Functional characterization of glycine effects on oligodendroglial cell lines

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Abbreviations

BrdU = bromodeoxyuridine
CNPase = 2′,3′-cyclic nucleotide 3′-phosphodiesterase
CNS = central nervous system
DAB = diaminobenzidine
DMEM = Dulbecco's Modified Eagle Medium
DTT = dithiothreitol
eGFP = enhanced green fluorescent protein
FACS = fluorescent-activated cell sorting
FBS = fetal bovine serum
FCS = fetal calf serum
GABA = gamma-aminobutyric acid
GalC = galactocerebroside
Gluc. Acid = gluconic acid
GlyR = glycine receptor
GlyT = glycine transporter
MBP = myelin basic protein
MEM = minimum essential medium
MeOH = methanol
MS = multiple sclerosis
NCAM = neural cell adhesion molecule
NMDA = N-methyl-D-aspartic acid
OPC = oligodendrocyte precursor
P/S = penicillin/streptomycin
PDGFRα = platelet-derived growth factor receptor-α
PI = propidium iodide
PLL = poly-L-lysine
PLP = proteolipid protein
PSA = polysialylated
PVDF = polyvinylidene Difluoride
rpm = revolutions per minute
RPMI = Roswell Park Memorial Institute
RT = room temperature
SES = standard extracellular solution
SIS = standard intracellular solution
TM = transmembrane
VIAAT = vesicular inhibitory amino acid transporter

Abstract

In multiple sclerosis (MS) inflammatory reactions damage the oligodendrocytes and their myelin sheaths. Even though oligodendroglial progenitors are present in MS lesions, the remyelination and differentiation fails. Several studies have shown that glycine levels are altered in the cerebrospinal fluid and plasma of MS patients. A link between the failing of remyelination and the altered glycine levels could lead to new insights and therapeutic approaches in treating this disease. Like for other neurotransmitters there is growing evidence that glycine, beside its classical role in synaptic transmission, can have can influence on cell proliferation, differentiation and migration in the central nervous system. Studies have indeed shown that glycine can affect these processes in neuronal progenitors; however for oligodendroglial cells the picture is far less clear. It has been suggested that glycine can influence the development of oligodendroglial progenitors by inducing a depolarization via cys-loop ionotropic glycine receptors (GlyRs) and sodium (Na^+)-dependent transporters (probably glycine transporters, GlyTs). However, those studies were conducted in an oligosphere culture model and never received a clear confirmation in other models. Therefore we addressed this question using oligodendroglial cell lines. Patch-clamp results suggest that in the used cell lines mainly Na^+-dependent-like transporters are responsible for the glycine-induced inward current. In the MO3.13 and OLN-93 cells there was also a small contribution of GlyT2a's to the currents. When looking at the effect of glycine on cell proliferation, there was only an inhibitory effect on the MO3.13 cells. Since the glycine-induced current in the MO3.13 cells is approximately six times larger than those induced in the HOG and OLN-93 cells, it is likely that the currents in the latter two are too small to trigger any downstream events (proliferation). These results suggest that there can be a role for glycine in the proliferation of some, but not all, oligodendroglial cell lines. Verification with primary cell cultures will give more information concerning the use of these cell lines as model for oligodendendrocyte progenitors and the effect of glycine on oligodendroglial cell proliferation.

1 Introduction

The central nervous system (CNS), comprising the brain and the spinal cord, is a complex network which is composed of different cell types that can communicate with each other by means of several types of molecules. One type of molecules are the soluble signalling factors known as neurotransmitters. In the CNS, neurotransmitters principally function as soluble factors that mediate the communication between pre- and postsynaptic membranes of neurons. They are necessary for the correct propagation of action potentials down nerves to their target cell/organ and cell-cell communication, events that are essential for the correct functioning of the organism. Neurotransmitters are stored in vesicles in the presynaptic cell. Upon a change in the electrical potential of the cell membrane, the neurotransmitters are released by exocytosis and diffuse across the synaptic cleft to interact with molecules present on the post-synaptic cell membrane. This interaction will induce an effect (an electrical change) in the post-synaptic cell. After dissociation, the synaptic action of the transmitter is terminated by its degradation or reuptake in presynaptic nerve terminals and/or neighbouring glial cells.

The effect of neurotransmitters can be excitatory, inhibitory or modulatory. This depends on the neurotransmitter itself, the receptor it will bind and the ion concentrations in the post-synaptic cell and the extracellular environment. Besides their function in neuron-neuron communication, neurotransmitters could also have some extra-synaptic functions in the CNS. In this study we will focus on the ionotropic receptors (ligand-gated ion channels) and specific, high-affinity transporters of the neurotransmitter glycine.

1.1 Glycine as neurotransmitter

Of all the amino acids glycine is the smallest and simplest one. There are three sources for glycine in cells. The first possibility is that it is simply taken up from the extracellular environment. A second source, which is intracellular, is 3-phosphoglycerate, an intermediate of the glycolytic pathway. This can lead in a three-step reaction to serine which can be converted into glycine, with serine hydroxymethyltransferase as key enzyme. A third way to yield glycine is a condensation reaction that is regulated by glycine oxidase. Besides its functions in peptide and protein structures glycine also exerts functions in the central nervous system. Like gamma-aminobutyric acid (GABA), glycine is one of the main inhibitory neurotransmitters of the CNS. It mediates fast synaptic inhibition in the CNS by binding to and activating chloride (Cl^-) permeable, ligand gated-ionotropic receptors. But glycine can also participate in excitatory transmission as obligatory co-agonist of glutamate for the activation of the N-methyl-D-aspartate (NMDA) receptor (1, 2).

Once synthesized, glycine is stored in small, clear vesicles in the presynaptic cell. A change in the electrical potential (depolarization) of this neuron triggers the release of glycine into the synaptic cleft

by exocytosis. The transmitter can diffuse across this cleft to interact with molecules present on the membrane of the postsynaptic cell. To end its actions, glycine is removed from the synaptic cleft by transporters. Glycine can interact with its specific cys-loop ionotropic receptor and Na^+-dependent transporters (1, 3-5).

1.1.1 Glycine receptor

The inhibitory glycine receptor is a member of the nicotinicoid receptor superfamily, also known as cys-loop proteins. They are called cys-loop because of the presence of a conserved 13 amino acid signature sequence, flanked by cysteines. These cysteine residues form a disulfide bond which results in a characteristic loop in the extracellular ligand-binding domain of the receptor subunit structure (6, 7). Other members of this family are the nicotinic acetylcholine receptor and GABA type A receptor. All functional receptors of this superfamily are composed of five subunits and mediate fast synaptic transmission in the CNS. After binding of the ligand to the receptor, their channel transiently opens which allows passive movement of ions down their electrochemical gradient (7, 8). Embryonic and neonatal GlyRs are mainly α2 homomers (5*α2). After birth there is a switch to the adult receptor isoform, which are mainly α1-β heteromers (2:3 or 3:2 α1β). Studies on the GlyR showed that the receptor can contain two different subunits, the α (48-kDa) and β (58-kDa) polypeptides. There are four isoforms of the α subunit (α1-4), but the β subunit is only expressed in one form throughout the entire CNS. Alternative splicing of receptor RNA creates further diversity in the subunit structure. Different α subunits form functionally different GlyRs what results in different subconductance levels and such. Only the α subunit is required for ligand binding since the N-terminal of this subunit contains the binding sites for glycine. The β subunit is essential for linking the receptor to the anchoring protein gephyrin (3, 4, 8-13). Gephyrin is a 93-kDa cytoplasmic protein responsible for the segregation of the GlyRs. It functions as a postsynaptic intracellular scaffold as it can bind to the large intracellular (transmembrane domain 3-4) loop of the β subunit. In this way gephyrin anchors the receptor to the cytoskeleton as it binds to microtubules and allows aggregation in clustering on the postsynaptic membrane (Fig. 1). The different subunits are assembled into a GlyR due to assembly boxes in their N-terminal extracellular domains (7-9). Each subunit of the GlyR has four transmembrane (TM) domains; of which the TM2 domain forms the pore of the ionic channel. The extremities of TM2 transmembrane domain have flanking arginine residues that have been shown to determine the anion selectivity, namely chloride (Cl^-) (9). Binding of a ligand is coupled to the activation of the channel via interactions of membrane proximal loops of the extracellular domain with surface loops that link the TM segments. After binding of an agonist a part of the extracellular binding region rotates and passes through the cys-loop. This leads to contact between the β1-β2 loop and the TM2-TM3 loop which bends the TM2 region and opens the channel (4, 7, 14).

