Universidade de Lisboa

Faculdade de Ciências

Departamento de Química e Bioquímica

GABAergic transmission impairment promotes the glycinergic phenotype

Catarina Reis Orcinha

Dissertação de Mestrado em Bioquímica
Especialização em Bioquímica Médica

2012

Universidade de Lisboa

Faculdade de Ciências

Departamento de Química e Bioquímica

GABAergic transmission impairment promotes the glycinergic phenotype

Catarina Reis Orcinha

Dissertação de Mestrado em Bioquímica
Especialização em Bioquímica Médica

Dissertação orientada pela Doutora Cláudia Valente
e pelo Doutor Pedro Lima

2012

O trabalho experimental que conduziu á elaboração desta dissertação foi realizado no Instituto de Farmacologia e Neurociências da Faculdade de Medicina de Lisboa e Unidade de Neurociências do Instituto de Medicina Molecular.

INDEX

Acknowledgements .... **v**

Abbreviations .... **vii**

Resumo .... **xi**

Abstract .... **xiii**

List of Figures .... **xv**

List of Tables .... **xvii**

1. Introduction **1**

1.1. Central Nervous System 1

1.2. Inhibitory transmission 1

1.2.1. The GABAergic System 1

1.2.1.1. GABA 1

1.2.1.2. GABAergic Synapse 3

1.2.2. The Glycinergic System 7

1.2.2.1. Glycine 7

1.2.2.2. Glycinergic Synapse 8

1.3. Glycinergic transmission as a potential therapeutic target for Epilepsy 11

2. Aim **13**

3. Materials and Methods **15**

3.1. Reagents 15

3.2. Animals 15

3.3. Biological Samples 15

3.3.1. Neuronal Cortical Cultures 15

3.3.2. Hippocampal Synaptosomes 17

3.4. Pharmacological Treatments 17

3.4.1. Neuronal Cortical Cultures 17

3.4.2. Hippocampal Synaptosomes 18

3.5. Immunofluorescence Assay 18

3.5.1. Neuronal Cortical Cultures 18

3.5.2. Hippocampal Synaptosomes 19

3.5.2.1. Immunofluorescence Assay 19

3.5.2.2. Quantification of the immunofluorescence images 19

3.6. Western Blot Assay 20

3.6.1. Cell Lysis 20

3.6.2. SDS-PAGE....20
3.7. Immunoprecipitation 21
3.8. Antibodies....22
3.9. Quantitative Real-Time PCR (qPCR)....23
3.10. Patch Clamp Recordings....24
3.11. Data analysis (Statistics)....25
4. Results 27
4.1. Primary neuronal cortical cultures 27
4.1.1. Evaluation of cellular health and functionality....27
4.1.1.1. Electrophysiological recordings 27
4.1.1.2. Immunofluorescence assays 28
4.1.2. Expression pattern of glycinergic transmission markers....29
4.1.3. Transcript expression pattern of glycinergic transmission markers....31
4.2. Hippocampal synaptosomes....33
4.2.1. Characterization of synaptosomes prepared from rat hippocampal slices by immunofluorescence 33
4.2.2. Characterization of synaptosomes prepared from hippocampal homogenates by immunofluorescence 33
4.2.3. Evaluation of the ratio GABAergic/glycinergic terminals in hippocampal synaptosomes....34
4.2.4. Assessment of a potential physical interaction between GlyT2 and VIAAT....36
5. Discussion 39
5.1. Changes in the glycinergic phenotype in insulted cortical primary neuronal cultures 39
5.2. Changes in the ratio of glycinergic *vs* GABAergic terminals in rat hippocampal synaptossomes 43
5.3. Interaction between vesicular and membrane glycine transporters, GlyT2 and VIAAT..44
6. General Conclusions....47
7. Future perspectives 49
8. References....51
9. Appendix 61
9.1. qPCR standard and melting curve analysis....61

ACKNOWLEDGEMENTS

Em primeiro lugar, gostaria de agradecer aos meus orientadores, Doutora Cláudia Valente de Castro e Doutor Pedro Lima. Á Doutora Cláudia Valente, pela oportunidade de poder trabalhar sob a sua supervisão. Por todo o conhecimento transmitido, pela paciência, pela sua disponibilidade em discutir o meu trabalho e todo o apoio que me deu. Por toda a sua simpatia e boa disposição. Ao Doutor Pedro Lima, pela sua disponibilidade e prestabilidade.

Gostaria também de agradecer ao Professor Joaquim Alexandre Ribeiro e à Professora Ana Maria Sebastião por me terem recebido na Unidade de Neurociências e por me terem concedido a oportunidade de desenvolver as minhas aptidões laboratoriais com esta equipa fantástica.

A todos os colegas do laboratório, obrigada por me terem feito sentir tão bem recebida desde o primeiro dia. Aos meus vizinhos de bancada, Rita e André, pela amizade, pela disponibilidade em ajudar sempre que precisei e pela paciência em responder às minhas perguntas, por mais ridículas que fossem. À Raquel, Mariana e Joana, pela vossa amizade, simpatia constante, bom humor e pela ajuda com o Patch.

A todos os meus amigos. Em especial, quero agradecer à Catarina, por estares sempre presente, à Belinha, ao Rodrigo, à Bagulho, à Rita e à Inês, pela vossa amizade e carinho.

E finalmente, um agradecimento muito especial à minha família. À minha mãe, ao meu avô, ao meu irmão e á minha irmã, pelo amor incondicional, força e inesgotável confiança em mim.

ABBREVIATIONS

3-MPA – 3-Mercaptopropionic Acid

5-HT3 – 5-hydroxytryptamine receptor

aCSF – Artificial cerebrospinal fluid

AEDs – Antiepileptic drugs

ANOVA – Analysis of variance

Ara C - Cytosine arabinoside

ATP – Adenosine 5'-triphosphate

BSA – Bovine serum albumin

cDNA – Complementary DNA

CNS – Central nervous system

DAPI - 4',6-diamidino-2-phenylindole

DIV – Days *in vitro*

DMSO – Dimethylsulfoxide

DNA – Deoxyribonucleic acid

DTT– 1,4-dithiothreitol

E – Embryonic day

EDTA – Ethylenediaminetetraacetic acid

FBS – Fetal bovine serum

GABA – γ-Aminobutiric acid

$GABA_AR$ – GABA receptor A

$GABA_BR$ – GABA receptor B

$GABA_cR$ – GABA receptor C

GABA-T – GABA transaminase

GAD – Glutamate decarboxylase

GATs – GABA transporters

GAT-1 – GABA transporter 1

GAT-2 – GABA transporter 2

GAT-3 – GABA transporter 3

GCS – Glycine cleavage system

GFAP – Glial fibrillary acidic protein

Gly - Glycine

GlyR – Glycine receptor

GlyT1 – Glycine transporter 1

GlyT2 – Glycine transporter 2

HBSS – Hank's balanced salt solution

HEPES – N-2-hydroxyehtylpiperazine-N'-2-ethanesulfonic acid

HRP – Horseradish peroxidase

ILAE - International League Against Epilepsy

IP - Immunoprecipitation

KCC2 - K^+–Cl^- co-transporter 2

KHR – Krebs-HEPES-Ringer

MAP2 – Microtubule associated protein 2

mEPCs – Miniature excitatory postsynaptic currents

mRNA – Messenger ribonucleic acid

NKCC1 - Na^+–K^+–Cl^- co-transporter

NMDA – N-metil-D-aspartate

PBS – Phosphate buffered saline

PBS-T – Phosphate buffered saline Tween-20

PCR – Polymerase chain reaction

PDL – Poly-D-lysine

PFA – Paraformaldehyde

PMSF - Phenylmethylsulfonyl fluoride

PVDF - Polyvinylidene fluoride

qPCR – Quantitative PCR

RIPA – Radio immunoprecipitation assay

RNA - Ribonucleic acid

RT – Room temperature

SDS - Sodium Dodecyl Sulfate

SDS - PAGE –sodium dodecyl sulfate-polyacrylamide-gel electrophoresis

SKF89976a - 1-(4,4-Diphenyl-3-butenyl)-3-piperidine-carboxyliacidhydro-chloride)

TBS – Tris buffered saline

TBS-T – Tris buffered saline Tween-20

TCA – Tricarboxilic acid cycle

TEMED – 1,2-bis(dimethylamino)ethane

TLE - Temporal Lobe Epilepsy

TMD – Transmembrane domain

Tris – Tris-hydroxymethyl-aminomethane

VGAT – Vesicular GABA transporter

VIAAT – Vesicular amino acid transporter

WHO – Worldwide Health Organization

RESUMO

A transmissão inibitória desempenha um papel importante na regulação e estabilização da actividade neuronal e é essencial para diversas funções cerebrais como a cognição, percepção, movimento e emoção. As sinapses inibitórias, GABAérgica e glicinérgica, e a sua distribuição, apresentam diferenças no sistema nervoso central dos mamíferos (CNS). A maioria das sinapses inibitórias no cérebro são GABAérgicas, e as glicinérgicas, predominantes na espinal medula e tronco cerebral, tem sido bastante negligenciadas no cérebro.

A glicina exerce a sua função através do receptor ionotrópico da glicina (GlyR), um canal pentamérico composto por dois tipos de subunidades (α e β) permeável a iões cloreto e localizado na membrana do terminal pós-sináptico. Os transportadores da glicina 2 (GlyT2) pertencem à família de transportadores dependentes de Na^+/Cl^-. Estão presentes na membrana dos terminais pré-sinápticos glicinérgicos, assegurando a remoção da glicina da fenda sináptica e permitindo a inserção do neurotransmissor em vesículas sinápticas.

O presente estudo tem como principal objectivo investigar quais os principais intervenientes na aquisição do fenótipo glicinérgico.

Para isso, efectuou-se uma abordagem farmacológica, em culturas primárias de neurónios, com o propósito de avaliar o fenótipo glicinérgico mediante o comprometimento da transmissão GABAérgica. Os resultados obtidos por western blot e por PCR quantitativo (qPCR) revelaram que a expressão de GlyR e de GlyT2 aumentava significativamente, após tratamento das células com antagonistas do receptor ionotrópico de GABA $GABA_A$ ($GABA_AR$) ou do transportador de GABA GAT-1, gabazina e SKF89976a, respectivamente. Em sinaptossomas obtidos de cérebro, a dupla detecção por imunofluorescência, de GlyT2 (marcador de neurónios glicinérgicos) e GAD (marcador de neurónios GABAérgicos) revelou igualmente que, na presença de SKF89976a, a razão entre terminais GABAérgicos e glicinérgicos se apresentava alterada. O comprometimento do sistema GABAérgico resultou no aumento de terminais glicinérgicos puros e mistos, com a consequente diminuição de terminais GABAérgicos. Neste trabalho, a interacção entre o transportador vesicular de aminoácidos inibitórios (VIAAT) e o GlyT2 foi igualmente explorada por ensaios de imunoprecipitação.

Os resultados obtidos nesta tese evidenciam, pela primeira vez, que o comprometimento da neurotransmissão GABAérgica induz um aumento dos marcadores da transmissão mediada pela glicina, nomeadamente GlyR e GlyT2, sugerindo assim um mecanismo de compensação entre os dois sistemas inibitórios no cérebro.

Palavras-chave: Transmissão inibitória, glicina, GABA, cérebro, GlyR, GlyT2

ABSTRACT

The inhibitory transmission plays an important role in the regulation and stabilization of brain network activity and is essential for a number of brain functions such as cognition, perception, movement and emotion. GABAergic and glycinergic inhibitory synapses, and their distribution, are very different in the mammalian central nervous system (CNS). Most inhibitory synapses in the brain are GABAergic, and glycinergic ones, predominant in the most caudal regions of the CNS, have been largely disregarded in the brain.

Glycine exerts its action through glycine receptors (GlyR), which belong to the superfamily of ligand-gated ion channels, are localized in the postsynaptic membrane and form pentameric channels composed of two different subunits (α and β) permeable to chloride ions. Glycine transporters 2 (GlyT2) belong to the family of Na^+/Cl^--dependent transporter proteins. They are located in the membrane of glycinergic neurons and are responsible for terminating glycine-mediated neurotransmission by uptaking glycine into glycinergic nerve terminals, allowing for neurotransmitter reloading of synaptic vesicles.

The present study aims to investigate which are the principal mediators for the acquisition of a glycinergic phenotype.

A pharmacological approach, in primary neuronal cultures, was pursued in order to evaluate the glycinergic phenotype upon a GABAergic transmission impairment. Western blot analysis and quantitative real-time PCR (qPCR) revealed that GlyR and GlyT2 expression increased significantly after treating the cultures with blockers for either $GABA_A$ receptor or GABA transporter GAT-1, gabazine and SKF89976a, respectively. In brain synaptosomes, double immunofluorescence of GlyT2 (marker of glycinergic neurons) and GAD (marker of GABAergic neurons) also revealed that, in the presence of SKF89976a, the ratio of GABAergic *vs* glycinergic terminals changed. GABAergic impairment caused an increase in mixed (GABA and glycine-containing) and pure glycinergic terminals, with a concomitant decrease in GABA-containing boutons. Furthermore, a physical interaction was assessed between Vesicular Inhibitory Amino Acid Transporter (VIAAT) and GlyT2 by immunoprecipitation assays.

These results obtained in this thesis have elucidated, for the first time, that impairment in GABA-mediated neurotransmission induces an increase in glycine-

mediated transmission components, namely GlyR and GlyT2, and suggest a compensatory mechanism between the two inhibitory systems in the brain.

Keywords: Inhibitory transmission, glycine, GABA, brain, GlyR, GlyT2

LIST OF FIGURES

Figure 1	During brain development, the role of GABA switches from excitatory to inhibitory neurotransmitter.	2
Figure 2	The GABAergic synapse.	3
Figure 3	Schematic representation of GABA and glycine uptake from the extracellular space to the cytosol and from the cytosol into synaptic vesicules.	4
Figure 4	GABA receptors and their subunit composition.	6
Figure 5	Biosynthesis of glycine, from the amino acid serine, by the enzyme serine hydroxymethyltransferase.	7
Figure 6	The glycinergic synapse.	9
Figure 7	Membrane topology of GlyTs.	10
Figure 8	Brain dissection from E17-18 rat embryos.	16
Figure 9	Patch-clamp recordings.	27
Figure 10	Double staining of MAP2 and GFAP in primary rat cortical neurons.	28
Figure 11	Double staining of GAD6 and VIAAT in primary rat cortical neurons.	29
Figure 12	Expression of inhibitory transmission related markers, when cells are treated with different concentrations of gabazine, at different times.	30
Figure 13	Expression of inhibitory transmission related markers, when cells are treated with different concentrations of SKF89976a, at different times.	31
Figure 14	Transcript expression profile of GlyT2.	32
Figure 15	Transcript expression profile of GlyR subunits α1, α2, α3 and β.	32
Figure 16	Double staining of GFAP and MAP2 in synaptosomes obtained from hippocampal slices.	33
Figure 17	Double staining of GFAP and MAP2 in synaptosomes obtained from hippocampal homogenates.	34
Figure 18	Double staining of GlyT2 and GAD6 in synaptosomes obtained from hippocampal homogenates.	35

Figure 19	Illustration of the method used for the quantitative analysis.	35
Figure 20	Changes in terminal phenotype in hippocampal synaptosomes.	36
Figure 21	Representative immunoblots of the immunoprecipited proteins, VIAAT (55kDa) and GlyT2 (60kDa).	37
Figure 22	qPCR standard and melting curves analysis for β-actin – endogenous control.	61
Figure 23	qPCR standard and melting curves analysis for GlyT2.	62
Figure 24	qPCR standard and melting curves analysis for GlyRα1.	62
Figure 25	qPCR standard and melting curves analysis for GlyRα2.	63
Figure 26	qPCR standard and melting curves analysis for GlyRα3.	63
Figure 27	qPCR standard and melting curves analysis for GlyRβ.	64

LIST OF TABLES

Table I Drugs used in this work. 18

Table II Primary antibodies used in this work. 22

Table III Secondary antibodies used in this work. 22

Table IV Description of the primers used for qPCR. 24

1. INTRODUCTION

1.1. CENTRAL NERVOUS SYSTEM

The Central Nervous System (CNS) is composed of the brain and spinal cord. The brain is constituted by a large number of different types of neurons, as well as several types of non neuronal cells, such as astrocytes, oligodendrocytes and microglia, which communicate and form complex circuits. This communication is highly relevant for the correct functions of the brain.

The neuron is an electrically excitable cell that processes and transmits information by electrical and chemical signaling. This process is also known as synapse. In the case of chemical synapses, a presynaptic neuron releases, by exocytose, signaling chemical molecules – the neurotransmitters. These neurotransmitters interact with specific receptor proteins located at the surface of postsynaptic neurons and hence induce cellular responses (Kandel et al., 2000). The neurotransmitters can be classified into two groups, excitatory and inhibitory, depending on the nature of effects elicited in the target cells.

The inhibitory neurotransmission in the CNS is mediated by GABA (γ-aminobutyric acid) and by glycine. GABA is established as the most important inhibitory neurotransmitter in the brain (Bowery and Smart, 2006), while glycine acts predominantly in the spinal cord and brain stem (Kirch, 2006).

In contrast, the main excitatory transmission system is mediated by glutamate and aspartate (Kandel et al., 2000).

1.2. INHIBITORY TRANSMISSION

1.2.1. The GABAergic System

1.2.1.1. GABA

The amino acid GABA has long been considered to be the main inhibitory neurotransmitter in the adult mammalian CNS. GABA was first identified in the mammalian brain during the 1950s (Roberts and Frankel, 1950). It regulates the neuron's ability to fire action potentials either through hyperpolarization of the membrane potential or through shunting of excitatory inputs. Recent studies have

shown that GABA shifts its action through development. In immature neurons, due to a high intracellular chloride concentration ensured by the expression of an inwardly directed Na^+–K^+–Cl^- co-transporter (NKCC1), GABA action upon ionotropic $GABA_A$ receptor causes depolarization and often excitatory actions, while in mature neurons owing to the higher expression of the outwardly directed K^+–Cl^- co-transporter (KCC2), GABA action leads to hyperpolarization and inhibitory actions (Figure 1) (Cherubini et al., 1991; Ben-Ari et al., 2012).

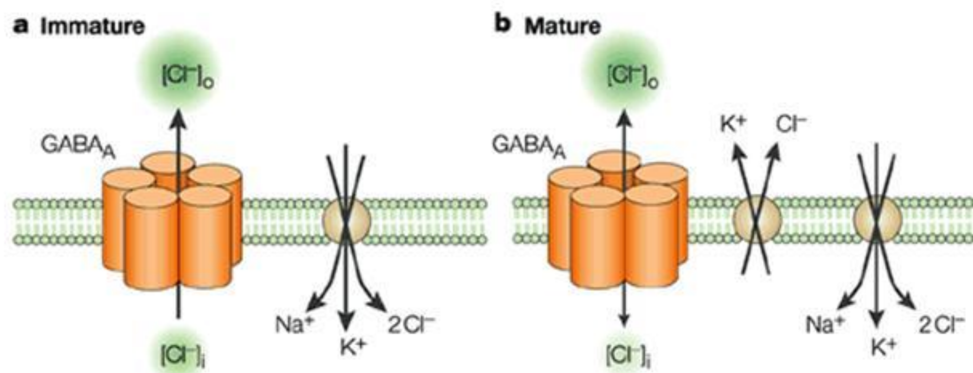


Figure 1. During brain development, the role of GABA switches from excitatory to inhibitory neurotransmitter. **(a)** In immature neurons, an inwardly directed Na^+–K^+–Cl^- co-transporter (NKCC1) acts to maintain relatively high intracellular chloride concentrations. **(b)** In mature neurons, intracellular chloride concentration is decreased by the expression of an outwardly directed K^+–Cl^- co-transporter (KCC2), thereby diminishing the driving force for chloride flux in response to $GABA_A$ receptor activation. Adapted from Owens and Kriegstein, 2002.

GABA also acts as a trophic factor during nervous system development to influence events such as proliferation, migration, differentiation, synapse maturation and cell death. GABA mediates these processes by the activation of ionotropic $GABA_A$ receptors or metabotropic $GABA_B$ receptors, localized in synaptic and extrasynaptic places (Owens and Kriegstein, 2002).

