the role of the GlyRa2 in interneuronal development at the start of their development in the GE. Analysis of our results suggested no defect at the level of proliferation in the absence of GlyRa2. Further experiments should consider the process of early interneuron differentiation before the start of their tangential migration to their destination in the developing brain.

In addition, hippocampal interneuronal subtypes have been observed. First, Dlx5/6-GFP expression was used to see the total amount of GABAergic interneurons residing in the hippocampus. For striatum, this transgene mouse line could not be used because the expression of Dlx5/6 is not specific for striatal interneurons. In addition, the GABAergic Medial spiny neurons (MSNs) cells are also expressing the transcription factor Dlx5/6 (79). Furthermore, the amount of Dlx5/6 positive cells were decreased in *GlyRa2*KO but their distribution remained unchanged. In parallel to the striatum, PV and SST positive interneurons were visualized and quantified: PV+ interneuronal population were decreased while the amount of SST+ interneurons remained unchanged in the absence of *GlyRa2*. Interestingly SST+ interneuronal number is not altered in the hippocampus. A possible explanation can be found in the existence of divers classes of SST+ cells and their distribution in the brain. To Clarify, SST positive hippocampal interneurons could be divided into different subtypes of SST+ interneurons. This classification is based on the coexpression of other different neurochemical markers like CB or NPY (94, 95).

Currently, it is now well established that PV+ interneurons and SST+ interneurons are generated both in the MGE where the tangential migration brings them to their place of destination. Additionally, experiments with the use of MGE explants have provided the information that SST+ interneurons mainly originate from the dorsal part of the MGE whereas PV+ interneurons are originated from ventral MGE(44, 96-98). To translate these findings to our results: proliferation of interneurons demands complex spatial and temporal regulation. It is hypothesized, with frequent BrdU experiments, that MGE presents similar proliferation patterns as the cortical VZ (99). Particularly, similarities are found in the lengthening of the G1 phase (100). Interestingly disruption of cell cycle progression in the MGE has been associated with the imbalance of certain interneurons, generated from this region. Suggesting that possible disruption mechanisms (like *GlyRa2*KO) could interfere with these interneuronal populations (majority SST+ and PV+ interneurons) (99).

4.4 The number of mitotic figures in the MGE remains unchanged in *GlyRa2*KO mice.

In the past, GABA and Glutamate function were studied in the focus of cell proliferation. Studies demonstrate that these neurotransmitters act before cell cycle (44, 101). Furthermore, experiments have now discovered the presence of GlyR in progenitor cell pools of the VZ and with the suggesting that they influence the proliferation rate in the VZ and SVZ in the dorsal part of the telencephalon where MGE is located (73). It is interesting that immunohistochemistry with the use of mitotic marker PHH3 showed no difference between genotypes at the peak of interneuronal proliferation. Nevertheless, presence of the *GlyRa2* has been shown in the ventral telencephalon and could be related to fate cell determination in these regions (51). In addition, to fully exclude a crucial role of *GlyRa2* in interneuronal cell proliferation we suggest performing BrdU labelling with the combination of different markers for proliferation (for example KI67+) and interneurons (*nkx2.1*, GFP-Dlx) at different time points to obtain complete information about cell cycle and proliferation rate. However it will not allow us to distinguish proliferation patterns between the different interneuronal subtypes: a rise could only give information about the ChAT positive

interneurons in the striatum meanwhile a decrease in proliferation rate could explain the decrease of PV positive interneurons located at hippocampus and striatum as well as the SST positive interneurons residing in the striatum. Possible alternative experiments could involve pulse and chase experiments; injection of BrdU at the age of E13 and stainings in early postnatal stages: P0, P7 and P14 with colabeling of interneuronal markers.