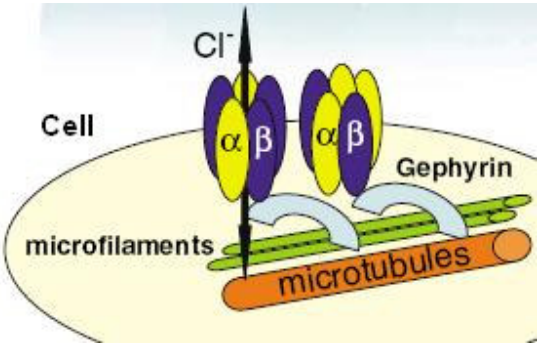


Figure 1. *The glycine receptor.* The GlyR is a pentameric protein that gates a Cl^- channel. The α subunit is necessary for ligand binding, the β subunit for linking the receptor to gephyrin. Gephyrin anchors the receptor to the cytoskeleton of the cell. Adapted from Bowery, 2006 (3).

The GlyR is thus a ligand-gated Cl^- channel. Binding of glycine to this receptor leads to conformational changes that increase the Cl^- conductance and result in a Cl^- current. Depending on the chloride reversal potential of the cell this will result in membrane depolarization or hyperpolarization. β-alanine and taurine are two known agonists for the GlyR. While strychnine acts as a high-affinity antagonist, binding the α subunit on a site overlapping the glycine binding site (4, 8, 9).

1.1.2 Glycine transporter

After glycine dissociates from the GlyR, the synaptic action of the transmitter is terminated by its reuptake in presynaptic nerve terminals and/or neighbouring glial cells. The neurotransmitter is cleared from the synaptic cleft by means of specific transporter systems that are located in neuronal and glial plasma membranes. Some of these transporters are members of the Na^+ and Cl^--dependent neurotransmitter transporter family (1, 9, 15). There are two known genes for high-affinity Na^+/Cl^- -coupled glycine transporters, GlyT1 and GlyT2, and each of the transporters has a their own isoforms (1, 2, 5, 9, 16). The GlyT1 is mainly expressed by glial cells, whereas the GlyT2 is mainly expressed by neurons (5, 9). The GlyTs are characterized by twelve putative transmembrane domains, intracellular hydrophilic amino- and carboxyl-termini and a large extracellular glycosylated loop (Fig. 2). The binding of glycine, Na^+ and Cl^- to the transporter induces a conformational change that allows interconversion and translocation of glycine and the ions to the other side of the membrane. The stoichiometry of these Na^+/Cl^--coupled transporters results in an electrogenic operation of the system that is strongly dependent on the ion gradients and membrane potential. The GlyT1 transporter has a stoichiometry of $2Na^+/1Cl^-/1$glycine; this presumes that it can reverse its mode of action and also export glycine depending on the intracellular Na^+ concentration. The GlyT2 transporter has a stoichiometry of $3Na^+/1Cl^-/1$glycine, which makes it unlikely that it will reverse in function and export glycine by changes in intracellular ion concentration (1, 2, 5, 9, 16-18). Since the reuptake of glycine is coupled to the electrochemical gradient of Na^+ it is a secondary active process. The plasma membrane Na^+/K^+-ATPase generates and maintains this Na^+ concentration gradient. Research of Zafra

et al. suggested that regulatory interactions between neurons and glial cells are necessary to modulate the expression of plasma membrane transporters (19). The sarcosine derivative ALX-5407 is a potent and selective inhibitor of the GlyT1 and the tricyclic antidepressant amoxapine selectively inhibits the GlyT2a in a dose-response manner (5, 20, 21).

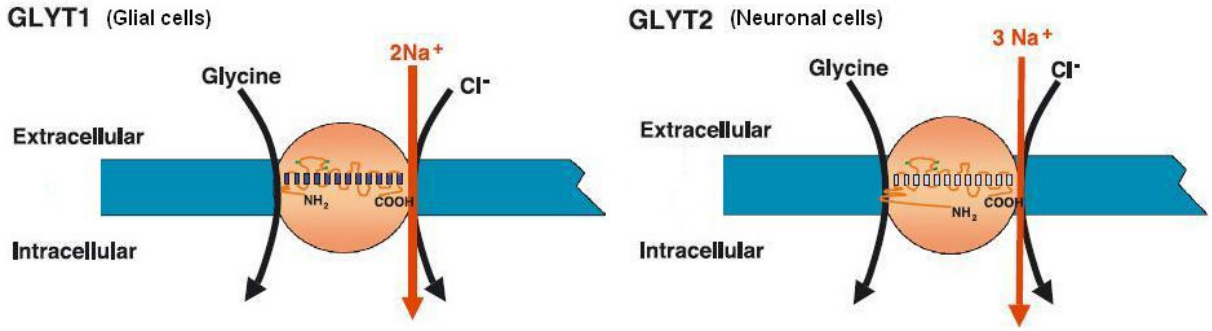


Figure 2. *The Na^+/Cl^--coupled glycine transporters.* GlyT1 is mainly expressed by glial cells and the GlyT2 mainly by neuronal cells. Both GlyTs transfer glycine together with Na^+ and Cl^- into the cell. Adapted from Aragón, 2003 (2).

Once glycine is taken up by the neuron or glial cell, it can be re-packaged into synaptic vesicles or hydrolysed via the glycine cleavage system. The vesicular inhibitory amino acid transporter (VIAAT) is the carrier protein that is responsible for the storage and re-packaging of glycine in presynaptic vesicles. VIAAT is located in the vesicular membrane at the nerve endings of glycine-releasing neurons. It is a secondary active transporter that exchanges protons (H^+ ions) for cytosolic glycine. The H^+ ions are pumped from the cytosol into the vesicle by another vesicular membrane protein, the V-type H^+-ATPase pump (1, 3).

1.2 Oligodendrocyte features and development

Oligodendrocytes are the myelinating cells of the CNS. Their primary function is to provide myelin sheaths around neuron axons which insulates the axon and increases the conduction of nerve impulses (22-24). One oligodendrocyte extends multiple processes; each process repeatedly wraps a part of an axon and this multi-spiral membrane-forming myelin subsequently condensates. The myelin sheath is periodically interrupted which leaves a small part of the axon exposed, the node of Ranvier, which results in the salutatory conduction of the nerve impulses down the axon. In the CNS one oligodendrocyte provides myelin sheaths for different neuron axons, so one axon is myelinated by several oligodendrocytes. Besides forming myelin sheaths for axons, the oligodendroglial cells also have other functions. They induce clustering of Na^+ channels at the node of Ranvier, participate in regulating axonal diameter and the maintenance of axons and are able to inhibit axonal growth and regeneration (22). Oligodendrocytes are also involved in maintaining the homeostatic environment of the CNS, balancing ion concentrations and recycling neurotransmitters and they play a role in the pH regulation and the iron metabolism of the brain (22, 25).

The oligodendrocyte precursors (OPC) originate from neuroepithelial cells of ventricular zones at early stages in embryonic life. In the spinal cord the oligodendrocytes originally arise in ventral regions of the neural tube and afterwards migrate dorsally. During the following development these dorsal regions gain the capacity for oligodendrogenesis. This early restricted localization of OPC in the ventral plate of the neural tube is also observed in the mid- and forebrain, later in the development oligodendrocytes originating from dorsal sources replace these ventral derived progenitors. The subventricular zone, which is present in the late gestational and early postnatal forebrain, is also a region where OPC arise from neuroepithelial cells (22, 26-28). Subsequently the OPC migrate and populate the developing CNS before they differentiate into myelin-forming oligodendrocytes. For the control of their migration and process extension the oligodendrocytes need a number of extracellular matrix molecules, like matrix metalloproteinases, and the expression of specific molecules, like the polysialylated (PSA) form of the neural cell adhesion molecule (NCAM) (22, 29).