Its biosynthesis in neurons mainly involves decarboxylation of glutamate (Figure 2) yielding GABA and CO_2 via the enzyme glutamate decarboxylase (GAD) (EC 4.1.1.15) (Roberts and Kuriyama, 1968).

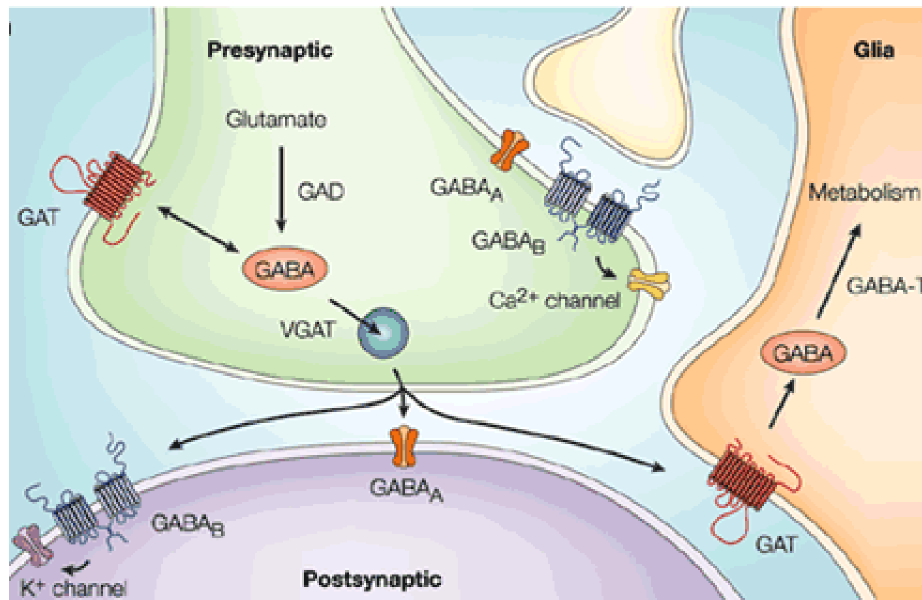


Figure 2. The GABAergic synapse. GABA is synthesized from glutamate by glutamic acid decarboxylase (GAD) and stored in vesicles located in the presynaptic terminal. It is then released into the synaptic cleft and interacts with postsynaptic $GABA_A$ receptors opposed to GABA releasing sites. The $GABA_A$ receptor is linked to the postsynaptic membrane by anchoring proteins, such as gephyrin. GABA is removed from the synaptic cleft by specific transporters, GABA transporters (GATs), located on nerve endings and adjacent glial cell membranes. In glial cells, GABA is again converted into glutamate by GABA transaminase (GABA-T) and glutamate can be further metabolized to glutamine, which is more easily taken up by neurons *via* amino acid transporters and again reused for GABA synthesis. Metabotropic $GABA_B$ receptors, located on GABAergic nerve terminals, suppress the release of GABA, by inhibiting Ca^{2+} influx. Adapted from Owens and Kriegstein, 2002.

The glutamate can be obtained by neurons from two different sources, namely from glutamine derived from tricarboxilic acid (TCA) cycle in glial cells and from glutamine present in nerve terminals. There are two isoforms of GAD, GAD65 and GAD67. GAD67 is found ubiquitously in GABAergic neurons, whereas GAD65 is preferentially located in the GABAergic nerve endings and is thus considered a marker of GABAergic terminals. Based on these findings it has been suggested that GAD65 is specialized to readily synthesize GABA under short-term demand (Martin and Rimvall, 1993).

1.2.1.2. GABAergic Synapse

Like most neurotransmitters, GABA is packaged into presynaptic vesicles by a vesicular GABA transporter (VGAT) (McIntire et al., 1997), also known as vesicular inhibitory amino acid transporter (VIAAT) (57kDa), since is also implicated in glycine vesicular uptake (Sagné et al., 1997). Thus, VIAAT is a transporter located in the

membranes of presynaptic vesicles (Sagné et al., 1997) and is composed of 12 transmembrane spanning regions (Figure 3).

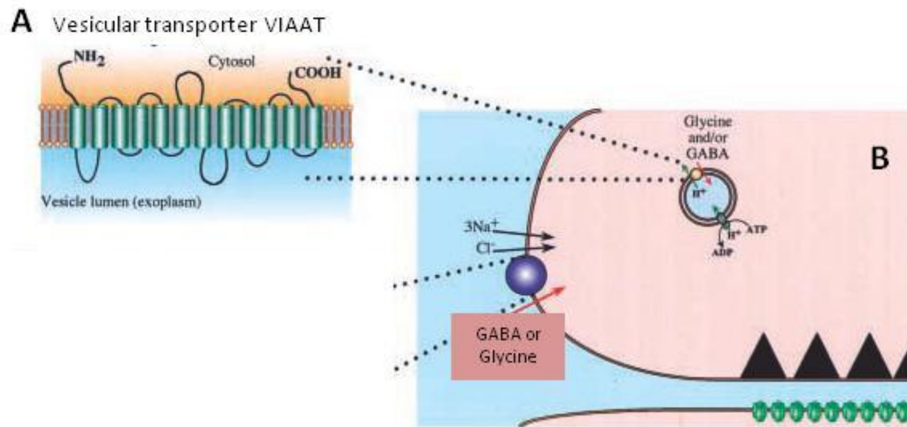


Figure 3. Schematic representation of GABA and glycine uptake from the extracellular space to the cytosol and from the cytosol into synaptic vesicules. (A) Vesicular transporter for GABA and glycine (VIAAT), with its 12 transmembrane spanning regions. (B) Membrane-specific neuronal transporters uptake, GABA or glycine from the extracellular space to the cytosol, where VIAAT ensures the exchange of luminal protons for cytosolic GABA or glycine, thus loading the vesicles. Adapted from Legendre, 2001.

The concentration of neurotransmitter transported into the vesicles is dependent of a vesicular-H^+-ATPase (V-ATPase). Using the energy generated by the hydrolysis of cytoplasmatic ATP (adenosine-5'-triphosphate), the V-ATPase creates a pH, or chemiosmotic, gradient by promoting the influx of protons into the vesicle (Dumoulin et al., 1999; Kandel et al., 2000). This mechanism allows the exchange luminal protons for cytosolic GABA or glycine, loading the vesicles. This transporter is therefore present in GABAergic, glycinergic and mixed (GABAergic/glycinergic) neurons and can be used as a marker of inhibitory presynaptic endings (Dumoulin et al., 1999). The role of VIAAT in the release of both GABA and glycine is supported by electrophysiological evidence from VIAAT deficient mice (Wojcik et al., 2006) and measurements of quantal release of glycine and GABA from VIAAT-transfected secretory cells, using a double-sniffer patch-clamp technique (Aubrey et al., 2007).

Furthermore, the complex formed by VIAAT and GAD65 appears to be necessary for efficient GABA synthesis and packaging into synaptic vesicles (Jin et al., 2003).

Upon stimulation, GABA is released from nerve terminals by calcium-dependent exocytosis (Gaspary et al., 1998). Once released, GABA freely diffuses

across the synaptic cleft and interacts with its appropriate receptors on the postsynaptic membrane.

The inhibitory neurotransmitter GABA activates two different types of receptors: ionotropic receptors, $GABA_A$ and $GABA_C$ receptors ($GABA_A$Rs and $GABA_c$Rs, respectively), and metabotropic receptors, $GABA_B$ receptors ($GABA_B$Rs) (Figure 4). The first ones mediate fast inhibitory responses, while the latter one mediates slow inhibitory responses (Owens and Kriegstein, 2002).

$GABA_A$Rs is a member of a superfamily to which nicotinic acetylcholine, glycine and 5- hydroxytryptamine (5-HT3) receptors also belong (Bowery and Smart, 2006). They are pentameric channels and consist of several subunits (e.g. α, β, γ, δ, ε, θ, π and ρ). The most common combination for $GABA_A$Rs is the triplet α1/β2/γ2, which is detected in various cell types in the CNS (McKernan and Whiting, 1996). These receptors are primarily permeable to chloride (Cl^-) ions, but other anions, such as bicarbonate (HCO_3^-) can also be carried by the channel pore, although with much lower efficiency. When GABA binds extracellularly to the $GABA_A$R, it induces a conformational change in the channel protein which increases the permeability of the ion pore to Cl^-.

$GABA_B$ receptors consist of transmembrane receptors that are coupled to G-proteins and activate Ca^{2+} and K^+ ion channels (Holopainen and Wojcik, 1993). Until now, two subtypes of the receptor have been identified, the $GABA_B$R-1 and $GABA_B$R-2, existing two isoforms of the $GABA_B$R-1 (R1a and R1b) (Kaupmann et al., 1998; Pierce et al., 2002). This receptor is an obligate heterodimer that is only functional when both the $GABA_B$R-1 and $GABA_B$R-2 are co-expressed in the same cell. $GABA_B$Rs are localized both pre- and postsynaptically, and they use different mechanisms at these locations to regulate cell excitability (Bettler et al., 2004). In the hippocampus, presynaptic $GABA_B$ receptors located both on inhibitory (GABAergic) and excitatory (glutamatergic) terminals are proposed to be tonically activated by ambient levels of GABA (Kubota et al., 2003).

$GABA_C$ receptors have prominent distributions on retinal neurons, but functionally and pharmacologically are less well-characterized than its counterpartners. This receptor is a Cl^--selective ion-channel but differs from $GABA_A$ receptor by having a smaller single channel conductance, meaning a longer lasting inhibition (Feigenspan and Bormann, 1994), and a higher affinity for GABA than the $GABA_A$ receptors (Feigenspan and Bormann, 1994; Wang et al., 1994). $GABA_C$R are believed to be

homo- or heteropentameric proteins that are composed of ρ-subunits, of which three subunits have been identified, the ρ1, ρ2 and ρ3 subunits (Cutting et al., 1991; Alakuijala et al., 2005).

Once GABA is removed from the synaptic cleft, the channel comes to a closed state and can, after desensitization, be re-opened.

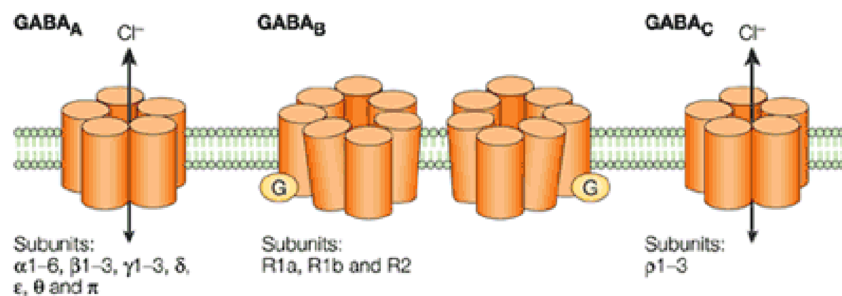


Figure 4. GABA receptors and their subunit composition. $GABA_A$ and $GABA_C$ receptors are closely related pentameric receptors that carry chloride; however, whereas $GABA_A$ receptors are composed of combinations of several subunit types, $GABA_C$ receptors are composed of single or multiple ρ-subunits. $GABA_B$ receptors are metabotropic receptors that exist as R1a, R1b and R2 isoforms, and are associated with G proteins. Native $GABA_B$ receptors are dimers composed of one R1 subunit and the R2 subunit. Adapted from Owens and Kriegstein, 2002.

After dissociation from the receptor complex, GABA is transported back into the presynaptic nerve terminal or into surrounding astrocytes *via* a high affinity GABA transport system thereby terminating GABA's inhibitory action (Iversen and Neal, 1968) and keeping the extracellular GABA concentrations under physiological levels. This reuptake from the synaptic cleft is mediated by several types of plasma membrane GABA transporters (GATs). There are four distinct GABA transporters (GATs 1-3 and a betaine/GABA transporter) that have been identified in mammalian tissues by molecular cloning techniques (Conti et al., 2004). GATs have unique anatomical distributions in the rodent CNS, and the major subtype, GAT-1, is considered the predominant neuronal GABA transporter, whereas the others show a more ubiquitous distribution (Guastella et al., 1990; Borden, 1996; Engel et al., 1998). GAT-1 is found in inhibitory axons and nerve terminals (Conti et al., 1998), and this organization is well suited for functions associated with GABA uptake. The GABA uptake *via* GAT-1 requires extracellular Na^+ and Cl^- since two Na^+ and one Cl^- ion are co-transported with each GABA molecule (Lester et al, 1994).

GABA can also be taken up by surrounding astrocytes where it is metabolized into succinic semialdehyde by GABA transaminase (GABA-T), and transformed into glutamate (Waagepetersen et al., 2003). Because GAD is not present in glia cells,

glutamate cannot be converted into GABA and is thus transformed by glutamine synthetase into glutamine (Waagepetersen et al., 2003; Schousboe and Waagepetersen, 2006). Glutamine is then uptaken back to neurons by specific transporters (Varoqui et al., 2000), where it can be converted by GAD to regenerate GABA (Schousboe and Waagepetersen, 2006).

1.2.2. The Glycinergic System

1.2.2.1. Glycine

Glycine is a non-essential amino acid and is the smallest of the 20 amino acids commonly found in proteins. It is biosynthesized in the body from the amino acid serine (Figure 5), a reaction catalyzed by the enzyme serine hydroxymethyltransferase (EC 2.1.2.1) (Nelson and Cox, 2000).

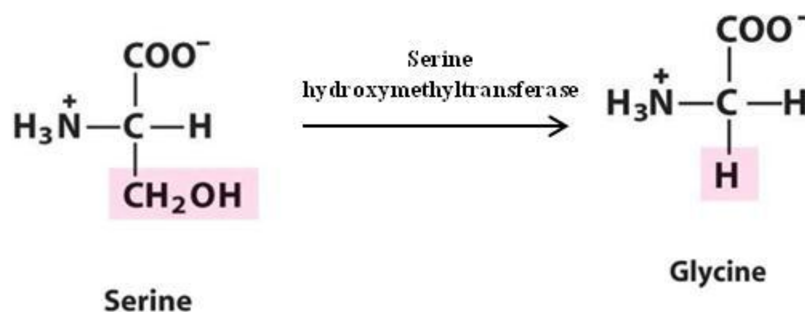


Figure 5. Biosynthesis of glycine, from the amino acid serine, by the enzyme serine hydroxymethyltransferase. The structural formulas show the state of ionization that would predominate at pH 7.0. The unshaded portions are those common to both amino acids; the portions shaded in pink are the R groups. Adapted from Nelson and Cox, 2000.

In neurobiology, it serves as the second major inhibitory neurotransmitter in the CNS, acting at more caudal regions.

High densities of glycinergic synapses are found in spinal cord and brain stem (Eulenburg et al., 2005) and are implicated in the control of many motor and sensory pathways (López-Corcuera et al., 2001). In addition, this amino acid functions as an excitatory neurotransmitter during embryonic development and is an essential co-

agonist at glutamatergic synapses containing the ionotropic N-methyl-D-aspartate (NMDA) subtype of glutamate receptors (Johnson and Ascher, 1987). Recent studies have shown that superfusion with 0.5-20μM glycine causes a potentiation of NMDA receptors currents in slice preparations (Berger et al., 1998). Furthermore, higher concentrations of glycine (≥100 μM) have been found to 'prime' NMDA receptors for internalization although this process is ultimately triggered by the activating agonist glutamate (Nelson, 1998).

1.2.2.2. Glycinergic Synapse

Glycine packaging into synaptic vesicles is mediated by VIAAT as happens to GABA (Figure 3). It is stored in high concentrations in presynaptic terminals (50-100mM) (Kandel et al., 2000), and released into the synaptic cleft upon cellular depolarization. At the synaptic cleft, it activates the ionotropic glycine receptor (GlyR).

GlyR is a heteropentameric ion channel permeable to chloride ions and shares many structural characteristics of the nicotinic acetylcholine receptor. Unlike $GABA_A$ receptor-mediated inhibition, glycine receptor-mediated inhibition is primarily postsynaptic (Mitchell et al., 1993; Todd et al., 1996). When activated, the channel serves to increase chloride conductance in the postsynaptic membrane leading to hyperpolarization and decreased excitability. GlyR is composed of α and β subunits, arranged around a central pore. Studies have shown evidence for both a 3α:2β (Becker et al., 1988; Kuhse et al., 1993), and more recently 2α:3β stoichiometry (Grudzinska et al., 2005). The α subunit of GlyR confers channel kinetics and pharmacology, and is the obligatory subunit which is capable of forming functional homomeric channels. The β subunit allows anchoring of the receptor to the membrane through binding of the auxiliary structure protein gephyrin (93kDa) (Triller et al., 1985; Schmitt et al., 1987; Betz et al., 2006). This GlyR-gephyrin interaction is reversible and highly dynamic (Meier et al., 2000), thus regulating the number of receptors at synapses. To date there are four known α subunit isoforms, named α1 -α4 (Grenningloh et al., 1990; Kuhse et al., 1990; Matzenbach et al., 1994) and a single β subunit isoform (Lynch, 2004). The diversity in GlyR subunits can be generated by alternative splicing, contributing for its heterogeneity (Kirsh, 2006). In the caudal regions of the CNS, α2 subunits predominate

at early stages, while α1 subunits predominate later (Kuhse et al., 1995). Some studies suggested that hippocampal neurons express α2 homomeric GlyRs (Chattipakorn and McMahon, 2002), while others stated that hippocampal GlyRs might also be composed of heteromeric αβ subunits (Danglot et al., 2004). Recently, it was shown that in mature hippocampus, although a few synaptic GlyRα1β can be detected in the dendritic layers, extrasynaptic α2/α3-containing GlyR and somatic localized GlyRα3 are the most abundant (Aroeira et al., 2011).

As stated before for GABA receptors (depicted in Figure 1), in immature neurons GlyR activation causes depolarization instead of the hyperpolarization observed in mature ones. This indicates that a shift, from glycine-mediated excitation to glycine-mediated inhibition, also occurs in GlyR function with development (Ben-Ari, 2002).

At the glycinergic synapse (Figure 6), termination of glycinergic transmission is achieved through the removal of glycine from the synaptic cleft by specific high affinity transporters: GlyT1, mainly present in glial cells, namely astrocytes (Guastella et al., 1992), and GlyT2, that can be found in the plasma membrane of glycinergic nerve terminals (Liu et al., 1993). Therefore, these transporters (GlyTs) regulate the effective synaptic glycine concentration (Eulenburg et al., 2005).

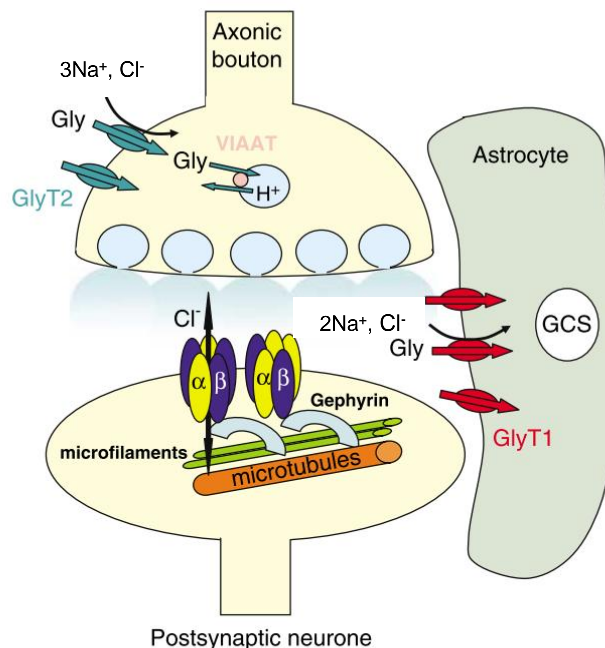


Figure 6. The glycinergic synapse. Schematic representation of a typical glycinergic synapse. The glycine receptors are shown as pentamers of stoichiometry 3α:2β and also the more recent preferred stoichiometry of 2α:3β. The receptors are anchored *via* the β subunits to gephyrin and thus to the microfilaments and microtubules. Presynaptic glycine is packaged into vesicles *via* VIAAT before release. After dissociation from the receptor, either of two discretely localized glycine transporters (GlyT1 or 2) sequester the glycine, which can then be re-packaged into synaptic vesicles or hydrolysed *via* the glycine cleavage system (GCS). Adapted from Bowery and Smart, 2006.