4.4 Glyc2R deletion has consequences in the expression of other different inhibitory receptors.

To look deeper in the expression of the inhibitory transporter, specific in the case of interneurons, studies have demonstrated that neurotransmitters actively control migration, influence interneuronal cell morphology, and are key in the start and formation of synaptogenesis (47, 102). Noteworthy, the important neurotransmitter GABA has an influencing role in diverse developmental phases but mice with a defect in the generation of this neurotransmitter showed no great defect in the cortex (6). In addition, the defect in the GABA_AR generation showed remarkably no lethal consequences (6, 103). Regarding these data, theories of possible compensational mechanisms have been taken into consideration (58, 92). However a few specific subunit GABA_AR Knock-out models carry out a severe phenotype and are related to epilepsy (104, 105)

To observe if possible compensational mechanisms are present in the development of hippocampus and striatum, qPCR-experiments for all the glycine subunits (α 1- α 4 and β) has been done as well as for five of the GABA_AR subunits (α 1- α 5) at two different time points: P7 and adult. This experiment showed only in P7 GABA_A α 4 a significant reduction in the *GlyRa2*KO samples and in adult a significant reduction in the expression of GABA_A α 1 in the hippocampus was present. No compensational mechanism was seen in the other glycine subunits or in striatal tissue at P7.

Along with the loss of expression in the *GlyRa2*KO, GAD65 expression is significantly reduced in the absence of GlyRa2 at Dlx5/6-GFP positive hippocampal interneurons. GAD65, one of the two isoforms of GAD (GABA synthesis machinery) is mainly associated with synaptic terminals and the expression of this enzyme is mainly restricted to neuronal cells (106-108). The expression of GAD65 serves as a good marker at GABAergic synaptic endings (108). The reduction of GAD65+boutons in the *GlyRa2*KO could be related to the decrease of interneurons. By meaning, less interneurons will cause a decrease in possible connections between other neurons, which will lead to a decrease in synapse formation where receptors are located in perisomatic boutons. Another suggestion, concluding from the mRNA expression, would implement that mRNA expression decrease of specific GABA_AR could implement the significant reduction in receptors localized in the interneuronal synapses in the hippocampus. Further experiments will need to address these findings. Suggestions for further experiments would be implementing techniques which will more look into the cell physiology and the ion currents such as patch clamp recordings and calcium imaging.

5 Conclusion

Previous studies and data from our lab led us to hypothesize an important role of the GlyRa2 in the development of interneurons in embryonic and early postnatal stages. By the use of Dapi and Nissl stainings we observed morphological impairments in striatum and hippocampus at P7, P14 and P30 in parallel with previous data recorded in striatum and cortex at P0. To go deeper in neuron cell groups, different types of interneurons were analysed by means of immunohistochemistry in both striatum and hippocampus in early post-natal ages. Results demonstrated significant difference in specific interneuronal sub populations: analysis of the striatal cholinergic interneurons in striatum presented an increase in ChAT positive interneurons, while GABAergic subpopulations (PV+ and SS+) presented a significant increase in striatum as well as in the hippocampus (Dlx+ and PV+). By performing PHH3 staining at E12, we tried to investigate these findings at the level of interneuronal proliferation. Analysis presented no significant difference in the two littermates which suggests no key role in interneuronal proliferation for GlyRa2. Moreover, qPCR experiments were carried to obtain information about possible compensational mechanisms by either GABA_AR or GlyR subunits, which displayed a reduction of hippocampal GABAA a1 mRNA expression at P7 and hippocampal GABA_A a4 mRNA expression in adult. Finally GFP-Dlx positive hippocampal interneurons were images with the colabeling of GAD65+ to observe the amount of synaptic GABAAR expression, which demonstrated a significant decrease of GABAergic perisomatic boutons. These findings are demonstrating the existence of an important role for the GlyRa2 in interneuronal brain development. Although the exact mechanisms, by which specific interneuronal subpopulations are disrupted in the absence of GlyRa2, remains elusive. Further experiments on the level of proliferation are needed to obtain a clear view of the events at the level of interneuronal proliferation and cell cycle in the MGE with the use of multiple BrdU assays at different time points to visualize the whole interneuronal proliferation stream. It would be interesting to investigate the role of GlyRa2 in early differentiation processes and the interneuronal patterning across the brain.

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