The maturation of oligodendrocytes in the CNS (Fig. 3) mostly takes place in the early postnatal life and is under the control of different transcription factors (22). The earliest stage of oligodendrocyte development is the precursor stage that expresses the PSA-NCAM molecule and intermediate filament protein nestin; these cells have a high migratory and proliferative capacity. The progenitor cell, also known as the O-2A cell, represents the next stage. It is a bipolar cell expressing the A2B5 antigen (a glycolipid), the chondroitin sulphate proteoglycan NG2, the glycolipid GD3 and the platelet-derived growth factor receptor-α (PDGFRα). These cells proliferate actively and posses high migratory properties. The progenitors further mature into pre-oligodendrocytes, which are multiprocessed cells and have acquired expression of the O4 antigen (a sulfatide). At this stage the expression of nestin is downregulated, the cell becomes less motile and loses its mitogenic response to PDGF. The next stage in the oligodendrocyte development is that of immature oligodendrocyte. This stage is characterized by the loss of GD3 and A2B5 expression but acquirement of the appearance of galactocerebroside (GalC) and 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase). Next the cell further matures into a non-myelinating oligodendrocyte; at this stage other myelin-specific markers start to appear. Myelin basic protein (MBP) is expressed along with proteolipid protein (PLP) as the cell develops into a myelinating mature oligodendrocyte (22, 25, 26, 30).

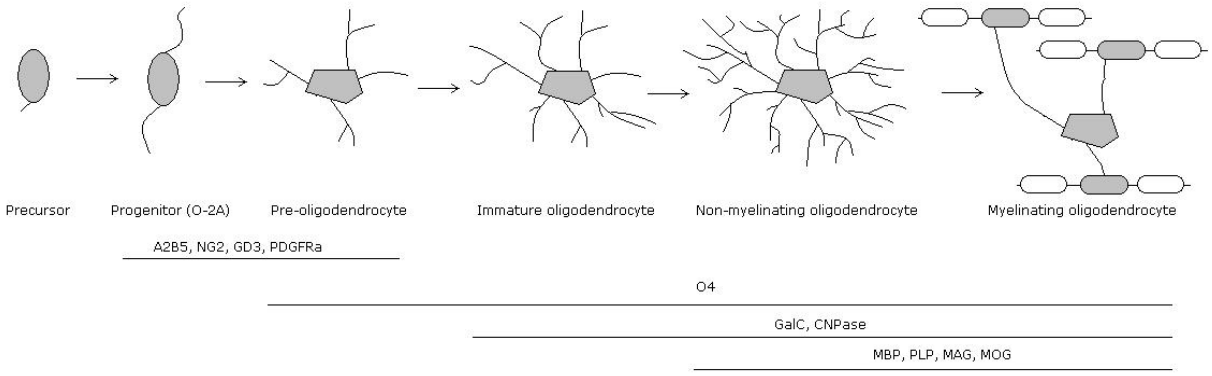


Figure 3. *Maturation of the cells of the oligodendrocyte lineage.* The cells become more arborised as they advance throughout cell development. Each stage is characterised by the expression of different markers. A2B2: ganglioside staining antibody; NG2: proteoglycan; GD3: glycolipid; PDGFRa: platelet-derived growth factor receptor-α; O4: sulfatide and glycolipid staining antibody; GalC: galactocerebroside; CNPase: 2',3'-cyclic nucleotide 3'-phosphodiesterase; MBP: myelin basic protein; PLP: proteolipid protein; MAG: myelin associated glycoprotein; MOG: myeling oligodendrocyte glycoprotein (22, 25, 30).

Oligodendrocyte progenitor cells remain present in the adult CNS, which allows oligodendrogenesis when needed. A part of the population of NG2-expressing cells is thought not to differentiate further along the oligodendrocyte lineage but to remain present in the mature CNS as an immature phenotype. This heterogeneous population, which is spread throughout the entire CNS, does not resemble embryonic or neonatal NG2 positive cells but represents another type of glial cell that can proliferate and develop into oligodendrocytes (28, 31). Beside this pool of NG2-expressing cells there are also germinal zones present in the adult CNS, the subgranular zone and the subvertricular zone, that contain oligodendroglial precursors (27, 32).

1.3 Glycine effects on oligodendroglial cells

Several studies have shown that neurotransmitters, beside their classical role in synaptic transmission, can influence neural cell proliferation, differentiation and migration. Some neurotransmitters, including glycine, have been shown to regulate different processes at specific stages of neuronal cell development by activating their ionotropic receptor(s) (10, 11, 13, 23, 33-37). For oligodendroglial cells however, the picture is far less clear.

Oligodendrocytes are non-excitable cells, as they are not capable of repeatedly firing action potentials, nevertheless it has been shown that these cells express a variety of voltage-gated and ligand-gated ion channels. There is some evidence that their electrophysiological properties could have an influence on their development. Several studies showed that oligodendroglial cells express GlyRs even before synaptic networks have been established in the CNS. Research of Belachew et al., on oligosphere-derived oligodendroglial precursor cells, identified presence of GlyRs on these cells and showed that this expression decreases with progressive differentiation of the oligodendroglial cells. They also

reported that the GlyRs that were activated in this model were pharmacological different from the neuronal isoform as they have two strychnine binding sites and are sensitive for picrotoxinin and cyanotriphenylborate, despite the expression of the β and α2 subunits (10, 35, 36).

Glycine is known as an inhibitory neurotransmitter but in oligodendrocytes it can induce a depolarization. Oligodendrocytes have a furosemide sensitive $Na^+/K^+/2Cl^-$ uptake system and a Cl^-/HCO_3^- exchanger that maintains a high intracellular Cl^- concentration. As a result the Cl^- reversal potential is more positive than the resting potential and as consequence the GlyR activation-induced increase of Cl^- conductance will result in a depolarization (10, 23, 33, 34, 38). Glycine can also interact with specific transporters. The uptake of glycine by Na^+ dependent transporters is an electrogenic uptake process that can also result in a depolarization (16, 34). Research of Belachew et al. on oligodendroglial cells derived from the oligosphere culture model has shown that glycine can induce a depolarization, caused by GlyRs and Na^+–dependent transporters, which can lead to an increase of intracellular calcium. Calcium is known to be an essential secondary messenger; it could trigger downstream events which are important for the generation of mature oligodendrocytes (34, 35, 39).

1.4 Goal of the project

Up to now, glycine has been suggested to influence the development of oligodendroglial cells by interacting with cys-loop ionotropic GlyRs and Na^+-dependent transporters, probably GlyTs. However, those studies were conducted in an oligosphere culture model and never received a clear confirmation in other models. Therefore we addressed this question in this project, using oligodendroglial cell lines and mouse primary cells.

The presence of GlyRs and GlyTs at the cell membrane surface will be determined by means of immunocytochemistry, biotinylation and subsequent Western blotting. The functionality of the identified membrane proteins will be determined by use of the whole-cell patch-clamp technique. Finally, the effect of glycine on oligodendroglial precursor proliferation will be examined. This proliferation will be quantified by bromodeoxyuridine (BrdU) incorporation. All experiments will be conducted on three oligodendroglial cell lines. The used cell lines are a model of oligodendrocytes arrested in an immature developmental stage. The HOG and MO3.13 are both human clonal oligodendroglial cell lines. The HOG cell line was established from a surgically removed oligodendroglioma. Whereas the MO3.13 cell line originates from a fusion of a 6-thioguanine-resistant mutant of the human rhabdomyosarcoma RD with adult human oligodendrocytes, which were cultured from surgical specimen. The OLN-93 cell line is derived from spontaneously transformed cells in primary rat brain glial cultures (40, 41). Because the cell lines are composed of immortalized/cancerous cells their origin can influence their results, because of this the results will

have to be verified in a primary culture. The primary culture will be derived from transgenic mice pups (1 day old) that express enhanced green fluorescent protein (eGFP) under the 2'-3'-cyclic nucleotide 3'-phosphodiesterase (CNP) promoter. This will allow creating a pure oligodendroglial culture by means of fluorescent-activated cell sorting (FACS) (42).

The results of this project will contribute to the academic study of the inhibitory neurotransmitter glycine in the CNS. They will lead to more insight into its role and function with respect to stimulation of oligodendrocytes and their progenitors. In this way the results can incite to new research that explores the newly discovered effects of this inhibitory neurotransmitter and aims at finding possible clinical applications, since it is known that oligodendrocyte progenitors remain present in the adult CNS. A role in the treatment of MS is an example of such application. MS is a chronic inflammatory disease of the CNS, which results in inflammatory damage to myelin, oligodendrocytes and neurons. Studies have shown that oligodendrocyte progenitors are present in the demyelinated lesions of MS patients, however remyelination fails and differentiation of these progenitors is impaired (43, 44). Other studies showed that the plasma and cerebrospinal fluid glycine levels were altered in MS patients (45, 46). A better understanding of the effect of glycine on oligodendrocyte progenitor proliferation could thus lead to more insight and help the development of better therapeutic strategies to treat the damage observed in MS.