Both GlyTs belong to a large family of Na^+/Cl^--dependent transporter proteins, which includes transporters for monoamines (serotonin, norepinephrine and dopamine) and GABA (Nelson et al., 1998). GlyT1 and GlyT2 share approximately 50% amino acid sequence identity, but differ in pharmacology and tissue distribution. They are characterized by a transmembrane topology with 12 transmembrane domains (TMDs) connected by six extracellular and five intracellular loops (Figure 7). The large second extracellular loop connecting transmembrane domains 3 and 4 is multiply N-glycosylated, and the N- and C-termini are located intracellularly. GlyT2 is a larger protein than GlyT1 due to a unique extended N-terminal domain of approximately 200 amino acids (Eulenburg et al., 2005).

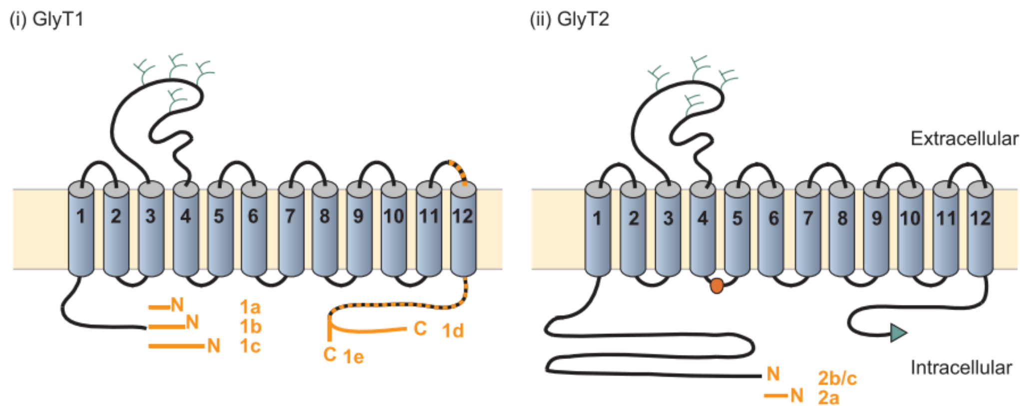


Figure 7. Membrane topology of GlyTs. GlyTs are characterized by 12 putative TMDs with intracellular N and C termini. Different splice variants are indicated in orange. For GlyT1, three N-terminal splice variants (a–c) and two C-terminal splice variants (d, e) have been identified. Alternate promoter usage generates three N-terminal GlyT2 isoforms (a–c) with eight additional amino acids for GlyT2a and shorter identical protein sequences for GlyT2b and c. Adapted from Eulenburg et al., 2005.

GlyTs have distinct functions at glycinergic synapses. Glial GlyT1 allows glycine transport into astrocytes together with two Na^+ and one Cl^- (Roux and Suplisson, 2000), where it’s hydrolyzed by an efficient system of degradation composed of several enzymes named glycine cleavage system (GCS), eliminating the excess intracellular glycine (Figure 6) (Sato et al., 1991). This transporter can be found in the cerebellum and olfactory bulb, but also in non-neuronal tissues (e.g. liver, pancreas and intestine) (Guastella et al., 1992). GlyT1 is also present in glutamatergic neurons and regulates the concentration of glycine at excitatory synapses containing NMDA receptors (Smith et al., 1992). Therefore, GlyT1 mediates both the clearance of glycine from the synaptic cleft of inhibitory synapses and participates in the regulation of glycine concentration at excitatory synapses (Eulenburg et al, 2005).

GlyT2 is highly expressed in CNS regions rich in glycinergic synapses such as spinal cord and brain stem, and has lower expression in the brain (Liu et al., 1993). Thus, this isoform is responsible for the termination of glycine neurotransmission, enhancing its efficacy by providing cytosolic glycine for vesicular release, with the co-transport of three Na^+ and one Cl^- (Roux and Suplisson, 2000). Glycine can then be recycled and repackaged into vesicles (Gomeza et al., 2003; Rousseau et al., 2008). Expression of VIAAT and GlyT2 alone have been shown to be sufficient for adequate glycine accumulation and release in model systems, more so than co-expression of VIAAT and GlyT1 (Aubrey et al., 2007). Moreover, immunostaining studies have shown that GlyT2 expression overlaps extensively with GlyR, the postsynaptic component of the glycinergic synapse, proving it to be a reliable marker for glycinergic neurons (Luque et al., 1994; Jursky and Nelson, 1995; Zafra et al., 1995; Poyatos et al., 1997; Spike et al., 1997; Betz et al., 2006).

1.3. GLYCINERGIC TRANSMISSION AS A POTENTIAL THERAPEUTIC TARGET FOR EPILEPSY

Epilepsy is one of the most prevalent neurologic disorders worldwide (Pitkänen and Sutula, 2002) affecting 0.4-1% of the world's population. According to Worldwide Health Organization (WHO), epilepsy accounts for 1% of global burden of disease, comparable to breast cancer in woman and lung cancer in man (Engel et al., 2008).

According to the International League Against Epilepsy (ILAE), epilepsy results from an electrical disturbance in the brain which is characterized by recurrent seizures (Fisher et al., 2005). The term 'epilepsy' includes several genetic and acquired neurological disorders which share the periodic and unpredictable occurrence of seizures, that is, episodes of excessive neuronal activity.

Epileptic activity may be conducted from all cortical areas to the hippocampus, a region of high vulnerability to seizures (Ben-Ari, 1985; Bengzon et al., 2002).

Although numerous antiepilectic drugs (AEDs) are currently available, 30% of patients still continue to experience seizures, and a subset suffer progression of the disease, with increasing seizure frequency and cognitive decline.

Furthermore, traditional AEDs are based on crucial CNS functions, such as GABAergic transmission, calcium channels and sodium channels and have strong side

effects. Many of these adverse effects have been reported in the immature brain, both in animals (Bittigau et al., 2002) and in children (Herranz et al., 1988; Calandre et al., 1990). There is still a growing concern about the current epilepsy treatment, and a need for more effective epilepsy therapy, especially in infants and children. It is therefore imperative to find novel and specific therapies aimed at preventing the onset and/or progression of this disorder.

Since GABAergic inhibitory transmission is impaired in this pathology, glycine-mediated transmission, through GlyR activation, could constitute an additional or alternate inhibitory mechanism to maintain the balance between neuronal inhibition and excitation.

Over the years, several reports have stated the potential anticonvulsant effect of GlyR activation and have described alterations in glycinergic transmission related elements on Temporal Lobe Epilepsy (TLE) patients.

Work *in vivo* demonstrated that exogenous application of glycine can depress seizure activity in an animal model of epilepsy (Cherubini et al. 1981). Other studies revealed that glycine can potentiate the action of some anticonvulsant (Seiler and Sarhan, 1984; Toth and Lajtha, 1984; Norris et al., 1994). Taurine, another GlyR agonist, has also been shown to depress epileptiform activity induced by Mg^{2+} removal in combined rat hippocampal-entorhinal cortex slices (Kirchner et al., 2003). Furthermore, GlyR subunits expression is altered in the brain of TLE patients corroborating an involvement of glycinergic transmission in this pathology (Eichler et al., 2008; Eichler et al., 2009).

In view of the above, if one wants to consider glycinergic transmission as a potential therapeutic target for epilepsy, a deep knowledge about the glycinergic synapse is imperious.

2. AIM

The objective of the present work is to investigate which are the contributors for the acquisition of a glycinergic phenotype.

In order to accomplish this general aim, the following specific objectives were pursued:

1. To promote the glycinergic phenotype by blockade of components of the GABAergic system, such as the $GABA_A$ receptor, the enzyme GAD65 and the GABA transporter GAT-1, in primary neuronal cultures.
    a. To evaluate, for each insult, the protein expression of glycinergic transmission markers, namely GlyT2 in the presynaptic boutons and GlyR in the postsynaptic density.
    b. To assess, for each insult, the transcript expression of GlyT2 and GlyR subunits.
2. To compare the ratio of glycinergic *vs* GABAergic terminals in synaptosomes prepared from control and insulted hippocampal slices.
3. Evaluate, in hippocampal synaptosomes, a potential physical interaction between GlyT2 and VIAAT.

3. Materials and Methods

3.1. Reagents

The reagents used in this work, unless stated otherwise, were obtained from Invitrogen (Carlsbed, CA, USA) and Sigma-Aldrich (St. Louis, MO, USA).

SKF89976a (1-(4,4-Diphenyl-3-butenyl)-3-piperidinecarboxylic acid hydrochloride) and Gabazine (SR-95531) (2-(3-Carboxypropyl)-3-amino-6-(4-methoxyphenyl) pyridazinium bromide) were obtained from Abcam (Cambridge, MA, USA). 3-Mercaptopropionic acid (3-MPA) was obtained from Sigma-Aldrich (St.Louis, MO, USA).

3.2. Animals

The described experiments used Sprague-Dawley rats obtained from Harlan Interfauna Iberia (Spain). For the neuronal primary cultures, cortical neurons were obtained from E17-E18 (embryonic day) embryos. For the preparation of the synaptosomal fraction, hippocampus was isolated from young-adult rats (3-5 weeks old).

The animals were handled according to the European Community guidelines (2010/63/EU) and Portuguese law concerning animal care. Animals were deeply anesthetized with halothane (2-Bromo-2-Chloro-1,1,1-Trifluoroethane) in an anesthesia chamber before being sacrificed by decapitation.

3.3. Biological Samples

3.3.1. Neuronal Cortical Cultures

Pregnant rats were anaesthetized and decapitated as previously described. Uterus containing the embryos was removed from the rat abdomen, as explained in Figure 8, and placed in cold Ca^{2+} and Mg^{2+} free Hank's Balanced Salt Solution supplemented with 0.37% glucose (HBSS-glucose). Under a laminar flow hood, embryos were rapidly removed from the uterus. After brain isolation from the embryo, meninges were gently removed from the hemispheres and the cortex was exposed. All cortices were collected in fresh HBSS-glucose and transferred to a 15 ml Falcon tube in a final volume of 2,7 ml. Trypsinization was carried out (0,350 ml of 2,5 % trypsin) at 37 ºC for 15 min in a water bath, in order to digest the proteins that promote adhesion between cells in the

tissue. Afterwards, trypsin solution was gently removed, 20 ml of HBSS supplemented with glucose and 30% Fetal Bovine Serum (FBS) was added and let stand for 5 min at room temperature (RT: 22-24ºC) to quench trypsin activity. The cortices were washed 3 more times in HBSS-glucose, and centrifuged each time at 200g for 4 minutes, allowing trypsin to diffuse from the tissue.

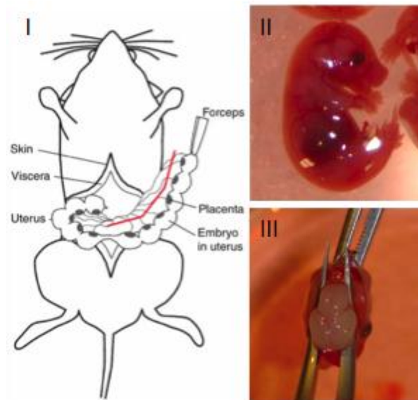


Figure 8. **Brain dissection from E17-18 rat embryos.** (**I**) The sacrificed pregnant rat is placed on its back to expose the abdomen and the abdominal cavity is open. The uterus containing the embryos is removed by lifting while cutting the mesometrium (red line). (**II**) Image of E17-E18 embryo after removal from the uterus. (**III**) The brain is dissected out by pressing the forceps gently against the skull, moving the bone down- and sidewise. Adapted from Fath, 2008.

The cortices were resuspended in Neurobasal medium (Neurobasal-B27: Neurobasal supplemented with 0.5 mM glutamine, 2% B27, 25 U/mL penicillin/streptomycin) and 25mM glutamic acid in a final volume of 40 mL. Dissociation was achieved by repeatedly pipetting the suspension up and down.

Cell suspension was filtered using a nylon filter (Cell Strainer 70µM, BD Falcon^{TM}) to remove cell clumps and tissue fragments. Cell density was determined by counting cells in a 0.4% trypan blue solution using a hemacytometer. Cells were plated according to the application to be performed: at $4.5x10^4$ cells/cm^2 in 12 and 24-well plates with glass coverslips (Marienfeld, Germany) coated with poly-D-lysine (PDL, 50 µg/ml) for immunofluorescence and electrophysiology assays; at $7x10^4$ cells/cm^2 in 6-well PDL-coated plates (TPP, Switzerland) for protein quantification and at $7x10^4$ cells/cm^2 in 100mm PDL-coated plates (Enzifarma, Portugal) for RNA extraction.

Cortical primary cultures were maintained for 8 days in an incubator with a humidified 37ºC and 5% CO_2 atmosphere with no media exchange.

3.3.2. Hippocampal Synaptosomes

Rats (3-5 weeks old) were anaesthetized and decapitated as previously described, the brain was rapidly removed and the hippocampus was dissected out. Whole hippocampi, or hippocampus derived slices (isolated from 8-10 animals) were collected in 5ml of a chilled 0.32M sucrose solution at pH 7.4 (1mM EDTA, 10mM HEPES and 1 mg/ml bovine serum albumin (BSA)). The tissue was homogenized with a Teflon piston and the volume was completed up to 15ml with ice-cold sucrose solution. After a first centrifugation at 1000g for 10 minutes, the supernatant was collected and centrifuged at 14000g for 12 minutes. The pellet was resuspended in 3ml of a Percoll solution, which contained 45% (v/v) Percoll in Krebs-HEPES-Ringer solution (KHR I: 140mM NaCl, 1mM EDTA, 10mM HEPES, 5mM KCl and 5mM glucose, pH 7.4). The suspension was centrifuged at 11000g for 2 minutes and the top layer, which corresponded to the synaptosomal fraction, was removed, washed two times with 1ml of KHR I solution and centrifuged each time at 11000g for 2 minutes.

The washed synaptosomal fraction was resuspended in KHR II solution at pH 7.4 (125mM NaCl, 10mM HEPES, 3mM KCl, 10mM glucose, 1.2mM $MgSO_4$, 1mM NaH_2PO_4, 1.5mM $CaCl_2$) and kept at 4ºC until use. All the centrifugations described in the protocol were performed at 4ºC.

The protein concentration in the synaptosomal fraction was assayed according to the Bradford method (Bradford, 1976), using BSA as standard.

3.4. Pharmacological Treatments

3.4.1. Neuronal Cortical Cultures

At 3 days *in vitro* (DIV), neurons were treated with 2µM of cytosine arabinoside (Ara C), by adding it to the Neurobasal-B27 maintenance medium. Ara C, an antimitotic drug, was used to reduce the presence of glia and other contaminating cells in neuronal cultures (Negishi et al., 2003; Rhodes et al., 2003).

At 7DIV, when the neurons have already specified the axon and primary dendrites (Dotti et al., 1988; Ziv and Smith, 1996), pharmacological treatments were performed. For this procedure, half of the medium was replaced with fresh Neurobasal-B27 medium with or without (control) the drugs in study. Three different compounds,

targeting components of the GABAergic synapse, were used in this work: SKF89976a, a selective GAT-1 inhibitor, 3-MPA, a glutamate decarboxylase inhibitor, and Gabazine, a selective and competitive $GABA_A$ receptor antagonist.

SKF89976a (50mM), 3-MPA (4,7mM) and Gabazine (5mM) were prepared as a stock solution in water. Stock solutions were aliquoted and stored at -20ºC until use. Dilutions of these stock solutions to the final concentration were prepared in Neurobasal-B27 at the day of the experiment. Different concentrations and exposure times were tested (Table I).

Table I. Drugs used in this work.

Drug	Concentrations tested (μM)	Time (h)
SKF89976a	20, 50, 100	2, 4, 24
Gabazine	10, 50, 100	2, 4, 24
3-MPA	10, 20, 100	6, 24

3.4.2. Hippocampal Synaptosomes

The synaptosomal suspension (300μl at 0.5μg of protein/μl in KHR II), was incubated at 37ºC in the presence of 20μM SKF89976a for 10, 20 and 30 minutes. Control experiments, for each exposure time, comprised the incubation at 37ºC, with no added drug. After the exposure, synaptosomes were washed with KHR II, in order to remove the drug, and kept on ice until further use.

3.5. Immunofluorescence Assay

3.5.1. Neuronal Cortical Cultures

At 8DIV, the cortical neurons plated in glass coverslips were washed with Phosphate Buffered Saline (PBS: 137mM NaCl, 2.7mM KCl, 8mM $Na_2HPO_4.2H_2O$ and 1.5mM KH_2PO_4) and fixed with 4% paraformaldehyde (PFA) in PBS at RT, for 15 minutes. The neurons were then washed two times with PBS. A glycine (0.1M in PBS) washing step, 10 minutes at RT, was used to remove any aldehyde traces. Cells were

permeabilized with 0.1% Triton X-100 (in PBS) for 5 minutes at RT and blocked with 10% FBS in PBS containing 0.05% Tween-20 (PBS-T) for 1 hour, also at RT.

Cells were then incubated with the primary antibodies (listed on Table II) overnight at 4ºC. Primary antibodies were rinsed off with PBS-T and the secondary antibodies coupled to fluorophores (listed on Table III) were applied to the cells for 1 hour at RT. All the antibodies were diluted to the working concentration with 10% FBS in PBS-T. The secondary antibodies were also rinsed off with PBS-T and nuclei were stained with Hoechst (2'-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1H-benzimidazole trihydrochloride hydrate, bisBenzimide), for 5 minutes at RT. Finally, the coverslips were mounted in Mowiol. Mowiol is a non-absorbing compound, has no autofluorescence, or light scattering, and is considered adequate for fluorescence microscopy.

In all immunofluorescence assays, specificity and absence of antibody cross-reaction were confirmed by omission of the primary antibodies.

Images were acquired with an inverted widefield fluorescence microscope (Zeiss Axiovert 200, Germany), with a 40x objective.

3.5.2. Hippocampal Synaptosomes

3.5.2.1. Immunofluorescence Assay

Immunofluorescence assays of synaptosomes were also performed. After incubating the synaptosomal suspension with the inhibitor SKF89976a (as described in section 3.4.2), 40µl of the suspension was placed on the center of a PDL-coated (50 µg/ml) glass coverslip and maintained for 3 hours at 37ºC and 5% CO_2. The plated synaptosomes were fixed with 4% PFA and the subsequent immunofluorescence protocol followed the one used for the neuronal cortical cultures (section 3.5.1), except for the incubation with Hoechst, which was not carried out.

Images were also acquired in an inverted widefield fluorescence microscope (Zeiss Axiovert 200, Germany) with a 40x objective.

3.5.2.2. Quantification of the immunofluorescence images

Five images per coverslip and two coverslips per experiment, in a total of 3 independent experiments, were quantified using an ImageJ mask. This mask was

performed in immunofluorescence images obtained from double staining of GAD and GlyT2. Binary masks were created for both red and green channels using a cut-off intensity threshold value for each staining, defined as the minimum intensity corresponding to specific staining above background values. Labeling was identified in each binary mask image using the Particle Analyzer function of ImageJ. Red and green clusters were considered to co-localize if they had at least one pixel in common.

3.6. WESTERN BLOT ASSAY

3.6.1. Cell Lysis

Western blot analysis was performed in order to address protein expression changes in the components of the glycinergic synapse, specifically GlyT2 and GlyR.

After exposure to the different drugs (section 3.4.1), and during different time periods, cultured neurons were washed twice with PBS. Cell lysis was performed in 70µl of ice-cold RIPA buffer (RIPA: 50mM Tris pH 8, 150mM NaCl, , 1mM EDTA (Ethylenediamine Tetraacetic Acid), 1% Nonidet P40 substitute (Nonyl phenoxylpolyethoxylethanol, Fluka), 0.1% SDS (Sodium Dodecyl Sulfate), 10% glycerol and protease inhibitors (EDTA-free Protease Inhibitor Cocktail Tablets, Roche). After 15 minutes on ice, cells were detached from the plate with a cell scraper. Subsequently cells were transferred to an eppendorf tube, sonicated and incubated at 4ºC with slow agitation for 15 minutes. The suspension was centrifuged at 11000g for 10 minutes and 4ºC. The supernatant was collected and frozen at -20ºC until further use.

The protein concentration was assayed according to the Lowry method, following the instructions described in the manual (Bio-Rad DC Protein Assay, Bio-Rad). The absorbance was read at 750 nm, using BSA as standard.

3.6.2. SDS-PAGE

After protein quantification, loading buffer (350mM Tris pH 6.8, 30% glycerol, 10% SDS, 600mM DTT (1,4-dithiothreitol) and 0.012% bromophenol blue) was added to the samples. Samples (50µg of total protein/lane) and the molecular weight marker (Precision Plus Protein Standards, Bio-Rad) were then run on a standard 12% sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to

PVDF (Polyvinylidene Fluoride, Millipore) membranes. Membranes were blocked with 5% non-fat dry milk dissolved in 0.1% TBS-T (Tris Buffered Saline) (0.2mM Tris, 137mM NaCl with 0.1% Tween-20) for 1 hour, washed with TBS-T and incubated with the primary antibody (Table II) overnight at 4ºC. All primary antibodies were diluted in 3% BSA in TBS-T with 0.02% $NaNO_3$.

Membranes were further incubated with the appropriate secondary antibody coupled to horseradish peroxidase (Table III) for 1 hour at RT. Chemoluminescent detection was performed with ECL-Plus Western Blot Detection Reagent (GE Healthcare, UK) using X-Ray films (Fujifilm). Quantifications were attained by densitometric scanning of the films, performed with the Image J software. β-Actin density was used as the loading control.

3.7. IMMUNOPRECIPITATION

The immunoprecipitation experiments were performed in synaptosomes from hippocampus, obtained as described in section 3.3.2. Synaptosomal suspension (0.5 µg of protein per µl) was centrifuged at 11000g for 2 minutes, the supernatant was discarded and the remaining synaptosomes were resuspended in 500 µl of lysis buffer (RIPA buffer without SDS). After 15 minutes incubation at 4ºC with slow agitation, the suspension was centrifuged at 11000g for 10 minutes and 4ºC and the supernatant was collected. The supernatant was incubated overnight at 4ºC with 15 µl of the appropriate primary antibody, either against GlyT2 or VIAAT (see Table II).

Protein G beads (Protein G Sepharose, Fast flow), previously washed three times with lysis buffer, were added (30 µl) to the protein suspension and the mixture was incubated overnight at 4ºC. After a final centrifugation for 2 minutes at 11000g, the supernatant was discarded, the immunoprecipitates were collected and washed five times with 1 ml of lysis buffer. Fifteen minutes at 100ºC, in the presence of loading buffer, were used to elute the immunoprecipitated proteins from the beads. The supernatant was then collected and Western Blot was performed.

3.8. ANTIBODIES

The following tables describe the primary and secondary antibodies used in this work for immunofluorescence assays (IF), western blot (WB) and immunoprecipitation (IP).

Table II. Primary antibodies used in this work.

Primary Antibody	Host	Supplier	Dilution	Technique
GAD-6	Mouse monoclonal supernatant	DSHB	1:20	IF
Gephyrin	Rabbit polyclonal antibody	Abcam	1:250	IF
GFAP	Rabbit polyclonal antibody	Sigma	1:250	IF
GlyT2	Rabbit anti-rat antiserum	Acris Antibodies	1:20	IF
MAP2	Mouse monoclonal antibody	Chemicon	1:200	IF
VGAT	Rabbit polyclonal antiserum	Synaptic Systems	1:200	IF
GlyR	Mouse monoclonal antibody	Synaptic Systems	1:250	IF, WB
GAD-65	Mouse monoclonal antibody	Abcam	1:500	WB
GlyT2	Rabbit polyclonal antibody	Gift from Dr. Manuel Miranda, USA	1:1000	WB
VGAT	Rabbit polyclonal antibody	Gift from Dr. Bruno Gasnier, France	1:800	WB
β-Actin	Rabbit polyclonal antibody	Abcam	1:5000	WB
GlyT2 (L-20)	Goat polyclonal IgG	Santa Cruz	-	IP
VGAT (D-18)	Goat polyclonal antibody	Santa Cruz	-	IP

Abbreviations: GAD – Glutamate decarboxylase; GFAP – Glial fibrillary acidic protein; GlyT2 – Glycine transporter 2; MAP2 – Microtubule-associated protein 2; VGAT – Vesicular inhibitory amino acid transporter; GlyR – Glycine receptor.

Table III. Secondary antibodies used in this work.

Secondary Antibody	Supplier	Dilution	Technique
AlexaFluor 488 Goat anti-mouse IgG (H+L)	Invitrogen	1:500	IF
AlexaFluor 568 Goat anti-rabbit IgG (H+L)	Invitrogen	1:500	IF
Goat anti-Rabbit IgG-HRP	Santa Cruz	1:10000	WB
Goat anti-Mouse IgG-HRP	Santa Cruz	1:10000	WB
Goat anti-Rabbit-HRP Light Chain Specific	Jackson	1:20000	WB
Goat anti-Mouse-HRP Light Chain Specific	Jackson	1:20000	WB

3.9. QUANTITATIVE REAL-TIME PCR (QPCR)

RNA was isolated from cortical cultured neurons (obtained as described in section 3.3.1), following the instructions of the isolation kit (RNAspin Mini RNA Isolation Kit, GE Healthcare) and the quantification of total RNA was performed using Nanodrop (ND-1000 Spectrophotometer). *In vitro* transcription reaction used 2µg of total RNA and was carried out according to the manufacturer's recommendations (SuperScript First Strand, Invitrogen), with the SuperScript II Reverse Transcriptase (EC 2.7.7.49, Invitrogen). For each RNA sample, a reverse transcription reaction was carried out in the absence of reverse transcriptase, in order to ensure that product amplification did not arise from genomic DNA.

cDNA was then amplified in the presence of SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and 0.2 µM of each specific gene primer (see Table IV). The amplification was performed in a Rotor-Gene 6000 real-time rotary analyzer thermocycler (Corbett Life Science, Hilden, Germany), with the following program: 94ºC for 2 minutes, 50 cycles at 94ºC for 30 seconds, 60ºC for 90 seconds and 72ºC for 60 seconds, followed by a melting curve to assess the specificity of the reactions. The threshold cycle (Ct) and the melting curves (see Appendix) were acquired with Rotor-Gene 6000 Software 1.7 (Corbett Life Science). In order to determine the PCR efficiency (E) for each gene, which is needed for the relative quantification by comparative Pfaffl method (explained in Appendix), a qPCR with cDNA samples from 5-fold sequencial dilutions of a CTL cDNA was performed for each set of primers. The relative qPCR establishes the cDNA expression level by normalization with an internal control gene. β-actin was the internal control gene used as a reference gene for normalization. For each gene, replica reactions were performed. Two types of negative controls, 'no reverse transcription' and 'no template', were run with samples.

Table IV. Description of the primers used for qPCR.

Gene	Primer sequence (5'-3')	Fragment Size (bp)
β-Actin	Forward: AGCCATGTACGTAGCCATCC	228
	Reverse: CTCTCAGCTGTGGTGGTGAA	
GlyT2	Forward: TCCGTCCTCATAGCCATCTA	295
	Reverse: TCACTCCCGCTGACAAATG	
α1	Forward: ACTCTGCGATTCTACCTTTGG	300
	Reverse: ATATTCATTGTAGGCGAGACGG	
α2	Forward: CAGAGTTCAGGTTCCAGGG	330
	Reverse: TCCACAAACTTCTTCTTGATAG	
α3	Forward: GTGAGACACTTTCGGACACTAC	353
	Reverse: GATGGGTCGAGGTCTAATGAATC	
β	Forward: CTGTTCATATCAAGCACTTTGC	223
	Reverse: GGGATGACAGGCTTGGCAG	

3.10. Patch Clamp Recordings

The patch-clamp technique is characterized by several configurations. In this work, it was applied the whole-cell configuration which was used to study the ensemble response of all ion channels within the cell's membrane and to assess about the viability and health of the cultured cortical cells.

Whole-cell patch-clamp recordings were obtained from cortical neurons with 8DIV, which were visualized with an upright microscope (Zeiss Axioskop 2FS) equipped with infrared video microscopy and differential interference contrast optics. The coverslips where the cells were previously plated (as described in section 3.3.1) were placed in the recording chamber, fixed with a grid, continuously superfused by a gravitational superfusion system, with artificial cerebrospinal fluid (aCSF: 124 mM NaCl, 3 mM KCl, 1.25 mM NaH2PO4, 26 mM $NaHCO_3$, 1 mM $MgSO_4$, 2 mM $CaCl_2$ and 10mM glucose, pH 7.4) gassed with 95% O_2 and 5% CO_2. Recordings were performed at RT

The patch pipettes, used in the setup, were made from borosilicate glass capillaries (1.5 mm and 0.86 mm, outer and inner diameters, respectively, (Harvard Apparatus, Germany) in two stages on a pipette puller (PC-10 Puller, Narishige Group). They were characterized by a resistance of 4–7 MΩ (it is usually a difficult task to break through the whole-cell configuration when the microelectrodes have too high resistance

values), when filled with an internal solution, which depended on the type of currents that were recorded. For this work, it was recorded miniature excitatory postsynaptic currents (mEPSCs). For mEPSCs, the internal solution is composed by: 125mM K-gluconate, 11mM KCl, 0.1mM $CaCl_2$, 2mM $MgCl_2$; 1mM EGTA, 10mM HEPES, 2 mM MgATp, 0.3mM NaGTP and 10mM phosphocreatine, pH 7.3, adjusted with 1 M NaOH.

The recording currents were performed in the voltage-clamp mode (Vh = -70mV) set up by an Axopatch 200B (Axon Instruments) amplifier. The offset potentials were nulled before the giga-seal formation. After establishing whole-cell configuration, it was possible to determine the membrane potential of the neurons in the current-clamp mode, before starting the recording miniature currents. Firing patterns, obtained in response to current injection through the recording electrode, were also recorded.

The described technique was performed by Raquel Dias and the analysis of the firing patterns and mEPSCs was done with the Clampfit 10.2 software.

3.11. Data analysis (Statistics)

The values presented are mean±Standard Error of the Mean (S.E.M.) of n independent experiments. Statistical significance was determined with GraphPad software (Prism, 5.00 for Windows). To evaluate multiple comparisons, two-way analysis of variance (ANOVA) followed by Bonferroni correction was used. When only two means were compared, the Student's t-test was used. Values of $p<0.05$ were considered to represent statistically significant differences (*$p<0.05$;**$p<0.01$;***$p<0.001$).

4. RESULTS

4.1. PRIMARY NEURONAL CORTICAL CULTURES

4.1.1. Evaluation of cellular health and functionality

Primary neuronal cultures have become a popular research tool since they allow easy access to individual neurons for electrophysiological recording and stimulation, pharmacological manipulations and also microscopy analysis.

Electrophysiological recordings and immunofluorescence assays were used in this work in order to ensure that the cortical neurons were healthy and functional.

4.1.1.1. Electrophysiological recordings

Whole cell current clamp recordings and electrically-evoked excitatory postsynaptic currents were performed in 8DIV, showing that the neurons were responsive and functional (Figure 9-B and C). In terms of morphology, all cultured neurons depicted normal features (Figure 9-A).

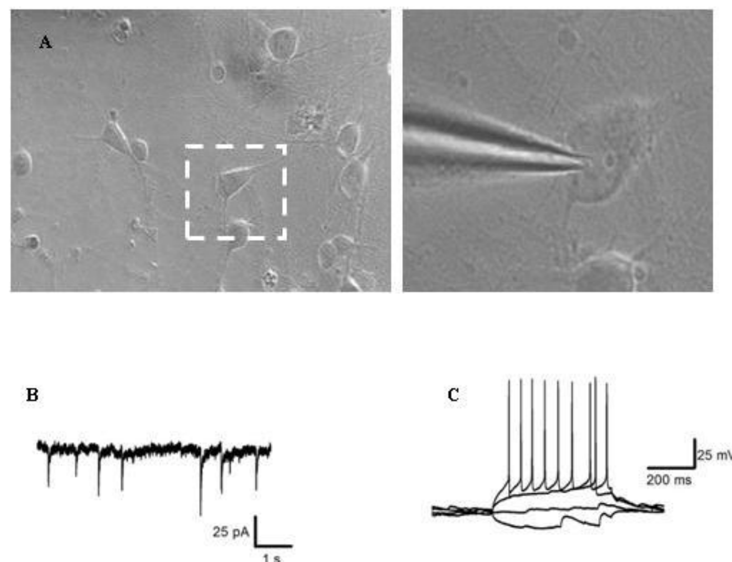


Figure 9. Patch-clamp recordings. Cells were visually identified by their characteristic morphological features (A) and functionally, by monitoring miniature excitatory post-synaptic currents (mEPSCs) (B) and by firing patterns obtained in response to current injection through the recording electrode (C), as described in Materials and Methods.

4.1.1.2. Immunofluorescence assays

Two types of double immunofluorescence assays were performed. For the assessment of neurons's normal morphology and development, and also to verify the presence of astrocytes, a double immunolabelling with Microtubule-associated protein 2 (MAP2) and Glial fibrillary acidic protein (GFAP) was performed. MAP2 is a neuronal marker, while GFAP is a marker for mature astrocytes. As can be observed in Figure 10, the majority of the cells is MAP2^{+} and depicts the characteristic neuronal feature: a long process, the axon, and many small dendritic processes. On the other hand, no GFAP^{+} cells were detected in the cultures, which confirm the culture's high purity.

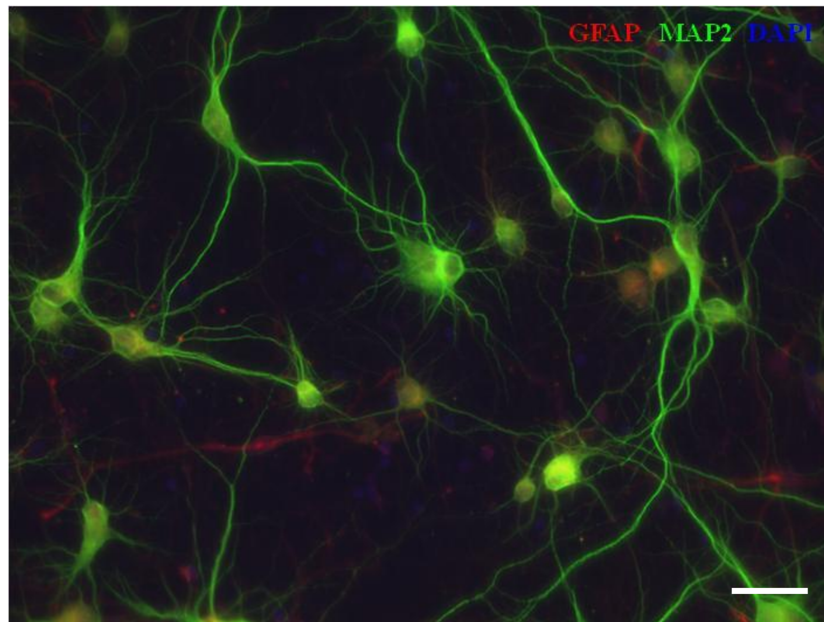


Figure 10. Double staining of MAP2 and GFAP in primary rat cortical neurons. Immunofluorescence staining of primary neuronal cultures at 8 DIV: neurons were stained with mouse anti-MAP2 antibody (green) and astrocytes were stained with rabbit anti-GFAP antibody (red). Nuclei were stained with DAPI (blue). Image was acquired with a 40x objective in a Axiovert 200 microscope from Zeiss. Scale bars 15µm.

A double immunostaining with VIAAT and GAD was also performed. As depicted in Figure 11, VIAAT and GAD show a punctuate staining that delineates all neuronal processes. VIAAT co-localizes with most GAD^{+} neurons (in yellow), which indicates that the majority of inhibitory neurons are GABAergic. A few VIAAT^{+}GAD^{-} neurons (in green) can also be found, indicating the presence of glycinergic presynaptic boutons. As happens in brain, cultures neurons also present a high ratio between GABAergic and glycinergic terminals.

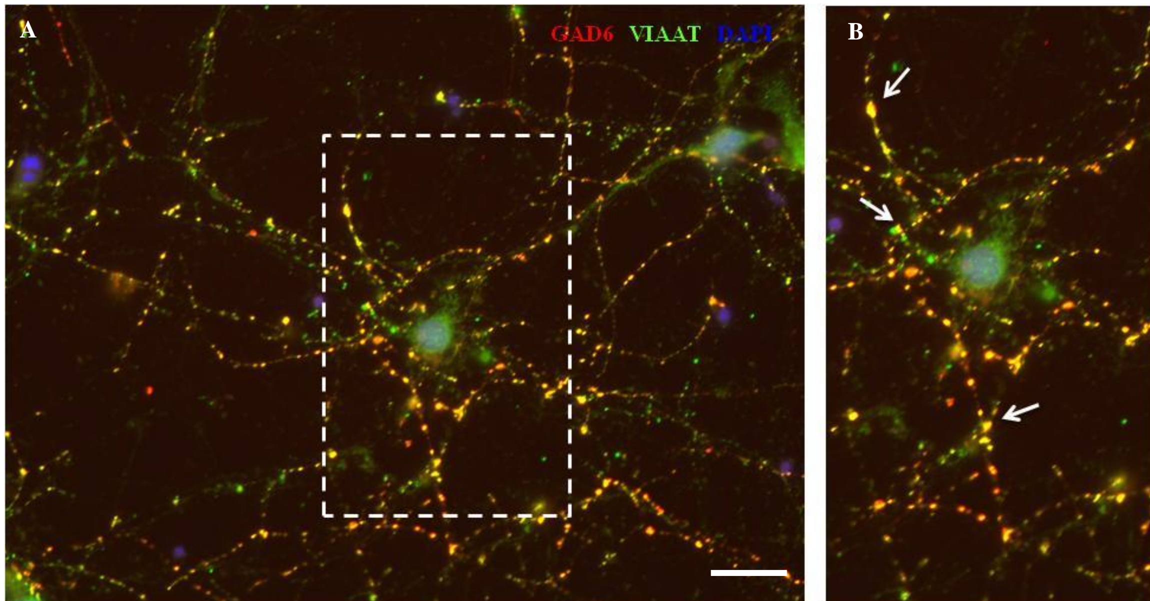


Figure 11. Double staining of GAD6 and VIAAT in primary rat cortical neurons. (A) Immunofluorescence staining of primary neuronal cultures at 8 DIV: neurons were stained with mouse anti-GAD6 antibody (red) with rabbit anti-VIAAT antibody (green). Nuclei were stained with DAPI (blue). (B) Amplification of the area indicated in the dashed box. Images were acquired with a 40x objective in a Axiovert 200 microscope from Zeiss. Scale bars 15µm.

4.1.2. Expression pattern of glycinergic transmission markers

To assess changes in glycinergic transmission components, namely GlyT2 and GlyR, a pharmacological treatment was performed as described in section 3.4.1. Cell homogenates were obtained, for each treatment and timepoint, and western blots were carried out. In the following figures cells that were not treated were named control (CTL). For comparison and positive control, spinal cord homogenates (from animals with 3 weeks old) were also analyzed (data not shown).

The identification of both proteins was made through interaction with specific antibodies, listed in Table II. The monoclonal antibody specific for GlyR (mAb4a) was previously described (Pfeiffer et al., 1984), while the antibody against GlyT2 was a gift from University of Texas.

Cultures were first treated with gabazine, an antagonist of $GABA_AR$. The immunoreactive bands of GlyT2 and GlyR, as well as the densitometry analysis are shown in Figure 12. A significant increase in GlyR protein levels at 2h post treatment with 50µM (p=0.0414) and 100µM (p=0.0002) was observed. Moreover, and even though no significance (p=0.0702) was reached, a tendency for an increase in GlyT2

expression was also evident (Figure 12B), at 2h and 4h, for all gabazine concentrations assessed.