2 Materials and methods

2.1 Cell lines

HOG and MO3.13 are both human clonal oligodendroglial cell lines, the OLN-93 cell line is rat-derived (40, 41). The cells were grown in 25 cm² culture flasks (Nunc™, Roskilde DENMARK) or on ø 12 mm glass coverslips (Thermo Scientific Menzel-Gläser, Braunschweig GERMANY), in a 90% air-10% CO_2 humidified atmosphere at 37°C. When they reached confluence they were passaged and they were harvested by using trypsin 0,25% (GIBCO®, Invitrogen™, Belgium). The MO3.13 and OLN-93 cell lines were cultured in glycine free minimum essential medium (MEM) (GIBCO®, Invitrogen™,Belgium) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Biochrom AG, Berlin, Germany) and 1% penicillin/streptomycin (P/S) (GIBCO®, Invitrogen™, Belgium). The MO3.13 cell line was cultured in a poly-L-lysine (PLL) 50µg/ml (diluted in sterile MilliQ) coated culture flask. When experiments started the medium was switched to MEM with 0% FCS. The HOG cell line was cultured in Roswell Park Memorial Institute (RPMI) medium (GIBCO®, Invitrogen™, Belgium), supplemented with 10% FCS and 1% P/S. For proliferation experiments, the cells were cultured in medium without glycine and 0% FCS.

2.2 Primary culture

The primary cell culture was prepared using a modified isolation procedure of McCarthy et al. 1980 (47). The growth medium used for the primary cell culture was O_2A medium; Dulbecco's Modified Eagle Medium (DMEM) (GIBCO®, Invitrogen™) supplemented with 10% FCS (not heat inactivated), 1% L-glutamine en 1% P/S. The primary culture was derived from brains isolated from CNP-mice pups at postnatal day 1 or 2. The brain stem, cerebellum and meninges were removed and the two cerebral hemispheres were transferred to a sterile container with 3 ml growth medium. Next the brain lobes were mechanically homogenized by cutting them with a small scissor and subsequently passing the tissue through an 18, 21 and 22 Gauge needle (each three times) in a little amount of culture medium. This homogenized tissue was transferred to a 15 ml falcon tube together with 5 ml culture medium. The cell suspension was centrifugated at 800 revolutions per minute (rpm) for 10 minutes at room temperature (RT). The cell pellet was resuspended in 10 ml growth medium and plated out in a 5µg/ml PLL-coated culture flask with screw-on cap without filter. Cells were grown in a 95% air-5% CO_2 humidified incubator at 37°C. The growth medium was changed every 4 days and at day 14 the shake-off procedure started. Culture flasks were shaken overnight for 18-24h at 240 rpm at 37°C to shake off the oligodendroglial cells. Subsequently the supernatant was collected in a petri dish that was placed in the incubator for 15 minutes. During this step the remaining astrocytes and microglial cells were removed by letting them adhere to the bacterial dish. Afterwards the supernatant was again collected and centrifuged at 800 rpm for 5 minutes at RT. The oligodendroglial cells were present in

the pellet. Since the cells were obtained from mice expressing eGFP under the CNP promoter, FACS was used to gather a pure oligodendroglial cell suspension.

2.3 mRNA isolation and PCR

Cells were allowed to grow in culture flasks and harvested; next the total RNA was isolated with the RNeasy Plus Mini kit (Qiagen), following the manufacturer's protocol. Cells were collected by trypsinisation and a pellet was obtained after centrifugation. Next the cells were disrupted by adding lysis buffer and genomic DNA was eliminated with the eliminator spin column. The sample was transferred to the RNeasy spin column and after consecutive washing steps the total RNA was obtained. cDNA was synthesized using the reverse-transcription system (Promega). PCRs were performed by using primers specific for the GlyR subunits, gephyrin, GlyT1 and GlyT2. PCR products were then visualized on a 2% Agarose TAE gel containing ethidium-bromide.

2.4 Immunocytochemistry

Cells were grown on coverslips in medium supplemented with 10% fetal bovine serum (FBS) and fixed with 4% para-formaldehyde for 15 minutes. The cells were permeabilized with 0,5% Triton-PBS for 10 minutes. Next the cells were washed and blocked with 2% goat serum. The coverslips were then further incubated with either mAb4a (GlyR; 1:200) (Synaptic Systems GmbH, Goettingen GERMANY), Cl3B11 (gephyrin; 1:200) (Synaptic Systems GmbH, Goettingen GERMANY) or GlyT11-A (1:1000) (AB1770, Chemicon International Inc.) for 30~60 minutes. Afterwards they were washed and incubated with their respective secondary antibodies (1:1000) for 1 hour. Subsequently they were washed and mounted with ProGold Antifade (Invitrogen). The cover slips were placed on the stage of the Zeiss inverted Laser Scanning Confocal Microscope 510 META system attached to an Axiovert 200M for further imaging.

2.5 Electrophysiology

Cultures for the whole-cell patch-clamp recordings were seeded and grown on ø 12 mm glass coverslips. The coverslips were mounted onto a perfusion chamber and placed onto the stage of a Nikon (JAPAN) contrast microscope where they were held at RT. The bathing solution was gravity fed in the perfusion chamber which was continuously perfused, permitting the application of drugs. The volume of the bath was kept constant by suction by a needle that was connected to a vacuum discarding system. The reference electrode was placed in a separated part of the perfusion bath filled with extracellular solution and was connected to the recording part by means of an agar bridge. The recording and reference electrode were both made of chlorated silver wires. The microscope was placed onto a vibration free table in a Faraday cage. The patch electrodes were made of borosilicate

glass with a length of 100mm, outer diameter of 1,5mm and wall thickness of 0,315mm (Hilgenberg, Malsfeld GERMANY). The electrodes were pulled with a P-97 Flaming/Brown (Heat 555 – Pull 0 – Velocity 15 – Time 250 – Pressure 500) or a P-1000 type (Heat 564 – Pull 0 – Velocity 20 – Delay 1 – Pressure 600) micropipette puller (Sutter Instrument Company, USA). The resistance of the electrodes ranged between 2 and 5 MΩ. The electrodes were placed on the head-stage of the amplifier, and their movement was controlled by a hydraulic micromanipulator (Narishige, JAPAN). A fast application perfusion system was used to deliver the neurotransmitters to the cell. The exchange of solutions was achieved by rapidly moving the interface across the cell.

Oligodendroglial cells were perfused at RT, the standard extracellular solution (SES) contained (in mM): 145 NaCl; 5 KCl; 1,5 MgCl2; 2 CaCl2; 10 HEPES; pH 7,4; osmolarity 310 mOsm. The neurotransmitter glycine (Acros Organics, Geel BELGIUM) was applied by a microperfusion system at 10 mM in SES. In the Na^+-free extracellular solution NaCl was substituted with an equimolar concentration of Choline chloride and in the Cl^--free solution Cl^- was substituted with an equimolar concentration of gluconic acid. Again, glycine was applied at 10 mM in the Na^+-free and Cl^--free extracellular solution. ALX-5407 was applied at 10 nM (20), strychnine at 10 µM and 1 µM (10, 48) and amoxapine at 100µM (21, 48) in the extracellular solution. All blockers were purchased from Sigma (Sigma Aldrich, Steinheim GERMANY), unless mentioned otherwise. The inhibitors ALX-5407 and strychnine were a kind gift from Dr. J Van den Eynden. Recording pipettes were filled with standard intracellular solution (SIS). Depending on the cell line and/or type of patch-clamp experiment a different type of SIS was used. SIS 1 containing (in mM): 125 KCl; 5 NaCl; 2 $CaCl_2$; 10 HEPES; 10 EGTA; 2,5 MgATP; 2,5 Na_2ATP; pH 7,4; osmolarity 290 mOsm. Or SIS 2 containing (in mM): 130 CsCl; 1 $CaCl_2$; 1 $MgCl_2$; 1 MgATP; 10 HEPES; 10 EGTA; 10 Glucose; pH 7,2; osmolarity 299 mOsm. HOG and MO3.13 cells were patched with SIS 1 when testing the effect of the different blockers and extracellular solutions. The OLN-93 cells were patched with SIS 2 for the experiments with the blockers and extracellular solutions. For the making of the current-voltage (IV) relationship SIS 3 was used, this solution is the same as SIS 1 except KCl was replaced with an equimolar concentration of cesium chloride (Fig. 4).

Figure 4. *Protocol for the current-voltage relationship*. The control and 10 mM glycine test condition are applied in the same trace. The test potential increased with 20 mV after every trace, starting at -100 mV up to +80 mV.

Voltage-clamp recordings were performed with an EPC9 or EPC10 HEKA amplifier, using the whole-cell recording configuration. Series resistance were in the range of 5-20 MΩ and were electronically compensated. Current traces were digitized and stored on an AT-compatible computer system.
The effect of the tested substances was always compared to the control, being 10mM glycine.

2.6 Biotinylation

To differentiate between membrane bound and intracellular proteins, biotinylation of the cell surface proteins was performed with the Pierce® cell surface protein isolation kit (Thermo Scientific, Illinois USA). For the biotinylation the cells were grown in petri dishes for two days. Cells were biotinylated for 30 minutes at 4°C. This reaction was quenched by adding the quenching solution and cells were harvested with a cell scraper. Subsequently the cells were lysed for 30 minutes on ice and the biotinylated proteins were isolated from the intracellular, unbound proteins by means of the NeutrAvidinTM Resin (Fig. 5). The protein concentration in the samples was determined by use of the BCA protein assay (Pierce). The bound, biotinylated proteins were eluted with SDS-PAGE buffer and Dithiothreitol (DTT).

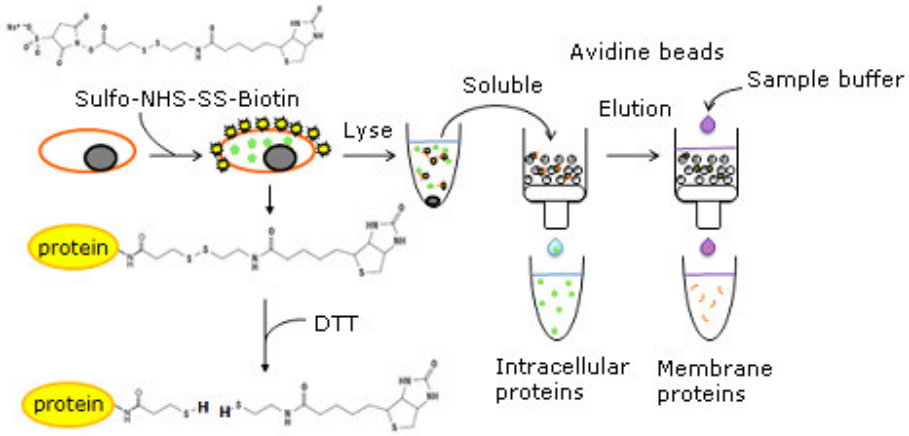


Figure 5. *Biotinylation protocol*. Biotin binds the membrane proteins and allows them to be separated from intracellular proteins by means of avidine beads. Adapted from S SahebAli and Pierce® cell surface protein isolation kit.

2.7 Western blotting

Whole cell lysates were made by dissolving cells in lysis buffer and incubating them for 30 minutes on ice. The protein concentration of the cell lysates were determined by using the BCA protein-assay (Pierce). The proteins were resolved on a SDS-PAGE gel and then transferred to a Polyvinylidene Difluoride (PVDF) membrane (Invitrogen). For the Western blot of the different fractions obtained with the biotinylation protocol, sample buffer was first added to the samples and then they were boiled for

10 minutes at 70°C prior to loading. Proteins were separated by a NuPAGE® Novex 4-12% Bis-Tris Gel (Invitrogen) and electro-transferred to a PVDF membrane. The membranes were washed with methanol and PBS before the membrane was blocked with milk solution, for 1 hour, to avoid non-specific protein binding. Subsequently the PVDF membranes were incubated for 1 hour with the primary antibody being either mAb4a (GlyR; 1:650) (Synaptic Systems GmbH, Goettingen GERMANY), Cl3B11 (gephyrin; 1:1400) (Synaptic Systems GmbH, Goettingen GERMANY), GlyT11-A (1:1000) (Alpha Diagnostic Intl. Inc, Texas USA) or β-actin (1:2000) (Santa Cruz Biotechnology, Inc.). After washing with PBS-Tween (0.1%) the blots were incubated for 1 hour with HRP-labeled secondary antibody: rabbit anti-mouse (1:1000) or goat anti-rabbit (1:2000) (Dako). Again the PVDF blots were washed with PBS-Tween (0.1%) and the bands were visualized using a diaminobenzidine (DAB) solution (Sigma Aldrich). The colour reaction was stopped by rinsing the blots with distilled water.

2.8 Proliferation assay

For the proliferation assay the cell proliferation ELISA, BrdU (colorimetric) kit of Roche Diagnostics GmbH was used. Cells were seeded in a flat bottomed 96 well mircotiterplate and allowed to grow for 1 day in their normal growth medium. Subsequently, the cells were stimulated with the test substance which contained 10 mM glycine or 1% PBS (control). The BrdU labelling solution was added to a final concentration of 10 µM. The MO3.13 cells were incubated for 17 hours and the HOG and OLN-93 cells for 4 hours with the BrdU solution. Afterwards the cells were fixed for 30 minutes and 45 minutes labelled with anti-BrdU-POD working solution, finally the substrate solution was added. The absorbance was measured with a FLUOstar OPTIMA plate reader (BMG Labtech) by using two excitation filters: 370 nm and 490 nm, no emission filters were used. Afterwards cells were washed with PBS and incubated in the dark with 4 µM propidium iodide (PI) for 15 minutes. After washing with PBS, the cells were excitated at 540 nm and emission was measured at 612 nm with the plate reader. The BrdU results were corrected for cell number by calculating the ratio of BrdU and PI staining.

2.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism software. The effect of the test substances was always compared to the control. All values are shown as mean ± standard error mean, with N being the number of experiments. Statistical tests with a p-value $< 0,05$ were considered significant. Asterisks where used to indicate the level of significance, * $p < 0,05$; ** $p < 0,01$ and *** $p < 0,001$.

3 Results and discussion

3.1 mRNA expression of glycine-associated proteins

To look for the presence of glycine-associated protein mRNA in the cell lines, total RNA was isolated from the cells and cDNA was synthesized. Primers specific for the GlyR subunits, gephyrin and GlyT genes were used to amplify their respective cDNAs. The PCR results (Fig. 6) showed presence of the GlyR α4 and β subunit mRNA transcripts in the OLN-93 cell line. For the HOG and MO3.13 cells mRNA transcripts for the GlyR subunits α2, α4 and β were present. In all three cell lines mRNA for gephyrin, a GlyR anchoring protein, and GlyT1 was detected. All cell lines were negative for GlyT2 mRNA. An extract from rat spinal cord was used as a positive control.

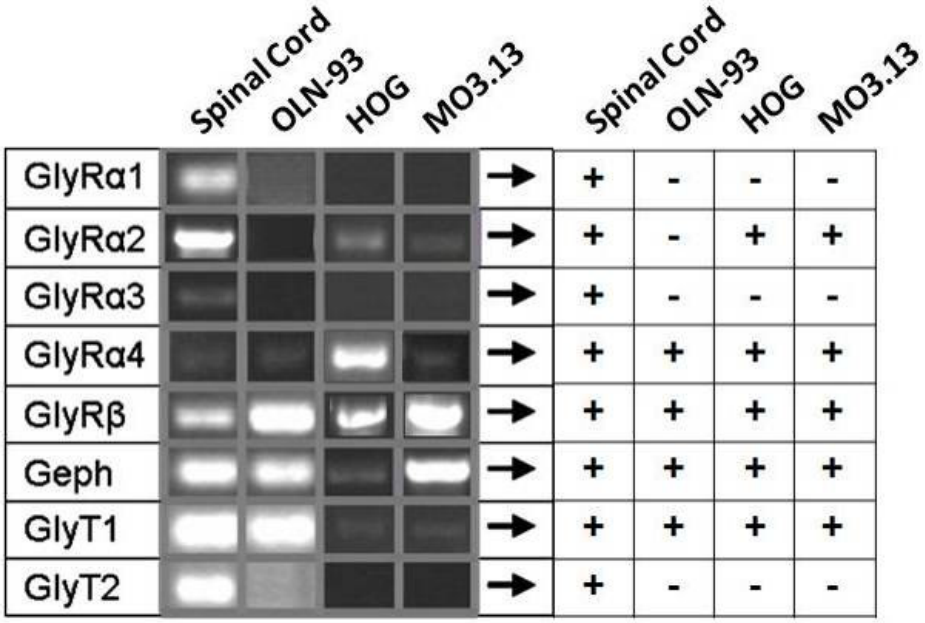


Figure 6. *PCR for glycine-associated proteins.* PCR reveals the presence of GlyR subunits α2, α4 and β, gephyrin and GlyT1 in the HOG and MO3.13 cell line and GlyR subunits α4 and β, gephyrin and GlyT1 in the OLN-93 cells. Adult rat spinal cord extract is used as a positive control.