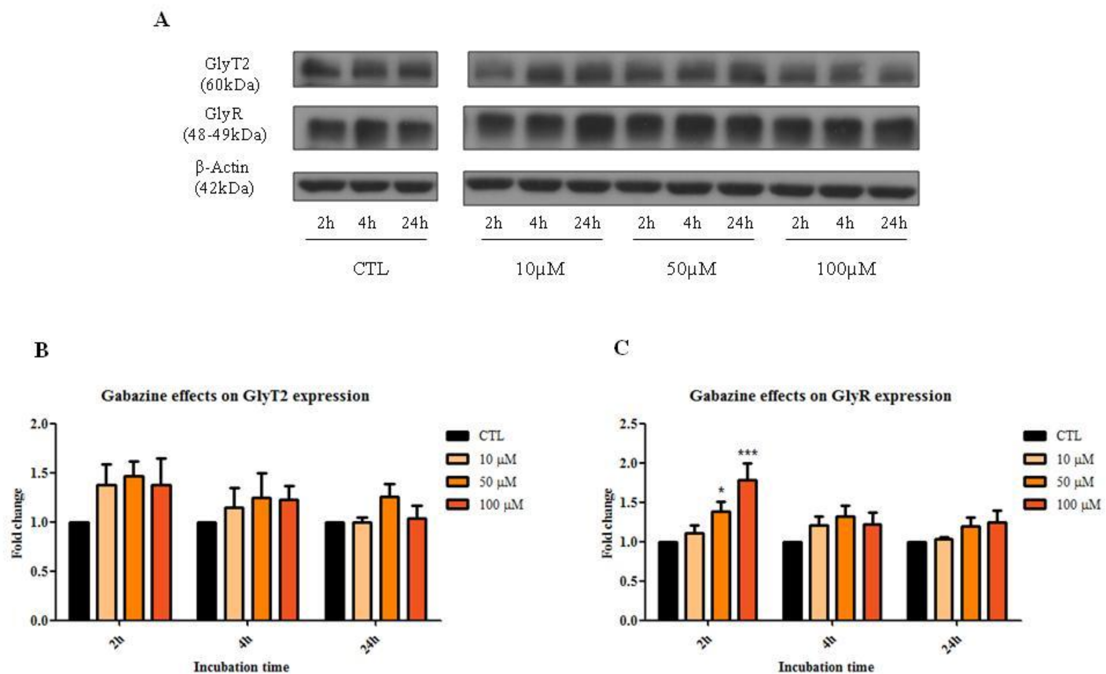


Figure 12. Expression of inhibitory transmission related markers, when cells are treated with different concentrations of gabazine, at different times. (A) Representative western blots of GlyT2 (60kDa), GlyR (48-49kDa) and β-actin (42kDa), for each condition. β-actin was used as a loading control. (B) and (C) Densitometry analysis of western blots for GlyT2 and GlyR, respectively. All values are mean±SEM, normalized to β-actin. *p<0.05, ***p<0.001 comparing with CTL. 5<n<7; Two-way ANOVA followed by Bonferroni's Multiple Comparison test.

SKF8996a, a GAT-1 antagonist, was also used to induce impairment in GABA-mediated transmission.

The representative immunoblots and expression pattern of GlyT2 and GlyR upon SKF89976a treatment, era depicted in Figure 13. In this case, GlyT2 and GlyR expression did not change significantly in comparison to CTL cultures, at any concentration or time tested. However, it is possible to observe a minor increasing trend in GlyR protein levels at all SKF89976a concentrations and incubation times evaluated. GlyT2 protein expression also depicts a minor increase for all SKF89976a concentrations, but only after 2h and 4h treatment.

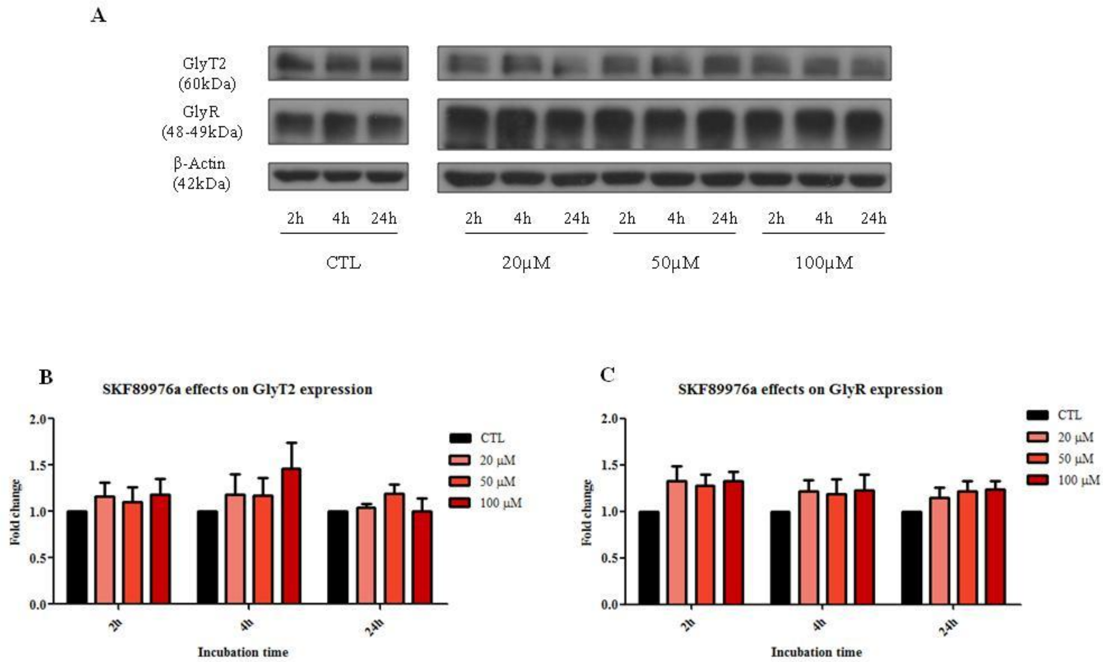


Figure 13. Expression of inhibitory transmission related markers, when cells are treated with different concentrations of SKF89976a, at different times. (A) Representative western blots of GlyT2 (60kDa), GlyR (48-49kDa) and β-actin (42kDa), for each condition. β-actin was used as a loading control. (B) and (C) Densitometry analysis of western blots for GlyT2 and GlyR, respectively. All values are mean±SEM, normalized to β-actin. $5<n<7$; Two-way ANOVA followed by Bonferroni's Multiple Comparison test in comparison to CTL.

The induction of GABAergic transmission impairment in cultured neurons was also tested with 3-MPA, the major inhibitor of GAD65 enzyme. Unfortunately, 3-MPA addition to the medium required a pH adjustment. Since the correct pH was very difficult to achieve, the neurons demonstrated to be seriously damaged soon after 3-MPA addition. Consequently, this line of investigation was not pursued.

4.1.3. Transcript expression pattern of glycinergic transmission markers

In order to assess the transcript expression of GlyT2 and the different GlyR subunits (α1, α2, α3 and β), a qPCR, as described in section 3.9, was performed. No PCR products were detected using cDNA synthesized in the absence of reverse transcriptase which ensured that amplification did not arise from contaminating genomic DNA. Moreover, other negative controls showed no signal amplification, which implies absence of genomic DNA, external contamination or other factors that could originate a non-specific increase in the fluorescence signal (data not shown).

Figure 14 represents the expression of GlyT2 mRNA after 2h and 24h treatment with 100μM SKF89967a and 100μM gabazine. GlyT2 transcript level shows a significant increase after 24h treatment (p=0.0059), when compared to control. Gabazine did not alter transcript levels.

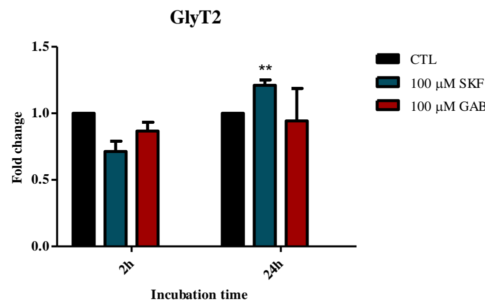


Figure 14. Transcript expression profile of GlyT2. Composition analysis of GlyT2 transcript in CTL and treated cells with SKF89976a and gabazine, by relative qPCR. All values correspond to mean±SEM (n=3). **p<0.01, when compared to CTL; Two-tailed Unpaired t-test.

The expression pattern of GlyR subunits mRNA is represented in Figure 15. Most GlyR subunits mRNA levels exhibited no significant increase, when compared with the control. However, GlyR subunit α1 (GlyRα1) showed a significant increase upon treatment with gabazine for 2h and 24h (p=0.043 and p=0.00051, respectively). GlyRα1 has also shown a tendency for increase when incubated with SKF89976a but no significance was attained.

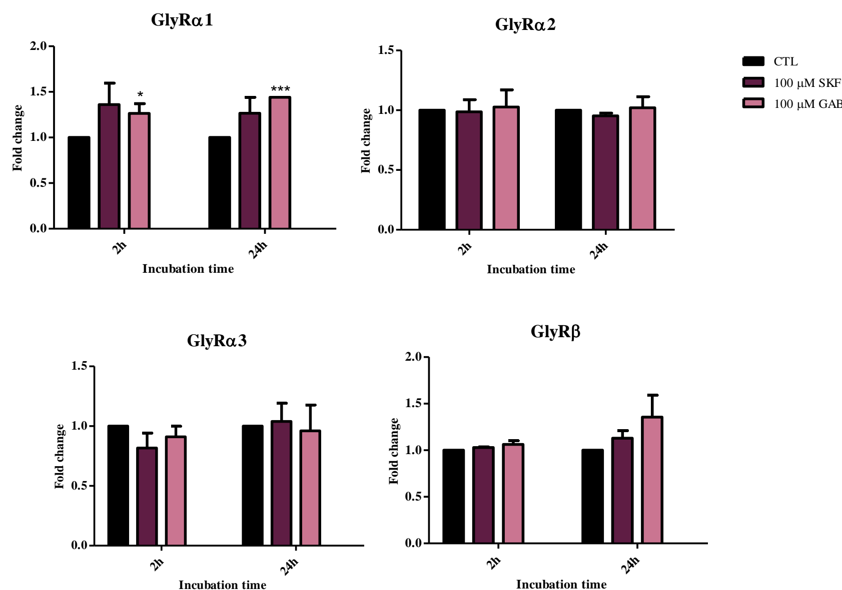


Figure 15. Transcript expression profile of GlyR subunits α1, α2, α3 and β. Composition analysis of GlyR subunit transcript in CTL and treated cells with SKF89976a and gabazine, by relative qPCR. All values correspond to mean±SEM (n=3). *p<0.05, ***p<0.001 when compared to CTL; Two-tailed Unpaired t-test.

4.2. HIPPOCAMPAL SYNAPTOSOMES

4.2.1. Characterization of synaptosomes prepared from rat hippocampal slices by immunofluorescence

Synaptosomal fractions were first obtained from hippocampal slices, either CTL or treated, according to the protocol described in section 3.5.2.

In order to verify the purity of these fractions, an immunofluorescence assay was performed. Thus, synaptosomes were prepared from hippocampal slices and plated onto PDL-coated glass coverslips. A double immunolabeling with MAP2 and GFAP was carried out and analysed by fluorescence microscopy, as shown in Figure 16.

As can be observed, purified synaptosomes exhibit an abundant GFAP labeling, when compared with MAP2, confirming the heavy gliosomal contamination in the synaptosomes preparation. This detail makes this protocol not suited for the current study. Therefore, the work was performed in synaptosomes prepared from the whole hippocampus.

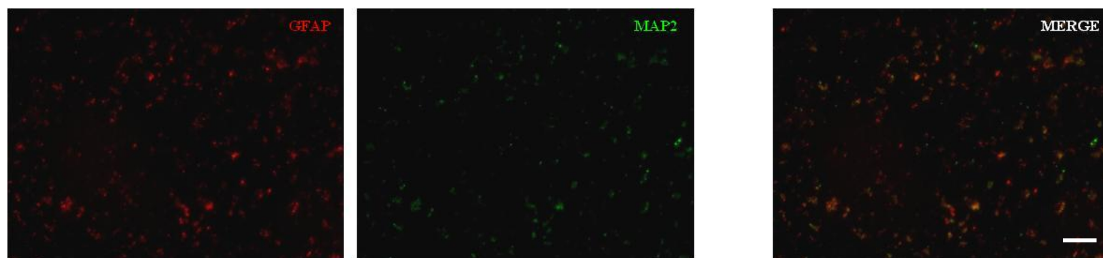


Figure 16. Double staining of GFAP and MAP2 in synaptosomes obtained from hippocampal slices. Immunofluorescence images of rat hippocampal synaptosomes stained with rabbit anti-GFAP antibody (red) and with mouse anti-MAP2 antibody (green). Images were acquired with a 40x objective in an Axiovert 200 microscope from Zeiss. Scale bars 15μm.

4.2.2. Characterization of synaptosomes prepared from hippocampal homogenates by immunofluorescence

In this section, once again to ensure that the sinaptosomal fraction was highly pure, synaptosomes obtained from rat hippocampal homogenates were plated onto PDL-coated glass coverslips. Figure 17 illustrates the fluorescence image obtained from the double immunolabeling with MAP2 and GFAP.

In this case, the majority of the synaptosomes is $MAP2^+$ and almost no $GFAP^+$ synaptosomes were detected. This assessment confirms that synaptosomes isolated from hippocampal homogenates are not contaminated with gliosomes.

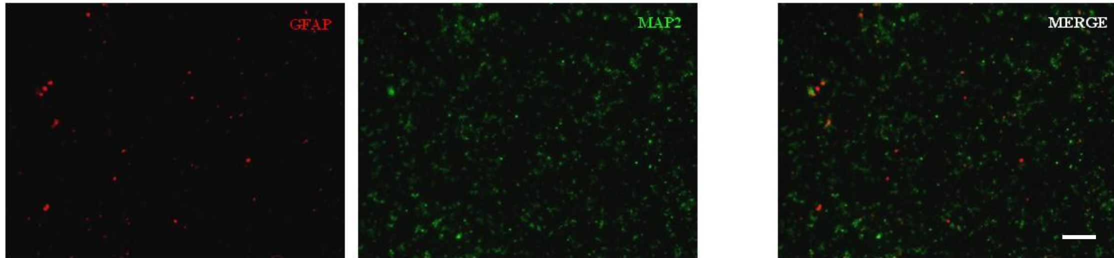
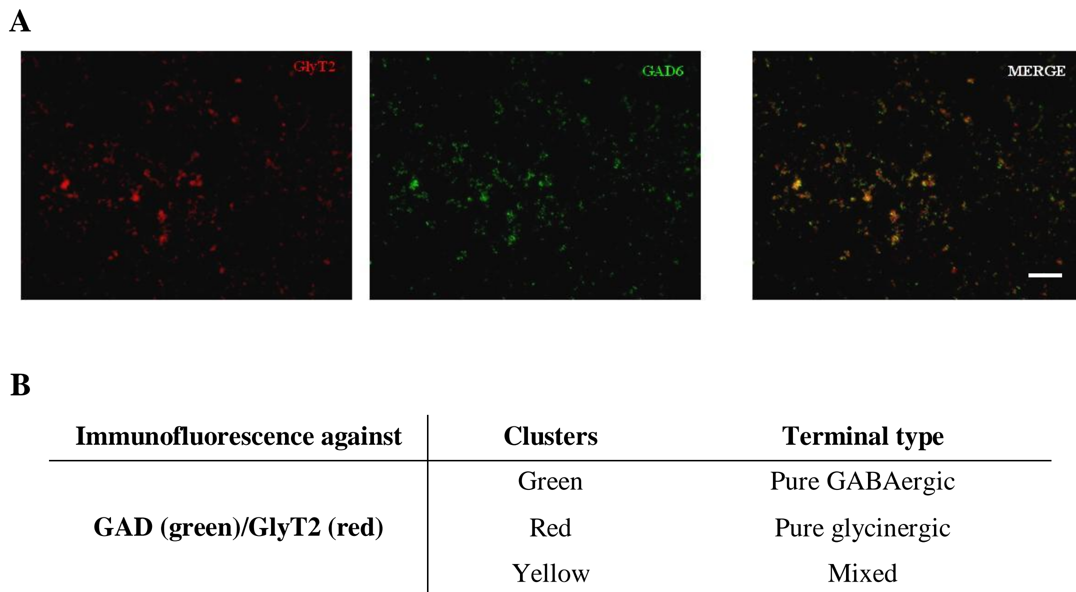


Figure 17. Double staining of GFAP and MAP2 in synaptosomes obtained from hippocampal homogenates. Immunofluorescence staining of rat hippocampal synaptosomes stained with rabbit anti-GFAP antibody (red) and with mouse anti-MAP2 antibody (green). Images were acquired with a 40x objective in a Axiovert 200 microscope from Zeiss. Scale bars 15µm.

4.2.3. Evaluation of the ratio GABAergic/glycinergic terminals in hippocampal synaptosomes

To assess changes in the ratio of GABAergic/glycinergic terminals upon GABA-mediated transmission impairment, pharmacological treatment was performed as described in section 3.4.2. After treatment with 20µM SKF89967a (or simple incubation in the case of CTL) synaptosomes were prepared, plated and the immunofluorescence assay was carried out.

A double immunolabelling of GAD and GlyT2 was performed. As referred before, GAD is a GABAergic terminal marker, while GlyT2 is considered a marker for glycinergic neurons. Figure 18 depicts an example of the final immunofluorescence images obtained from the microscope and identifies the types of terminals that can be identified.



Immunofluorescence against	Clusters	Terminal type
GAD (green)/GlyT2 (red)	Green	Pure GABAergic
	Red	Pure glycinergic
	Yellow	Mixed

Figure 18. Double staining of GlyT2 and GAD6 in synaptosomes obtained from hippocampal homogenates. (A) Immunofluorescence images of synaptosomes stained with rabbit anti-GlyT2 antibody (red) and with mouse anti-GAD6 antibody (green). Images were acquired with a 40x objective. Scale bars 15µm. (B) Types of terminals in the immunofluorescence images: GABAergic, glycinergic and mixed.

The quantitative analysis of the referred images, was carried out using a mask written for ImageJ software, as described in section 3.5.2.2. After running the mask in each image taken with the fluorescence microscope, a composite was created by the software, as illustrated in Figure 19.

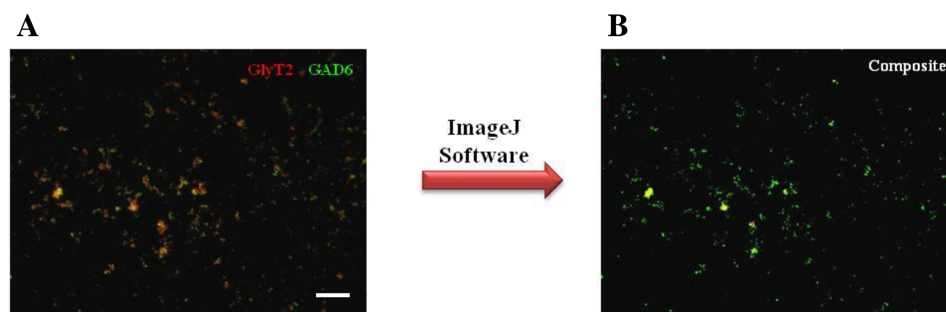


Figure 19. Illustration of the method used for the quantitative analysis. The immunofluorescence image (A) is converted to a composite image (B) by the ImageJ mask. Scale bars 15µm.

The described quantification method was then used to calculate the percentage of GABAergic, glycinergic and mixed terminals in each synaptosomal preparation, CTL or treated with 20µM SKF89967a for several time periods, as presented in Figure 20.