These results suggest that the mRNA transcripts of some glycine-associating proteins are present in the used cell lines. To confirm the production of their respective proteins, immunocytochemical staining and Western blot experiments were conducted next.

3.2 Glycine-associated protein expression in the oligodendroglial cell lines

To investigate whether the mRNA transcripts were translated into the corresponding proteins, the protein expression pattern of the GlyT1, GlyR and the anchoring protein gephyrin was visualized by means of Western blotting and immunostainings. The protein extracts derived from whole-cell lysates were labeled after Western blotting with antibodies specific for the different glycine-associated proteins. The protein extracts of the three tested cell lines could be labelled after Western blotting by antibodies recognising the GlyR α and β subunit, the anchoring protein gephyrin and GlyT1 (Fig. 7).

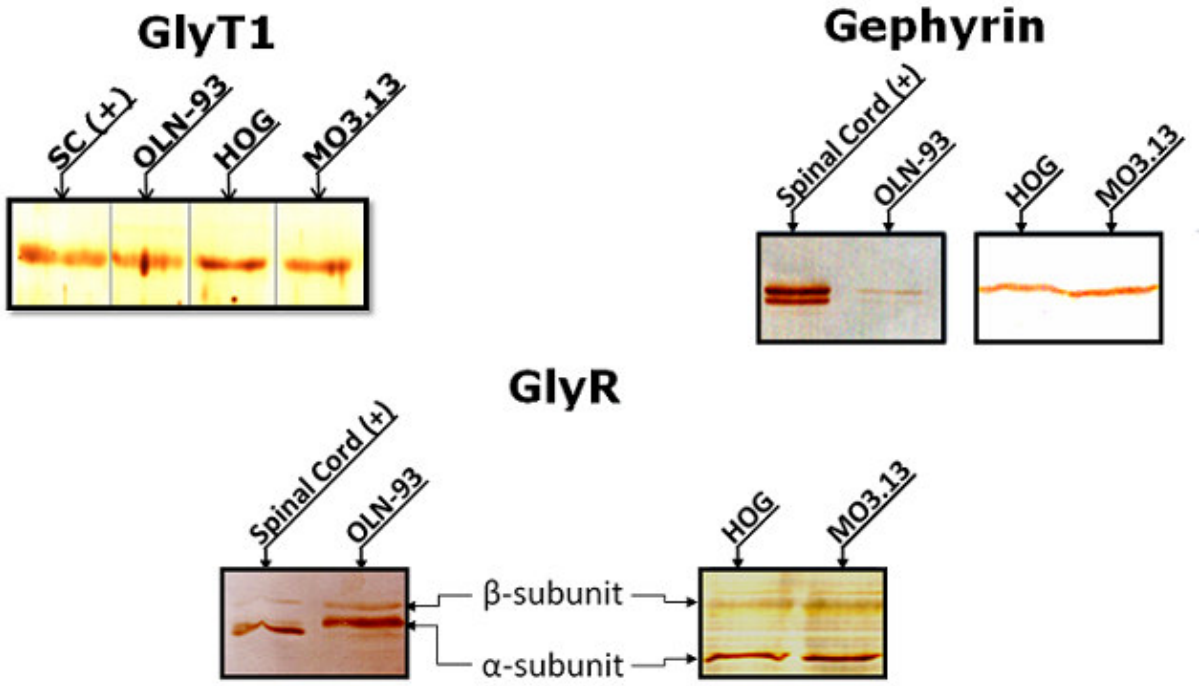


Figure 7. *Protein expression patterns of glycine-associated proteins*. All three cell lines express the GlyR α and β subunit, the GlyR anchoring protein gephyrin and the GlyT1. Rat spinal cord is used as a positive control.

These results suggest that the GlyR, gephyrin and GlyT1 proteins are present in the tested cell lines. For the immunocytochemical stainings, the cells were grown on coverslips and stained as described in the section material and methods. All three cell lines appeared positive for the tested antibodies (Fig. 8).

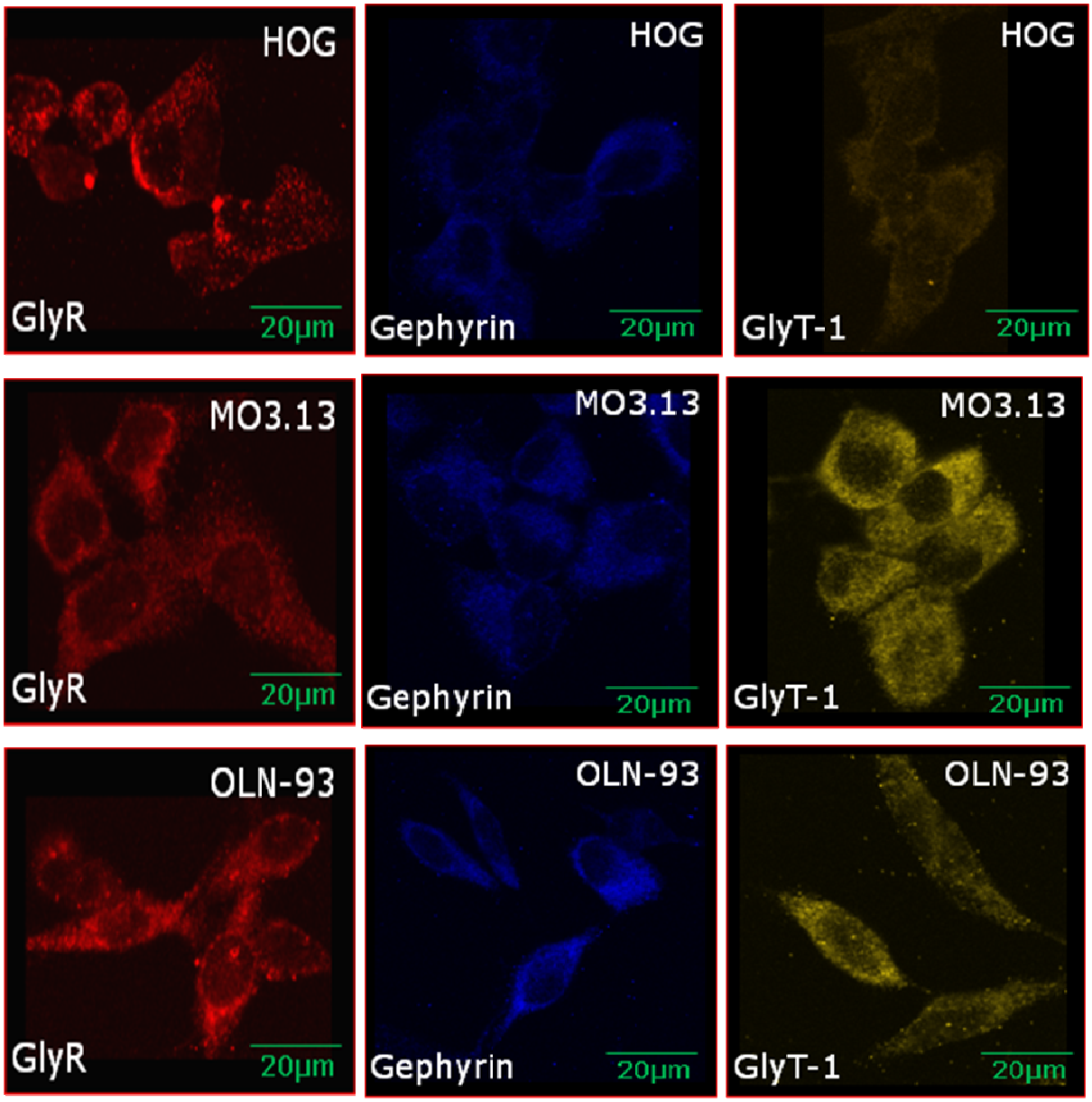


Figure 8. *Immunocytochemical staining of glycine-associated proteins*. Cells are respectively incubated with anti-GlyT1 (GlyT11-A), anti-GlyR (mAb4a) and anti-gephyrin (Cl3B11) antibodies. Binding of primary antibodies is visualized using a fluorescently labeled secondary antibody. All three cell lines appeared positive for the tested antibodies.

These results confirm the presence of the GlyR, gephyrin and GlyT1 proteins in the three cell lines. However no clear membrane localization was found for the GlyT1 and GlyR proteins with these two methods. Therefore the patch-clamp technique was applied next. This technique will allow determining whether or not these proteins are also functionally present on the cell lines.

3.3 Presence of Na^+-dependent-like transporters in the oligodendroglial cell lines

Immunocytochemical staining showed the presence of GlyR and GlyT1 proteins in the three cell lines. The patch-clamp technique was applied in the whole-cell configuration to test the functionality of the proteins detected with the preceding methods. For this, cells were allowed to grow for one or two days on glass coverslips. Afterwards they were placed on the perfusion chamber and patched as described in the section material and methods. The capability of glycine to induce a current was tested. The ability of different test substance to inhibit the glycine-evoked currents was used to characterize the protein(s) responsible for the currents. The effect of the test substance was always compared to the control, being 10 mM glycine.

3.3.1 HOG cell line

Application of 10 mM glycine induced a small, inward current of -3,4 ± 0,66 pA (or -0,28 ± 0,09 pA/pF) (N = 8) at a holding potential of -60 mV in cells of the HOG cell line (Fig. 9).

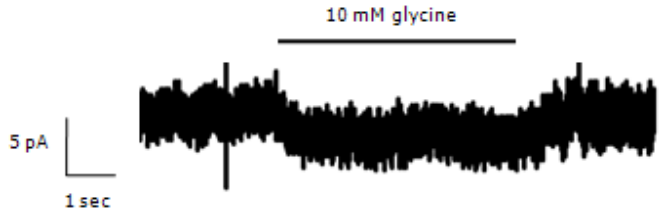
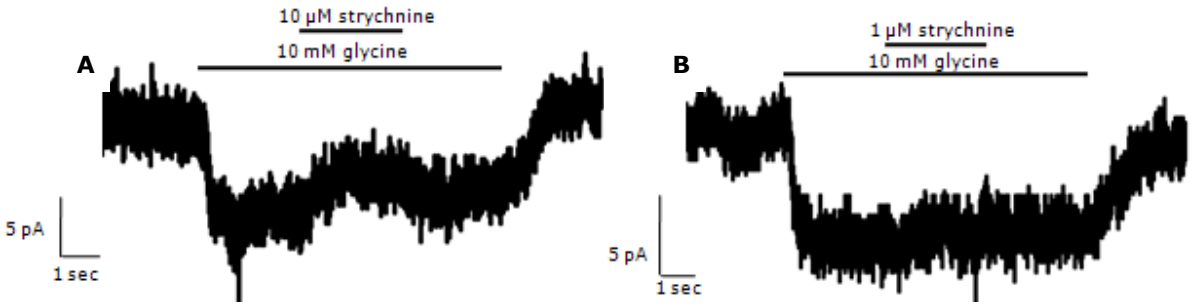


Figure 9. *Glycine-induced current in HOG cells*. A representative trace of a sustained inward current in HOG cells, evoked by 10mM glycine.

To see if the glycine-evoked current was GlyR-sustained the GlyR antagonist strychnine was applied at 10 µM and 1 µM. At a concentration of 10 µM, strychnine significantly reduced (t-test, p value = 0,0043) the glycine-induced currents to 62,8 ± 9,8% of the control (N = 10) (Fig. 10 A, C). There was however no significant effect of 1 µM strychnine (t-test, p > 0,05), as the currents were still 82,2 ± 9,1% of the control (N = 10) (Fig. 10 B, C).



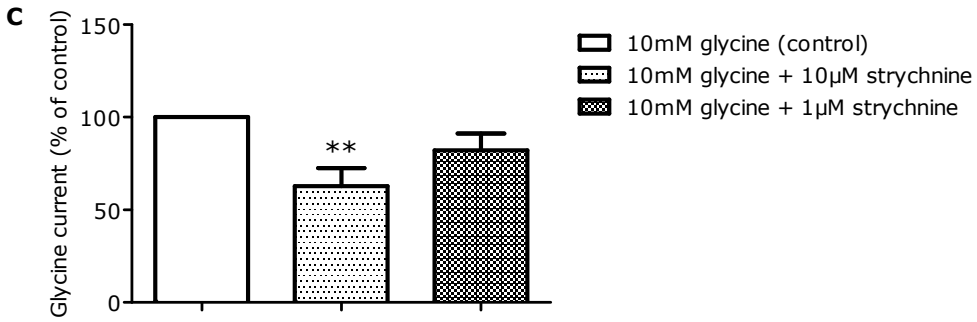
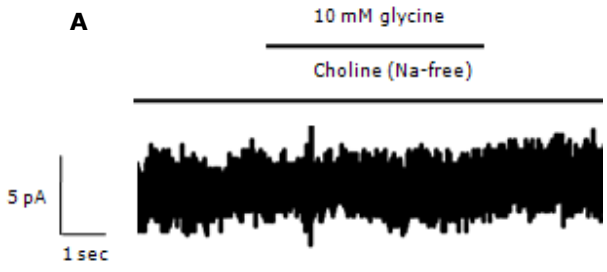


Figure 10. *Effect of the GlyR antagonist strychnine on the glycine-induced currents in the HOG cells*. A. A representative current induced by 10 mM of glycine in the HOG cells, which is sensitive to 10 µM strychnine. B. The glycine-evoked current in the HOG cells is insensitive to 1 µM strychnine. C. The glycine-evoked current is significantly blocked by 10 µM strychnine, whereas the current is insensitive to 1 µM of strychnine. * $p < 0,05$; ** $p < 0,01$ and *** $p < 0,001$.

This suggests that he glycine-evoked inward current is a non-receptor like sustained current since the GlyR specific concentration of 1 µM of strychnine has no effect. Another protein must be responsible for the glycine-evoked current.

To investigate the functional presence of GlyTs a Na^+-free SES was applied, as is known that these transporters are Na^+-dependent this will block them and thus inhibit the glycine-evoked currents. Substituting NaCl by choline chloride had a significant effect (t-test, $p < 0,0001$), the glycine-evoked currents were entirely inhibited (N = 6) and even reversed to -8 ± 5,7% of the control (Fig 11 A, D). The reversal of the current can be the result of blockage of other Na^+–dependent systems. Since removal of the extracellular Na^+ completely blocked the currents, the involvement of GlyT1s and/or GlyT2s was investigated by applying specific blockers. The sarcosine-derivative ALX-5407, a specific GlyT1 blocker, had no effect (t-test, $p > 0,05$) on the glycine-evoked currents, as they were still 83 ± 9,8% of the control (N =9) (Fig 11 B, D). Next the effect of 100 µM amoxapine, a GlyT2a blocker, was tested. This blocker also did not significantly reduce (t-test, $p > 0,05$) the glycine-induced currents, as they were still 85,6 ± 10,4% of control (N = 5) (Fig 11 C, D).