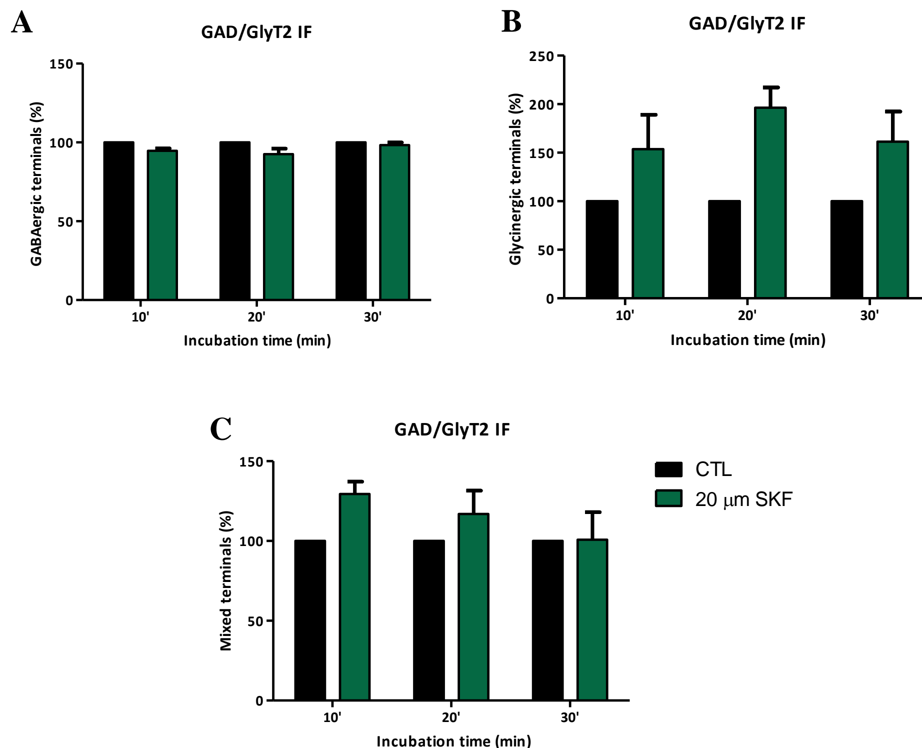


Figure 20. Changes in terminal phenotype in hippocampal synaptosomes. Relative percentage of (A) GABAergic, (B) glycinergic and (C) mixed terminals in synaptosomal preparations, non-treated (CTL) or treated with 20µM SKF89967a. over time. The values indicate the fold change when compared to the control (consider to be 100%). All values correspond to mean±SEM, n = 3.

As can be observed, no type of terminal changed significantly in any of the incubation times tried. However, glycinergic and mixed terminals have shown a great tendency to increase upon synaptosomes treatment with SKF89976a for 10 and 20 minutes. As to the GABAergic terminals, there's a slight overall decrease, which is in agreement with the referred increase in pure glycinergic and mixed terminals.

4.2.4. Assessment of a potential physical interaction between GlyT2 and VIAAT

The full understanding of the relationship between the plasma membrane transporter GlyT2 and the vesicular transporter VIAAT and how this dynamic interplay can influence the glycinergic phenotype is still poorly understood.

Given the fact that GlyT2 plays an important role in the recycling and refilling of synaptic vesicles and knowing that both VIAAT and GlyT2 cooperate to determine

the glycinergic phenotype (Aubrey et al., 2007), a further study was pursued in order to assess a potential physical interaction between the two glycine transporters.

For that, immunoprecipitation assays were preformed, as described in section 3.7.

To ensure that the pull down of the proteins of interest was efficient, western blot detection with specific antibodies against GlyT2 and VIAAT was performed in the immunoprecipitates (IP). As illustrated in Figure 21, both VIAAT and GlyT2 are efficiently pulled down since they were detected in the IP obtained with an antibody against itself, indicated in blue in Figure 21. Furthermore, VIAAT was detected in the IP obtained by pulling down GlyT2 and GlyT2 was detected in the IP obtained by pulling down VIAAT, shown in red in Figure 21. For comparison and positive controls, homogenates from spinal cord (3-weeks old animals) (SC) and from primary cortical neurons (8 DIV) (PC) were also analyzed.

The data shown in Figure 21, strongly suggests the interaction between VIAAT and GlyT2.

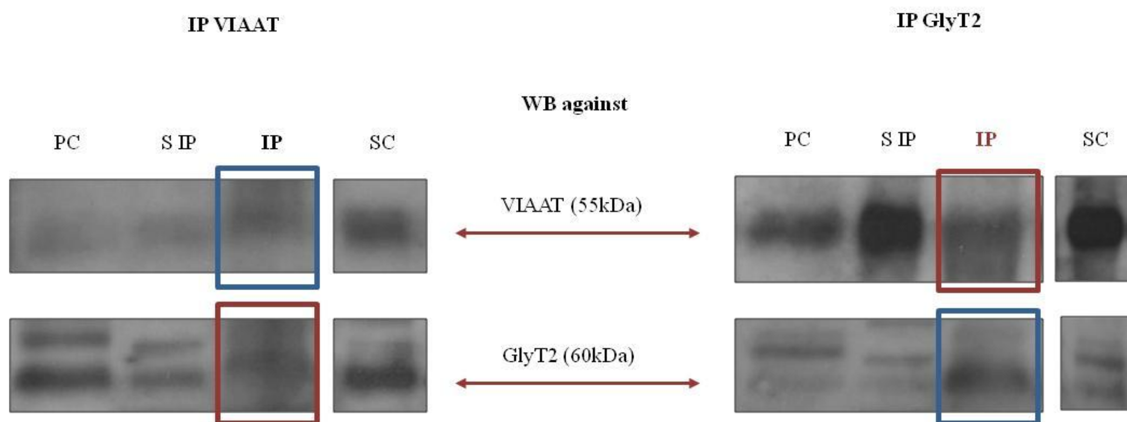


Figure 21. Representative immunoblots of the immunoprecipited proteins, VIAAT (55kDa) and GlyT2 (60kDa). Pull-down were carried out with goat polyclonal anti-VIAAT and goat polyclonal anti-GlyT2 antibodies. Immunoprecipitates were resolved by western blot with rabbit anti-VIAAT and rabbit anti-GlyT2 antibodies. (n = 2) PC (cells from primary cortical cultures); S IP (IP supernatant); IP (immunoprecipitate); SC (spinal cord).

5. Discussion

Given the crucial role of GABAergic transmission in the neuronal function of the cerebral cortex, an enormous research effort has been directed in the past decades at investigating it. On the other hand, studies related to glycine-mediated transmission in the brain, are considerably behind. Nevertheless, in recent years glycinergic transmission has gain importance, since many studies have suggested it as a possible therapeutic target for epilepsy. Because the balance between excitation and inhibition may be a key factor in the etiology of neurological and cognitive disorders, it is critical to understand how inhibitory synapses are formed, maintained, and modified. The present study allowed to profile the expression pattern of glycinergic transmission-related markers associated with an impaired GABAergic transmission in the brain.

5.1. Changes in the glycinergic phenotype in insulted cortical primary neuronal cultures

Recent studies showed that blocking neuronal glycine uptake for several hours markedly reduced glycinergic transmission, both in cultures and slices (Gomeza et al., 2003; Bradaia et al., 2004), but enhanced GABAergic transmission. The combination of these changes was sufficient to shift the neuron phenotype from mostly glycinergic to mostly GABAergic (Rousseau et al., 2008). Taking this evidence into account, the reverse approach was followed.

The $GABA_A$ receptors are the major receptors involved in GABA-mediated inhibitory signaling in the brain and changes in this receptor are associated with changes in the inhibitory tonus (Steiger and Russek, 2004).

In monoamine-releasing terminals, neurotransmitter transporters – in addition to terminating transmission by clearing released transmitters from the extracellular space – are the primary mechanism for replenishing transmitter stores and thus regulate presynaptic homeostasis. On this note, GAT-1 has a prominent role in both tonic and phasic $GABA_AR$-mediated inhibition (Bragina et al., 2008) and is the only GABA transporter that can contribute directly to GABA replenishment in terminals. When GAT-1 is inhibited via SKF89976a, GABA uptake is interrupted, which leads to an abnormal accumulation of GABA in the synaptic cleft. However, there are two more types of glycine transporters. GAT-2 transporter, which is primarily located in the

extrasynaptic region, and can be found both in neuronal and non-neuronal cells (Conti et al., 1999; Conti et al., 2004) and GAT-3 is localized to both neurons and astrocytes; being primarily localized to the latter cell type (Durkin et al., 1995; Minelli et al., 1996; Conti et al., 2004). Also GAT-3 has a relevant role in GABAergic transmission since an acute blockade of GAT-3 under resting conditions is fully compensated by GAT-1. This indicates that GAT-3 might provide an additional uptake capacity when neuronal activity and GABA release are increased (Kirmse et al., 2009). However, upon blockade of GABA transporters there is an alteration of both the amplitude and decay of the response mediated through 'slow', $GABA_B$ receptors (Isaacson et al., 1993), and of the decay phase of the 'fast' $GABA_A$ receptor-mediated response (Isaacson et al., 1993).

The above information suggests that, and given the fact that the purpose was to facilitate the glycinergic phenotype by damaging the GABAergic transmission, $GABA_AR$ and GAT-1 seemed good targets to pursue this objective. Therefore, the glycinergic phenotype was analyzed upon blockade of $GABA_A$ receptor or GABA transporter GAT-1, by specific antagonists, namely gabazine and SKF89976a, respectively.

In neuronal cultures, interfering with $GABA_AR$ mostly present in the postsynaptic density, through the use of gabazine, showed a significant increase in GlyR expression. This effect, observed after 2h of incubation, was shown to be concentration dependent. Also GlyT2 expression displayed a tendency for increasing after gabazine incubation, but in this case the effect is detected in all ranges of gabazine concentration and incubation time tested.

Upon blockade of GABA uptake with SKF89976a, densitometric analysis of western blots only showed an increasing trend in GlyR and GlyT2 expression, but without statistical significance, at any concentration or incubation time tested.

These results strongly suggest that the blockade of $GABA_AR$-mediated transmission or a deficient clearance of GABA from the synaptic cleft by blockade of GAT-1 leads to a compensatory mechanism through the increase of glycine-mediated transmission components, specifically GlyR in the postsynaptic density and GlyT2 in presynaptic membrane.

Moreover, it has been proven that a deficiency in VIAAT results in drastic reduction of GABA and glycine release from nerve terminals (Wojcik et al., 2006), suggesting that both transmitters compete for VIAAT-mediated vesicle loading. Although glycine and GABA vesicular uptake by VIAAT is presumably coupled to the

same exchange with $1H^+$ (Burger et al., 1991), their accumulation in synaptic vesicles may not be limited by the same thermodynamic or kinetic constraints if their cytosolic concentrations differ significantly, especially in the brain. Since GABA and glycine compete for VIAAT, a deficiency in GABAergic synapse-related components might abolish this competition and drive glycine loading, in detriment of GABA. This increase in glycine vesicular uptake induces the expression of glycinergic-related components.

As referred in section 4.1.2, the blockade of GAD65 enzyme with 3-MPA, the major inhibitor for this enzyme, was also tested. This was so due to the knowledge that GAD65, the enzyme responsible for the synthesis of GABA from glutamate for vesicular release, was necessary for efficient GABAergic neurotransmission (Latal et al., 2010). Also an association of VIAAT with GAD65 has been shown to provide a major kinetic advantage for the vesicular accumulation of newly synthesized GABA (Jin et al., 2003) and this association is likely to alter the relative accumulation of GABA in vesicles. Since a study in neuronal cortical cultures using 3-MPA was already described (Monnerie H and Le Roux, 2007), this appeared a possible approach. Unfortunately, cultured neurons are extremely sensitive to factors that alter the optimal conditions in which they were plated. Hence, before adding the 3-MPA a pH adjustment had to be done. Since this adjustment was not very successful, 3-MPA strongly induced cell death shortly after addition and this line of investigation could not pursued.

The use of real-time quantitative PCR was important to evaluate if the observed changes in the protein levels were accompanied by changes in the expression of the mRNA encoding for GlyT2 and GlyR. For this part of the work, the highest concentration of gabazine and SKF89976a was used - 100µM. The incubation time for both drugs was 2h and 24h.

The transcript expression profile seems to corroborate the GlyR expression findings. The antibody used for the detection of GlyR by western blot is characterized (Pfeiffer et al., 1984) and it recognizes all alpha subunits (α1, α2 and α3). A single band of 48-49kDa was obtained, which corresponds to both α1 (48kDa) and α2 (49kDa) subunits of the receptor. qPCR has shown a significant increase in GlyRα1 subunit when the cells were exposed to gabazine, and a strong increasing trend of the same subunit after exposure to SKF89976a, which indicates that GlyRα1 is the subunit responsible for the protein increase observed in the western blots. After 24h incubation, with both gabazine and SKF89976a, a minor increasing trend might also be observed in GlyRβ subunit.

GlyR is composed of α and β subunits, arranged around a central pore. The α subunit of GlyR, confers channel kinetics and pharmacology, and is the obligatory subunit which is capable of forming functional homomeric channels. The β subunit allows GlyR anchoring to the membrane through binding of the auxiliary structure protein gephyrin, a cytoplasmatic protein necessary for synaptic localization of GlyR (Triller et al., 1985; Schmitt et al., 1987; Betz et al., 2006). The increase in GlyRα1, concomitant with the trending increase in GlyRβ suggest a recruitment of heteromeric GlyRα1β to the postsynaptic density. However, this result is contradictory to what has been previously reported (Aroeira et al., 2011). Aroeira and co-workers postulated that in the mature brain, specifically in the hipocamppus, although a few synaptic GlyRα1β can be detected in the dendritic layers, extrasynaptic α2/α3-containing GlyR and somatic localized GlyRα3 are the most abundant. The authors pointed towards an important function of a slow tonic activation of extrasynaptic GlyR, over a fast phasic activation of synaptic GlyRα1β. However, the results indicate that GABA mediated-neurotransmission impairment affects the GlyRα1β already located in the synapse, probably to allow a quick response of the glycinergic system to low GABA levels in the terminals (Triller and Choquet, 2005).

Also, regarding the formation of inhibitory synapses, it is described that clusters of $GABA_AR$ are recruited at the postsynaptic location, followed by the association of gephyrin and GlyR with $GABA_AR$ at this site. $GABA_AR$ could be the initial element responsible for the recruitment of the other postsynaptic components in mixed synapses, by a mechanism similar to the one proposed by Kirsh and Betz (1998). The binding of GABA to synaptically clustered receptors would initiate a depolarizing Cl^- current that activates a voltage-dependent Ca^{2+} channel. Ca^{2+} would in turn aggregate gephyrin, which would stabilize $GABA_AR$ and allow the subsequent recruitment of GlyR (Dumoulin et al., 2000). On the view of the above, one can speculate that in the presented experiments gabazine blocks $GABA_AR$ and as a compensatory mechanism GlyR recruitment to the postsynaptic densities is induced. On the other hand, SKF89976a causes an increase in GABA in the synaptic cleft due to GAT-1 inhibition. This over activation of $GABA_AR$ and its consequent internalization, causes an induction of GlyR recruitment to the postsynaptic densities.

Regarding GlyT2 transcript expression, GlyT2 mRNA levels increased significantly after 24h treatment with SKF89976a, when compared to the control. Gabazine did not seem to affect GlyT2 transcript expression. These results do not

support the expression pattern obtained by western blot and point to the need of increasing the number of experiments to confirm the data and to draw any conclusions.

Furthermore, it is always important to have in mind, there are presumably at least three reasons for the poor correlations generally reported in the literature between the level of mRNA and the level of protein, and these may not be mutually exclusive. First, there are many complicated and varied post-transcriptional mechanisms involved in turning mRNA into protein that are not yet sufficiently well defined to be able to compute protein concentrations from mRNA; second, proteins may differ substantially in their in vivo half lives as the result of varied protein synthesis and degradation, and protein turnover can vary significantly depending on a number of different conditions; and/or third, there is a significant amount of error and noise in both protein and mRNA experiments that limit our ability to get a clear picture (Gygi et al., 1999; Greenbaum et al., 2002).

GlyT2 removes glycine from the synaptic cleft, thereby aiding the termination of the glycinergic signal and achieving the reloading of the presynaptic terminal. The task fulfilled by this transporter is fine tuned by regulating both transport activity and intracellular trafficking. Different stimuli such as neuronal activity or protein kinase C (PKC) activation can control GlyT2 surface levels although the intracellular compartments where GlyT2 resides are largely unknown. Moreover, it has also been published that inhibitory glycinergic neurotransmission is modulated by the GlyT2 exocytosis/endocytosis equilibrium, but the mechanisms underlying the turnover of this transporter remain elusive (Juan-Sanz J et al., 2011). Also, the available antibody used for GlyT2 detection by western blot did not always had a clear signal, making the densitometry analysis quite difficult. The above statements can explain the differences on the GlyT2 data obtained by western blot and qPCR.

Nevertheless, the results obtained point to an overall tendency for up-regulation of glycine-mediated neurotransmission markers, upon a GABAergic transmission impairment.

5.2. Changes in the ratio of glycinergic *vs* GABAergic terminals in rat hippocampal synaptossomes

To further explore the findings from the previous task, the ratio of glycinergic vs GABAergic terminals was investigated in hippocampal synaptosomes. This was

achieved, by immunostaining of GAD65 and GlyT2 with fluorescence labels. Synaptosomes are sealed presynaptic nerve terminals that hold all the necessary components to store, release and retain neurotransmitters. In addition, essentially all synaptosomes contain functional mitochondria (Kauppinen and Nicholls, 1986).

The initial objective was to perform the pharmacological treatments in hippocampal slices with both drugs, thus acting on pre- and postsynaptic sites. However, the sinaptosomal fractions obtained from the treated hippocampal slices were highly contaminated with gliosomes. Since the whole approach was based on the assessment of GlyT2 expression in neurons and this protein is also expressed in astrocytes (Raiteri et al., 2008), this would alter the final experimental data and was consider not suitable for the study.

Therefore, the work was carried out using synaptosomes obtained directly from hippocampal homogenates, followed by the incubation with 20µM of SKF89976a (as optimized in Vaz et al., 2008) for 10, 20 and 30 minutes.

The quantitative analysis was in agreement with what has been previously reported in the literature, related to the relative expression of GABAergic and glycinergic synapses in the brain (Kandel et al., 2000). In the non-treated synaptosomes, the results clearly show the predominance of GABAergic terminals (> 80%), when compared to both glycinergic and mixed terminals.

The results also show a change, although not statistically significant, in the relative percentage of GABAergic, glycinergic and mixed terminals in treated synaptosomes when compared to non-treated ones. In particular, glycinergic and mixed terminals depicted a tendency to increase upon treatment with SKF89976a for 10 and 20 minutes, and the percentage of GABAergic terminals tended to decrease. In spite the need to increase the number of experiments, these results are consistent with what was shown before.

5.3. Interaction between vesicular and membrane glycine transporters, GlyT2 and VIAAT

GlyT2 plays an important role in the recycling and refilling of synaptic vesicles and both VIAAT and GlyT2 cooperate to determine the glycinergic phenotype. Therefore, it was important to try to understand the relationship between these two proteins and how this dynamic interplay can influence the glycinergic phenotype.

Many fundamental aspects of the presynaptic contribution to the inhibitory phenotyps remain unclear. New and recycled synaptic vesicles are filled with high concentration of neurotransmitter by specific vesicular H^{+} antiporters (Sulzer and Pothos, 2000). Yet, despite the importance of this loading step for the completeness of vesicle recycling, little is known about its dynamics and the thermodynamics, kinetic and osmotic constraints that regulate the vesicular storage of neurotransmitters under physiological conditions. Although glycine is a ubiquitous intracellular metabolite, its basal synthesis cannot account for the 10- to 100-fold larger accumulation detected in glycinergic neurons (Daly, 1990). Thus, a local recapture is considered to be the main mechanism of glycine supply at terminals. In a model system of inhibitory transmission, it has been shown that the only requirement for glycine accumulation into vesicles is the coexpression of GlyT2 and VIAAT (Aubrey et al., 2007).

Concerning the GABAergic system, GABA homeostasis in presynaptic terminals is dominated by the activity of the GABA synthesizing enzyme (GAD65) and GAT-1-mediated GABA transport contributes to cytosolic GABA levels. In fact, GAD65 may be anchored to synaptic vesicles by forming a complex, known to include the vesicular GABA transporter VIAAT, an integral membrane protein of synaptic vesicles responsible for their filling. This may provide a structural and functional coupling between synthesis and vesicular packaging of GABA. Interestingly, [^{3}H]GABA newly synthesized from [^{3}H]Glu by synaptic vesicles-associated GAD is taken up preferentially into vesicles over cytosolic GABA (Jin et al., 2003).

Taking all these statements into account, it seemed interesting to explore a possible relationship between GlyT2 transported glycine and vesicle filling, as seen for GAD65 in the GABAergic system. Preliminary results point to a potential physical interaction between GlyT2 and VIAAT, but a truly accurate assessment of this finding requires more experiments.

6. General Conclusions

The major objective of the present work was to investigate which are the mediators for the acquisition of a glycinergic phenotype. The characterization was performed by promoting the glycine-mediated transmission by blockade of components of the GABAergic system, followed by the evaluation of the consequences in the glycinergic transmission markers, namely GlyT2 and GlyR. That assessment was made by studying the protein expression, by western blot, and mRNA levels, by RT-qPCR, of the main GlyR subunits and GlyT2. Furthermore, the ratio of glycinergic vs GABAergic terminals, in insulted synaptosomes, was compared by immunofluorescence assays. Finally, a potential physical interaction between GlyT2 and VIAAT was explored by immunoprecipitation.

In conclusion, this work strongly suggests that the loss of a functional GABAergic system leads to a compensatory mechanism, which recruits glycine-mediated transmission markers, namely GlyT2 and GlyR. Moreover, the assessment of an interaction between the membrane and vesicular glycine transporters, GlyT2 and VIAAT, still not described, came to reinforce the important role of both transporters for the glycinergic transmission.

One of the most pertinent issues that these results can raise is probably related to the physiological role of glycinergic transmission in the brain, where the inhibitory transmission is predominantly mediated by GABA. The discovery of the occurrence of mixed synapses in the brain, which allows the simultaneous release of both inhibitory neurotransmitters, contributed to the hypothesis of a possible joint action of GABA and glycine, leading to a more efficient and precise control of neuronal activity. Furthermore, the results presented along this study shows that the glycinergic transmission might be important upon the existence of a fault in GABAergic transmission, partially replacing it. However, at which level this compensation is enough to substitute GABA, still remains unknown. In addition, hypofunction of glycine signaling has been implicated in several pathologies such as neuromotor disorders or epilepsy and, recently, it was shown that some missense mutations in the gene encoding for GlyT2 cause hyperekplexia or startle disease in humans (Eulenburg et al., 2006). Therefore increasing the efficacy of inhibitory glycinergic neurotransmission would conceivably produce benefits in these disorders and it is reasonable to speculate that the modulation of GlyT2 activity might find therapeutic applications.

Thereby, the work here described has increased the knowledge about the glycine-mediated transmission in the brain. Still, many questions remain unanswered and further investigation must be pursued in this subject.

7. FUTURE PERSPECTIVES

The study described in this thesis clarifies some details which involves the acquisition of glycinergic phenotype in the brain. However, further understanding of the role of glycine in the brain is necessary. So, following the work already done and presented here, a deeper characterization should be pursued in organotypic hippocampal slice cultures (OHSC). In these cultures, many of the intrinsic properties of the tissue are maintained, including important aspects of connectivity, and the morphological organization of the hippocampus is well preserved (Stoppini et al., 1991). There is still proliferation of granule cells, outgrowth and organization of axons and dendrites. Also maturation of synapses and receptors resembles those seen in vivo. Therefore, OHSC are valuable models to study changes in cellular and molecular levels upon pharmacological manipulations, in more physiological conditions.

Studies in OHSC are imperative and will further increase the understanding about the glycinergic neurotransmission in the brain.

8. REFERENCES

Alakuijala A, Palgi M, Wegelius K, Schmidt M, Enz R, Paulin L, Saarma M, Pasternack M (2005) GABA receptor ρ subunit expression in the developing rat brain. Brain Res Dev Brain Res 154: 15-23

Aroeira RI, Ribeiro JA, Sebastião AM, Valente CA (2011) Age-related changes of glycine receptor at the rat hippocampus: from the embryo to the adult. J Neurochem 118: 339-53

Aubrey KR, Rossi FM, Ruivo R, Alboni S, Bellenchi GC, Le Goff A, Gasnier B, Supplisson S (2007) The transporters GlyT2 and VIAAT cooperate to determine the vesicular glycinergic phenotype. J Neurosci 27:6273-6281

Becker CM, Hoch W, Betz H (1988) Glycine receptor heterogeneity in rat spinal cord during postnatal development. EMBO J 7:3717–3726

Ben-Ari Y (1985) Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy. Neuroscience 14: 375-403

Ben-Ari Y (2002). Excitatory actions of GABA during development: the nature of nurture. Nat Rev Neurosci. 3:728–739

Ben-Ari Y, Khalilov I, Kahle KT, Cherubini E (2012) The GABA excitatory/inhibitory shift in brain maturation and neurological disorders. Neuroscientist XX(X): 1-20

Bengzon J, Mohapel P, Ekdahl CT, Lindvall O (2002) Neuronal apoptosis after brief and prolonged seizures. Prog Brain Res 135: 111-119

Berger AJ, Dieudonné S, Ascher P (1998) Glycine uptake governs glycine site occupancy at NMDA receptors of excitatory synapses. J Neurophysiol 80(6): 3336-3340

Bettler B, Kaupmann K, Mosbacher J, Gassmann M (2004) Molecular structure and physiological functions of GABA(B) receptors. Physiol Rev 84(3): 835-867

Betz H, Gomeza J, Armsen W, Scholze P, Eulenburg V (2006) Glycine transporters: essential regulators of synaptic transmission. Biochem Soc Trans 34: 55-58

Bittigau P, Sifringer M, Genz K, Reith E, Pospischil D, Govindarajalu S, Dzietko M, Pesditschek S, Mai I, Dikranian K, Olney JW, Ikonomidou C (2002) Antiepileptic drugs and apoptotic neurodegeneration in the developing brain. Proc Natl Acad Sci U.S.A 99: 15089-15094

Borden LA (1996) GABA transporter heterogeneity: pharmacology and cellular localization. Neurochem Int 29(4): 335-356

Bowery NG, Smart TG (2006) GABA and glycine as neurotransmitters: a brief history. Br J Pharmacol 147: S109-S119

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254

Bradaia A, Schlicher R, Trouslard J (2004) Role of glial and neuronal glycine transporters in the control of glycinergic and glutamatergic synaptic transmission in lamina X of the rat spinal cord. J Physiol 559: 169-186

Bragina L, Marchionni I, Omrani A, Cozzi A, Pellegrini-Giampietro DE, Cherubini E, Conti F (2008) GAT-1 regulates both tonic and GABA(A) receptor-mediated inhibition in the cerebral cortex. J Neurochem 105: 1781-1793

Burger PM, Hell J, Mehl E, Krasel C, Lottspeich F, Jahn R (1991) GABA and glycine in synaptic vesicles: storage and transport characteristics. Neuron 7: 287-293

Caceres A, Banker GA, Binder L (1986) Immunocytochemical localization of tubulin and microtubule-associated protein 2 during the development of hippocampal neurons in culture. J Neurosci 6: 714-722

Calandre EP, Dominguez-Granados R, Gomez-Rubio M, Molina-Font JA (1990) Cognitive effects of long-term treatment with phenobarbital and valproic acid in school children. Acta Neurol Scand 81: 504-506

Chattipakorn SC, McMahon LL (2002) Pharmacological characterization of glycine-gated chloride currents recorded in rat hippocampal slices. J Neurophysiol 87: 1515-25

Cherubini E, Bernardi G, Stanzione P, Marciani MG, Mercuri N (1981) The action of glycine on rat epileptic foci. Neuroscience Lett 21: 93–97

Cherubini E, Gaiarsa JL, Ben-Ari Y (1991) GABA: an excitatory transmitter in early postnatal life. Trends Neurosci 14: 515-519

Conti F, Melone M, De Biasi S, Minelli A, Brecha NC, Ducati A (1998) Neuronal and glial localization of GAT-1, a high-affinity gamma-aminobutyric acid plasma membrane transporter, in human cerebral cortex: with a note on its distribution in monkey cortex. J Comp Neurol 396 (1): 51-63

Conti F, Minelli A, Melone M (2004) GABA transporters in the mammalian cerebral cortex: localization, development and pathological implications. Brain Res Rev 45: 196-212

Conti F, Zuccarello LV, Barbaresi P, Minelli A, Brecha NC, Melone M (1999) Neuronal, glial, and epithelial localization of gamma-aminobutyric acid transporter 2, a high-affinity gamma-aminobutyric acid plasma membrane transporter, in the cerebral cortex and neighboring structures. J Comp Neurol 409: 482-494

Cutting GR, Lu L, O'Hara BF, Kasch LM, Montrose-Rafizadeh C, Donovan DM, Shimada S, Antonarakis SE, Guggino WB, Uhl GR et al. (1991) Cloning of the γ-aminobutyric acid (GABA) ρ1 cDNA: a GABA receptor subunit highly expressed in the retina. Proc Natl Acad Sci U.S.A 88: 2673-2677

Daly E (1990) The biochemistry of glycinergic neurons. In: Glycine neurotransmission (Ottersen O, Storm-Mathisen J, eds). Chichester, UK: Wiley

Danglot L, Rostaing P, Triller A, Bessis A (2004) Morphologically identified glycinergic synapses in the hippocampus. Mol Cell Neurosci 27: 394-403.

Dotti CG, Sullivan CA, Banker GA (1988) The establishment of polarity by hippocampal neurons in culture. J Neurosci 8: 1454-1468

Dumoulin A, Lévi E, Riveau B, Gasnier B, Triller A (2000) Formation of mixed glycine and GABAergic synapses in cultured spinal cord neurons. Eur J Neurosci 12(11): 3883-3892

Dumoulin A, Rostaing P, Bedet C, Levi S, Isambert MF, Henry JP, Triller A, Gasnier B (1999) Presence of the vesicular inhibitory amino acid transporter in GABAergic and glycinergic synaptic terminal boutons. J Cell Sci 112(6):811-823

Durkin MM, Smith KE, Borden LA, Weinshank RL, Branchek TA, Gustafson EL (1995) Localization of messenger RNAs encoding three GABA transporters in rat brain: an in situ hybridization study. Brain Res Mol Brain Res 33: 7-21

Eichler SA, Kirischuk S, Juttner R, Schafermeier PK, Legendre P, Lehmann TN, Gloveli T, Grantyn R, Meier JC (2008) Glycinergic tonic inhibition of hippocampal neurons with depolarising GABAergic transmission elicits histopathological signs of temporal lobe epilepsy. J Cell Mol Med 12: 2848–2866

Eichler SA, Forstera B, Smolinsky B, Juttner R, Lehmann TN, Fahling M, Schwarz G, Legendre P, Meier JC (2009) Splice-specific roles of glycine receptor α3 in the hippocampus. Eur J Neurosci 30: 1077–1091

Engel D, Schmitz D, Gloveli T, Frahm C, Heinemann U, Draguhn A (1998) Laminar difference in GABA uptake and GAT-1 expression in rat CA1. J Physiol 512: 643-649

Engel J, Pedley TA, Aicardi J, Dichter MA (2008) Epilepsy: A Comprehensive Textbook (Vol. 2). Philapelphia: Lippincott Williams & Wilkins

Eulenburg V, Armsen W, Betz H, Gomeza J (2005) Glycine transporters: essential regulators of neurotransmission. TRENDS Biochem Sci 30(6):325-333

Eulenberg V, Becker K, Gomeza J, Schmitt B, Becker CM, Betz H (2006) Mutations within the human GLYT2 (SLC6A5) gene associated with hyperekplexia. Biochem Biophys Res Commun 348(2): 400-405

Feigenspan A, Bormann J (1994) Differential pharmacology of $GABA_A$ and $GABA_C$ receptors on rat retinal bipolar cells. Eur J Pharmacol 288: 97-104

Fisher RS, van Emde Boas W, Blume W, Elger C, Genton P, Lee P, Engel J (2005) Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). Epilepsia 46: 470-472

Gaspary HL, Wang W, Richerson GB (1998) Carrier-mediated GABA release activates GABA receptors on hippocampal neurons. J Neurophysiol 80:270-281

Gomeza J, Ohno K, Hulsmann S, Armsen W, Eulenburg V, Richter DW, Laube B, Betz H (2003) Deletion of the mouse glycine transporter 2 results in a hyperekplexia phenotype and postnatal lethality. Neuron 40:797–806

Greenbaum D, Jansen R, Gerstein M (2002) Analysis of mRNA expression and protein abundance data: an approach for the comparison of the enrichment of features in the cellular population of proteins and transcripts. Bioinformatics 18: 585-596

Grenningloh G, Schmieden V, Schofield PR, Seeburg PH, Siddique T, Mohandas TK, Becker CM, Betz H (1990) Alpha subunit variants of the human glycine receptor: primary structures, functional expression and chromosomal localization of the corresponding genes. EMBO J 9: 771-776

Grudzinska J, Schemm R, Haeger S, Nicke A, Schmalzing G, Betz H, Laube B. (2005) The beta subunit determines the ligand binding properties of synaptic glycine receptors. Neuron 45: 727-739

Guastella J, Nelson N, Nelson H, Czyzyk L, Keynan S, Miedel MC, Davidson N, Lester HA, Kanner BI (1990) Cloning and expression of rat brain GABA transporter. Science 249: 1303-1306

Guastella J, Brecha N, Weigmann C, Lester HA, Davidson N (1992) Cloning, expression, and localization of a rat brain high-affinity glycine transporter. Proc Natl Acad Sci U.S.A. 89:7189–7193

Gygi SP, Rochon Y, Franza BR, Aebersold R (1999) Correlation between protein and mRNA abundance in yeast. Mol Cell Biol 19: 1720-1730

Herranz JL, Armijo JA, Arteaga R (1988) Clinical side effects of phenobarbital, primidone, phenytoin, carbamazepine, and valproate during monotherapy in children. Epilepsia 29: 794-804

Holopainen I, Wojcik WJ (1993) A specific antisense oligodeoxynucleotide to mRNAs encoding receptors with seven transmembrane spanning regions decreases muscarinic m_2 and γ-aminobutyric $acid_B$ receptors in rat cerebellar granule cells. J Pharmacol Exp Ther 264: 423-430

Isaacson JS, Solis JM, Nicoll RA (1993) Local and diffuse synaptic actions of GABA in the hippocampus. Neuron 10: 165-175

Iversen LL, Neal MJ (1968) The uptake of [^{3}H]GABA by slices of rat cerebral cortex. J Neurochem 15: 1141-1149

Jin H, Wu H, Osterhaus G, Wei J, Davis K, Sha D, Floor E, Hsu CC, Kopke RD, Wu JY (2003) Demonstration of functional coupling between γ-aminobutyric acid (GABA) synthesis and vesicular GABA transport into synaptic vesicles. Proc Natl Acad Sci U.S.A. 100:4293-4298

Johnson JW, Ascher P (1987) Glycine potentiates the NMDA response in cultured mouse brain neurons. Nature 325:529-531

Juan-Sanz J, Zafra F, López-Corcuera B, Aragón C (2011) Endocytosis of the neuronal glycine transporter GLYT2: role of membrane rafts and protein kinase C-dependent ubiquitination. Traffic 12(12): 1850-1867

Jursky F, Nelson N (1995) Localization of glycine neurotransmitter transporter (GLYT2) reveals correlation with the distribution of glycine receptor. Journal of Neurochemistry 64: 1026-1033

Kandel E, Schwartz JH, Jessell TM (2000) Principles of Neural Science, 4ª Ed., McGraw-Hill, E.U.A.

Kaupmann K, Malitschek B, Schuler V, Heid J, Froestl W, Beck P, Mosbacher J, Bischoff S, Kulik A, Shigemoto R, Karschin A, Bettler B (1998) GABA(B)-receptor subtypes assemble into functional heteromeric complexes. Nature 396: 683-687

Kauppinen RA, Nicholls DG (1986) Synaptosomal bioenergetics. The role of glycolysis, pyruvate oxidation and responses to hypoglycaemia. Eur J Biochem 158: 159-165

Kim H, Binder LI, Rosendaum JL (1979) The periodic association of MAP2 with brain microtubules *in vitro*. J Cell Biol 80: 266-276

Kirmse K, Kirischuk S, Grantyn R (2009) Role of GABA transporter 3 in GABAergic synaptic transmission at striatal output neurons. Synapse 63: 921-929

Kirsch J (2006) Glycinergic transmission. Cell Tissue Res 326: 535-540

Kirsch J and Betz H (1998) Glycine-receptor activation is required for receptor clustering in spinal neurons. Nature 392: 717-720

Kirschner A, Breustedt J, Rosche B, Heinemann UF, Schmieden V (2003) Effects of taurine and glycine on epileptiform activity induced by removal of Mg2+ in combined rat entorhinal cortexhippocampal slices. Epilepsia 44(9): 1145-1152

Kubota H, Katsurabayashi S, Moorhouse AJ, Murakami N, Koga H, Akaike N (2003) $GABA_B$ receptor transduction mechanisms, and cross-talk between protein kinases A and C, in GABAergic terminals synapsing onto neurons of the rat nucleus basalis of Meynert. J Physiol 551: 263-276

Kuhse J, Laube B, Magalei D, Betz H (1993) Assembly of the inhibitory glycine receptor: identification of amino acid sequence motifs governing subunit stoichiometry. Neuron 11: 1049-1056

Kuhse J, Schmieden V, Betz H (1990) Identification and functional expression of a novel ligand binding subunit of the inhibitory glycine receptor. Journal of Biological Chemistry 265: 22317-22320

Kuhse J, Betz H, Kirsch J (1995) The inhibitory glycine receptor: architecture, synaptic localization and molecular pathology of a postsynaptic ion-channel complex. Curr Opin Neurobiol 5; 318-23. Review.

Latal A, Kremer T, Gomeza J, Eulenburg V, Hulsmann S (2010) Development of synaptic inhibition in glycine transporter 2 deficient mice. Mol Cell Neurosci 44(4): 342-352

Legendre P (2001). The glycinergic inhibitory synapse. Cell Mol Life Sci 58:760–793

Lester HA, Mager S, Quick MW, Corey JL (1994) Permeation propertiers of neurotransmitter transporters. Annu Rev Toxicol 34: 219-249

Liu, QR, López-Corcuera B, Mandiyan S, Nelson H, Nelson N (1993) Cloning and expression of a spinal cord-and brain-specific glycine transporter with novel structural features. J Biol Chem 268:22802–22808

López-Corcuera B, Geerlings A, Aragón C (2001) Glycine neurotransmitter transporters: an update. Mol Membr Biol 18(1): 13-20

Luque JM, Nelson N & Richards JG (1994) Cellular expression of glycine transporter 2 messenger RNA exclusively in rat hindbrain and spinal cord. Neuroscience 64: 525-535

Lynch, JW (2004) Molecular structure and function of the glycine receptor chloride channel. Physiol Rev 84:1051–1095

Martin DL, Rimvall K (1993) Regulation of γ-aminobutyric acid synthesis in the brain. J Neurochem 60:395-407

Matzenbach B, Maulet Y, Sefton L, Courtier B, Avner P, Guenet JL, Betz H (1994) Structural analysis of mouse glycine receptor alpha subunit genes. Identification and chromosomal localization of a novel variant. J Biol Chem 269: 2607-2612

McIntire SL, Reimer RJ, Schuske K, Edwards RH, Jorgensen EM (1997) Identification and characterization of the vesicular GABA transporter. Nature 389:870-876

McKernan RM, Whiting PJ (1996) Which GABAA-receptor subtypes really occur in the brain? Trends Neurosci 19: 139-143

Meier J, Meunier-Durmort C, Forest C, Triller A, Vannier C (2000) Formation of glycine receptor clusters and their accumulation at synapses. J Cell Sci 113(15): 2783-2795

Minelli A, DeBiasi S, Brecha NC, Zuccarello LV, Conti F (1996) GAT-3, a high-affinity GABA plasma membrane transporter, is localized to astrocytic processes, and it is not confined to the vicinity of GABAergic synapses in the cerebral cortex. J Neurosci 16: 6255-6264

Mitchell K, Spike RC, Todd AJ (1993) An immunocytochemical study of glycine receptor and GABA in laminae I- III of rat spinal dorsal horn. J Neurosci 13: 2371-2381

Monnerie H and Le Roux P (2007) Reduced dendrite growth and altered glutamic acid decarboxylase (GAD) 65- and 67-kDa isoform protein expression from mouse cortical GABAergic neurons following excitotoxic injury *in vitro*. Exp Neurol 205(2): 367-382

Negishi T, Ishii Y, Kyuwa S, Kuroda Y, Yoshikawa Y (2003) Primary cultura of cortical neurons, type-1 astrocytes, and microglial cells from cynomolgus monkey (Macaca fascicularis) fetuses. J Neurosci Methods 131(1-2): 133-140

Nelson DL, Cox MM (2000) Lehninger Principles of Biochemistry, 3ª Ed., Worth Publishers, E.U.A.

Nelson N (1998) The family of Na^+/Cl^- neurotransmitter transporters. J Neurochem. 71: 1785–1803

Norris DO, Mastropaolo J, O'Connor DA, Cohen JM, Deutsh SI (1994) A glycinergic intervention potentiates the antiseizure efficacies of MK-801, flurazepam, and carbamazepine. Neurochem Res 19(2): 161-165

Owens DF, Kriegstein AR (2002) Is there more to GABA than synaptic inhibition? Nat Rev Neurosci 3: 715-727

Pfeiffer F, Simler R, Grenningloh G, Betz H (1984) Monoclonal antibodies and peptide mapping reveal structural similarities between the subunits of the glycine receptor of spinal cord. Proc Natl Acad Sci U.S.A. 81: 7224-7227

Pierce KL, Premont RT, Lefkowitz RJ (2002) Seven transmembrane receptors. Nat Rev Mol Cell Biol 3: 639-650

Pitkänen A, Sutula TP (2002) Is epilepsy a progressive disorder? Prospects for new therapeutic approaches in temporal-lobe epilepsy. Lancet Neurol 1: 173-181

Poyatos I, Ponce J, Aragón C, Giménez C, Zafra F (1997) The glycine transporter GLYT2 is a reliable marker for glycine-immunoreactive neurons. Brain Res Mol Brain Res 49:63–70

Raiteri L. Stigliani S, Usai C, Diaspro A, Paluzzi S, Milanese M, Raiteri M, Bonanno G (2008) Functional expression of release-regulating glycine transporters GlyT1 on GABAergic neurons and GlyT2 on astrocytes in mouse spinal cord. Neurochem Int 52(1-2): 103-112

Roberts E, Frankel S (1950) γ-Aminobutyric acid in brain: its formation from glutamic acid. J Biol Chem 187:55-63

Roberts E, Kuriyama K (1968) Biochemical-physiological correlations in studies of the γ-aminobutyric acid system. Brain Res 8:1-35

Rhodes KE, Moon LD, Fawcett JW (2003) Inhibiting cell proliferation during formation of the glial scar: effects on axon regeneration in the CNS. Neuroscience 120(1): 41-56

Rousseau F, Aubrey KR, Supplisson S (2008) The glycine transporter GlyT2 controls the dynamics of synaptic vesicle refilling in inhibitory spinal cord neurons. J Neurosci 28: 9755-9768

Roux MJ, Supplisson S (2000) Neuronal and glial glycine transporters have different stoichiometries. Neuron 25:373–383

Sagné C, Mestikawy S, Isambert MF, Hamon M, Henry JP, Giros B, Gasnier B (1997) Cloning of a functional vesicular GABA and glycine transporter by screening of genome databases. FEBS Letters 417: 177-183

Sattilaro RF (1986) Interaction of microtubule-associated protein 2 with actin filaments. Biochemistry 25: 2003-2009

Sato K, Yoshida S, Fujiwara K, Tada K, Tohyama M (1991) Glycine cleavage system in astrocytes. Brain Res 567:64 –70

Schousboe A, Waagepetersen HS (2006) Glial modulation of GABAergic and glutamatergic neurotransmission. Curr Top Med Chem 6: 929-934

Schmitt B, Knaus P, Becker CM, Betz H (1987) The Mr 93,000 polypeptide of the glycine receptor is a peripheral membrane protein. Biochemistry 26:805-811

Seiler N, Sarhan S (1984) Synergistic anticonvulsant effects of a GABA agonist and glycine. Gen Pharmacol 15: 367–9

Smith, KE, Borden LA, Harting PR, Branchek T, Weinshank RL (1992) Cloning and expression of a glycine transporter reveal co-localization with NMDA receptors. Neuron 8:927-935

Spike RC, Watt C, Zafra F, Todd AJ (1997) An ultrastructural study of the glycine transporter GLYT2 and its association with glycine in the superficial laminae of the rat spinal dorsal horn. Neuroscience 77: 543-551

Steiger JL and Russek SJ (2004) $GABA_A$ receptors: Building the bridge between subunit mRNAs, their promoters, and cognate transcription factors. Pharmacol Ther 101: 259-81

Stoppini L, Buchs PA, Muller D (1991) A simple method for organotypic cultures of nervous tissue. J Neurosci Methods 37(2): 173-182

Sulzer D, Pothos EN (2000) Regulation of quantal size by presynaptic mechanisms. Rev Neurosci 11: 159-212

Todd AJ, Watt C, Spike RC, Sieghart W (1996) Colocalization of GABA, glycine, and their receptors at synapses in the rat spinal cord. J Neurosci 16: 974-982

Toth E, Lajtha A (1984) Glycine potentiates the action of some anticonvulsant drugs in some seizure models. Neurochem Res 9(12): 1711-1718

Triller A, Choquet D (2005) Surface trafficking of receptors between synaptic and extrasynaptic membranes: and yet they do move! Trends Neurosci 28(3): 133-139

Triller A, Cluzeaud F, Pfeiffer F, Betz H, Korn H (1985) Distribution of glycine receptors at central synapses: an immunoelectron microscopy study. J Cell Biol 101: 683-688

Varoqui H, Zhu H, Yao D, Ming H, Erickson JD (2000) Cloning and functional identification of a neuronal glutamine transporter. J Biol Chem 275: 4049-4054

Vaz SH, Cristóvão-Ferreira S, Ribeiro JA, Sebastião AM (2008) Brain-derived neurotrophic factor inhibits GABA uptake by the rat hippocampal nerve terminals. Brain Res 2019: 19-25

Waagepetersen HS, Sonnewald U, Shousboe A (2003) Compartmentation of glutamine, glutamate, and GABA metabolism in neurons and astrocytes: functional implications. Neuroscientist 9, 398-403

Wang TL, Guggino WB, Cutting GR (1994) A novel γ-aminobutyric acid receptor subunit (ρ2) cloned from human retina forms bicuculline-insensitive homooligomeric receptors in Xenopus oocytes. J Neurosci 14: 6524-6531

Wojcik S, Katsurabayashi S, Guillemin I, Friauf E, Rosenmund C, Brose N, Rhee JS (2006) A shared vesicular carrier allows synaptic release of GABA and Glycine. Neuron 50(4): 575-587

Zafra F, Aragon C, Olivares L, Danbolt NC, Gimenez C, Storm-Mathisen J (1995) Glycine transporters are differentially expressed among CNS cells. J Neurosci 15(5):3952–3969

Ziv NE, Smith SJ (1996) Evidence for a role of dendritic filopodia in synaptogenesis and spine formation. Neuron 17: 91-102

9. APPENDIX

9.1. qPCR STANDARD AND MELTING CURVE ANALYSIS

For the analysis of the transcript expression, it was used the Pfaffl relative quantification method. This method requires the crossing point (CP) determination indicated by the threshold (red line) in the normalized fluorescence *vs* cycle plot (panels A in each figure). CP is defined as the point at which the fluorescence rises appreciably above the background fluorescence. 5-fold serial dilutions of cDNA net solution were used to create a standard curve for each analyzed gene (β-actin, GlyT2, GlyRα1, GlyRα2, GlyRα3 and GlyRβ).The standard curves were created by plotting CP *vs* the log concentration of cDNA (ng/μL) (panels B). The parameters calculated using the standard curves are also indicated (panels C). The amplification efficiency for the Pfaffl relative quantification method, E ($E = 10^{(-1/slope)}$), is calculated from the slope (m) of the standard curve and should present a value between 3.1 and 3.7. E(Corbett) is a parameter determined by the software which is a measure of the overall efficiency of the reaction and should present a value between 0.85 and 1.10. R^2 value of a standard curve represents how well the experimental data fit the regression line; that is, how linear the data are. The assessment of the reaction specificity was also evaluated by melting curve analysis (panels D). The visualization of one single peak in the Derivate (dF/dt) vs Temperature plot indicates a specific amplification on the targeted gene. All plots were created using Corbett Software (Corbett Life Science). The following figures illustrate qPCR standards and melting curves analysis for each analyzes gene.

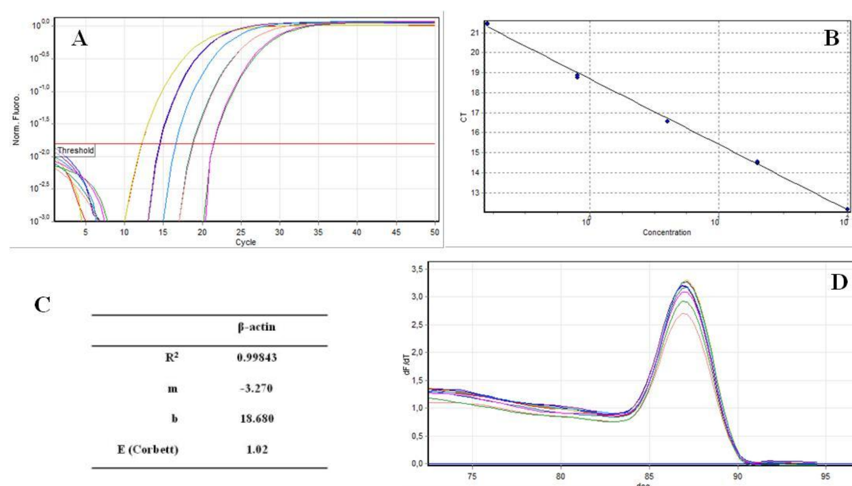
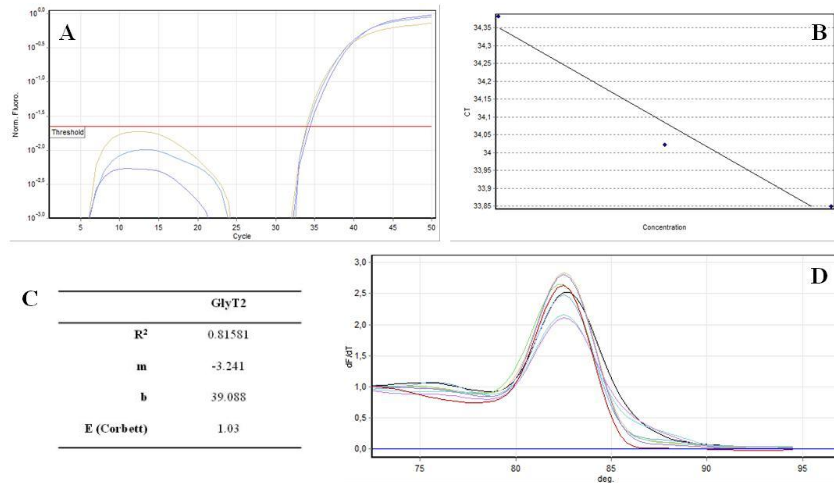
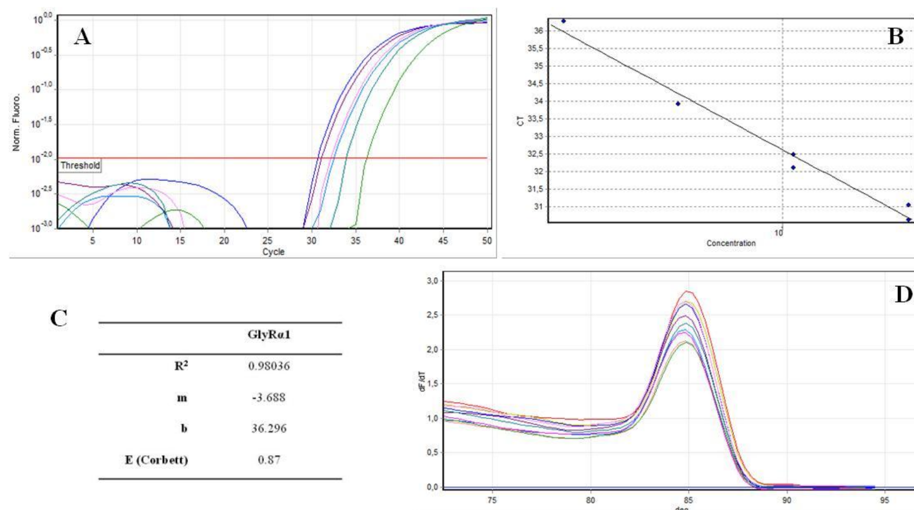


Figure 22. qPCR standard and melting curves analysis for β-actin – endogenous control. (A) PCR amplification plot for the β-actin gene. (B) Standard curve created by plotting CP vs the log concentration of cDNA (ng/μL). (C) Parameters calculated using the standard curve. (D) Assessment of the reaction specificity by melting curve analysis. All plots were created using Corbett Software (Corbett Life Science).



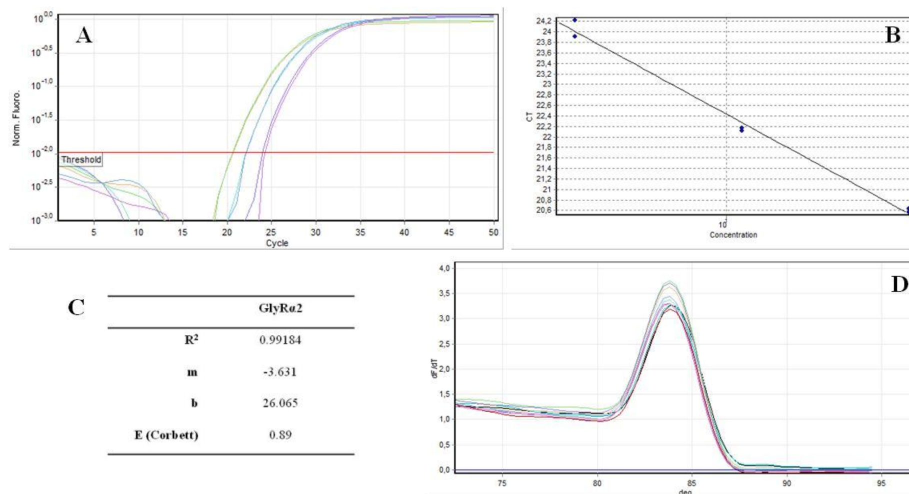
	GlyT2
R^2	0.81581
m	-3.241
b	39.088
E (Corbett)	1.03

Figure 23. qPCR standard and melting curves analysis for GlyT2. (A) PCR amplification plot for the GlyT2 gene. (B) Standard curve created by plotting CP vs the log concentration of cDNA (ng/μL). (C) Parameters calculated using the standard curve. (D) Assessment of the reaction specificity by melting curve analysis. All plots were created using Corbett Software (Corbett Life Science).



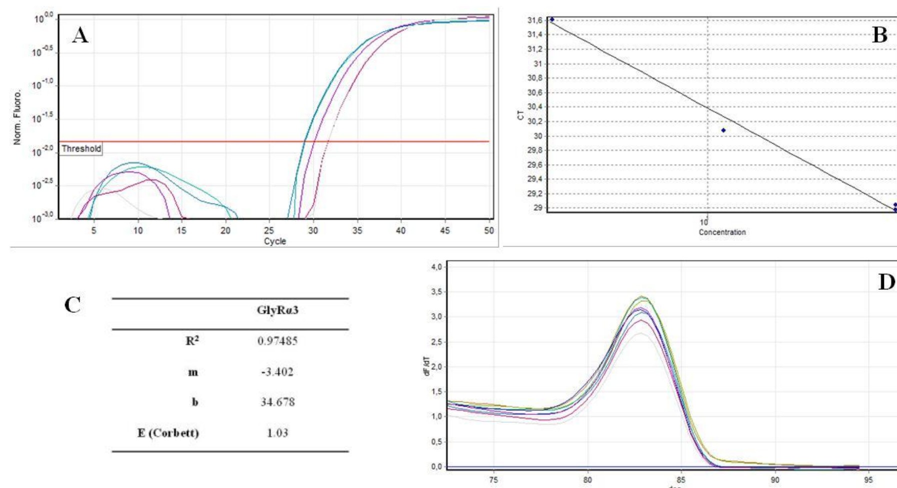
	GlyRα1
R^2	0.98036
m	-3.688
b	36.296
E (Corbett)	0.87

Figure 24. qPCR standard and melting curves analysis for GlyRα1. (A) PCR amplification plot for the GlyRα1 gene. (B) Standard curve created by plotting CP vs the log concentration of cDNA (ng/μL). (C) Parameters calculated using the standard curve. (D) Assessment of the reaction specificity by melting curve analysis. All plots were created using Corbett Software (Corbett Life Science).



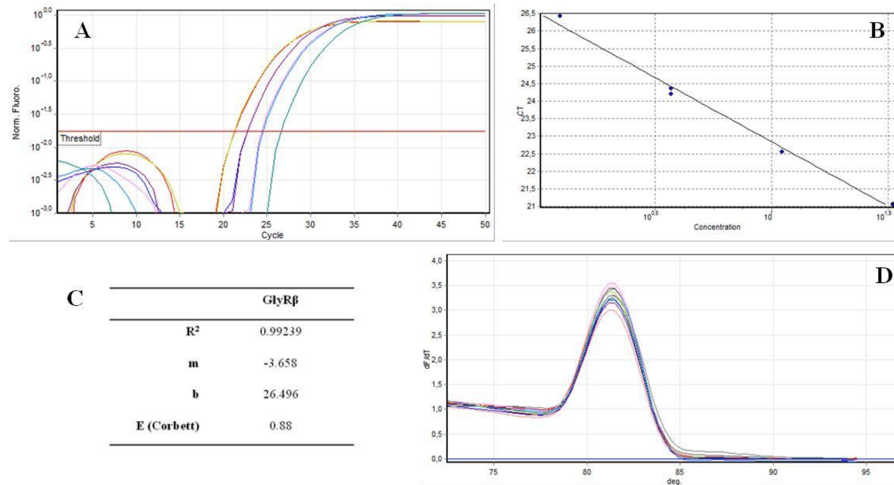
	GlyRα2
R^2	0.99184
m	-3.631
b	26.065
E (Corbett)	0.89

Figure 25. qPCR standard and melting curves analysis for GlyRα2. (A) PCR amplification plot for the GlyRα2 gene. (B) Standard curve created by plotting CP vs the log concentration of cDNA (ng/µL). (C) Parameters calculated using the standard curve. (D) Assessment of the reaction specificity by melting curve analysis. All plots were created using Corbett Software (Corbett Life Science).



	GlyRα3
R^2	0.97485
m	-3.402
b	34.678
E (Corbett)	1.03

Figure 26. qPCR standard and melting curves analysis for GlyRα3. (A) PCR amplification plot for the GlyRα3 gene. (B) Standard curve created by plotting CP vs the log concentration of cDNA (ng/µL). (C) Parameters calculated using the standard curve. (D) Assessment of the reaction specificity by melting curve analysis. All plots were created using Corbett Software (Corbett Life Science).



	GlyRβ
R^2	0.99239
m	-3.658
b	26.496
E (Corbett)	0.88

Figure 27. qPCR standard and melting curves analysis for GlyRβ. (A) PCR amplification plot for the GlyRβ gene. (B) Standard curve created by plotting CP vs the log concentration of cDNA (ng/µL). (C) Parameters calculated using the standard curve. (D) Assessment of the reaction specificity by melting curve analysis. All plots were created using Corbett Software (Corbett Life Science).