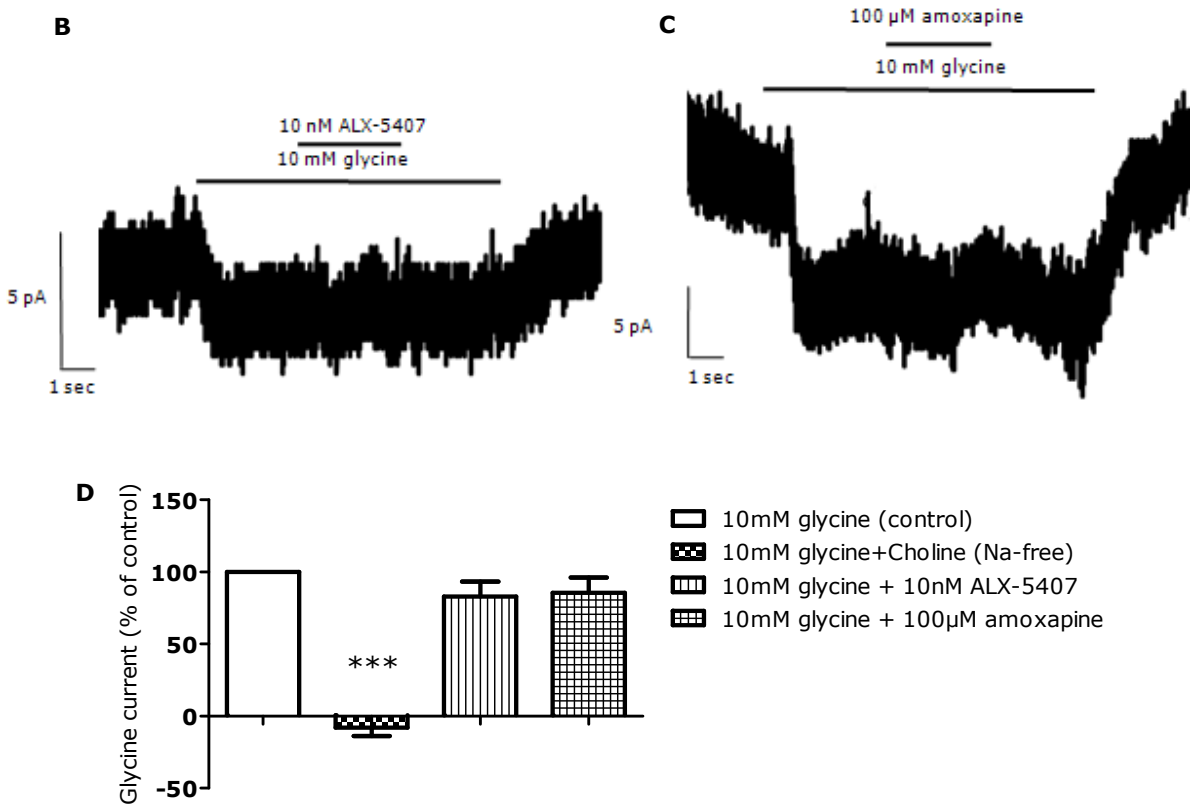


Figure 11. *Involvement of GlyTs in evoking the glycine current in the HOG cells*. A. Replacing NaCl by choline chloride significantly reduced the glycine-induced current. B. The glycine-evoked current is insensitive to the GlyT1 blocker ALX-5407. C. The glycine-evoked current is insensitive to the GlyT2 blocker amoxapine. D. Choline chloride significantly reduces the current; the specific GlyT blockers have no significant effect. * $p < 0,05$; ** $p < 0,01$ and *** $p < 0,001$.

There is no indication for the presence of functional GlyTs on the HOG cells since the glycine-evoked currents are insensitive to ALX-5407 and 100 µM amoxapine. The Na^+ -dependency indicates the presence of different transporters that also couple the transport of glycine to Na^+ and that also induce an inward current, like for instance Na^+-coupled neutral amino acid transporters.

Besides being dependent on Na^+ for the transportation of glycine are the GlyTs also Cl^- -dependent. Other transporter types like Na^+-coupled neutral amino acid transporters are only Na^+ -dependent. Substituting the Cl^- by gluconic acid could therefore allow discriminating between GlyTs and Na^+-coupled neutral amino acid transporters since it would only have an effect on the first transporter type (48, 49). Application of a Cl^- free SES did not significantly inhibit (t-test, $p > 0,05$) the glycine-evoked currents (Fig. 12 A, B), they were still 81,6 ± 8% of control (N = 8).

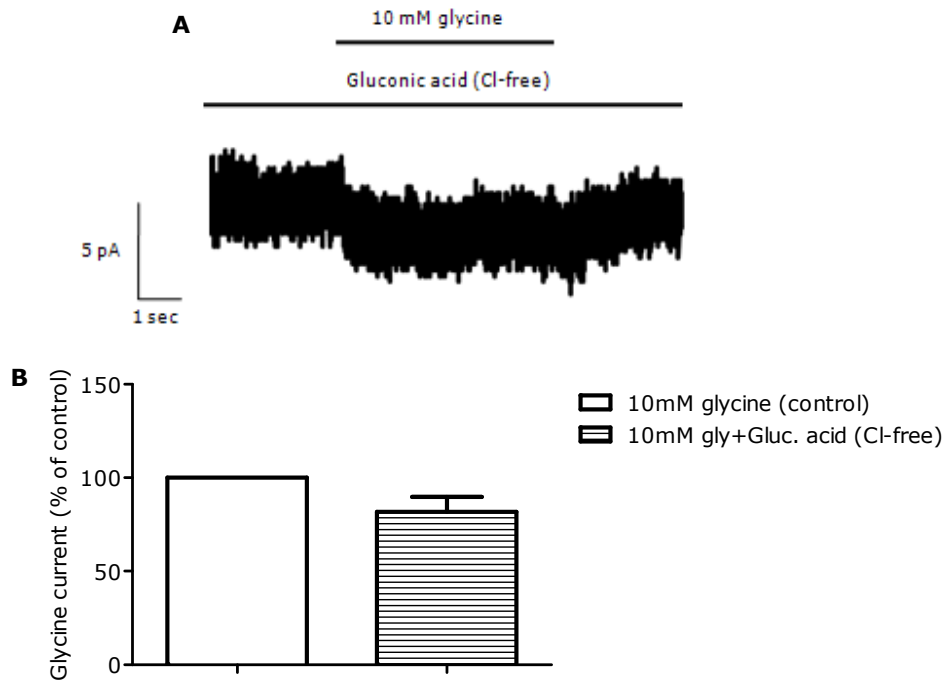


Figure 12. *Involvement of GlyTs in evoking the glycine current in the HOG cells*. A. A representative current induced by 10 mM of glycine in the HOG cells, which is insensitive to Cl^- substitution. B. Replacing Cl^- by gluconic acid does not significantly block the glycine-induced current. Gluc. acid = gluconic aid; gly = glycine. * $p < 0,05$; ** $p < 0,01$ and *** $p < 0,001$.

The glycine-evoked inward current is a non-receptor like sustained current. The insensitivity to 1 μM of strychnine rules out the possibility that the current is mediated by GlyRs. The insensitivity to the specific GlyT blockers and Cl^- independency implies that no GlyTs are involved. These results together with the complete Na^+-dependency suggest that the glycine-induced currents in the HOG cells are mainly the result of Na^+-dependent-like transporters, probably the system A subtype of the neutral amino acid transporters since they are Na^+-dependent and electrogenic (stoichiometry of 1 Na^+:1 glycine) (49, 50).

3.3.2 MO3.13 cell line

Application of 10 mM glycine induced an inward current of -25,63 ± 3,35 pA (or -1,14 ± 0,1 pA/pF) (N = 12) at a holding potential of -60 mV in the MO3.13 cells (Fig. 13).

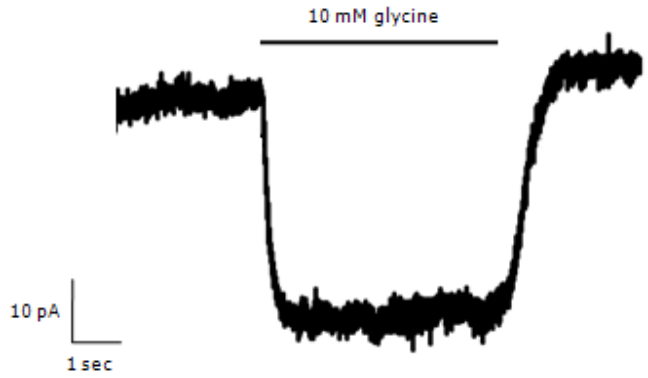


Figure 13. *Glycine-induced current in MO3.13 cells*. A representative trace of a sustained inward current in MO3.13 cells, evoked by 10mM glycine.

Since 10 mM glycine induced a large, inward current, the concentration of glycine that induces the half-maximal response, the EC50 value, and the Hill number were determined. The dose-response curve (Fig. 14) revealed an EC50 value for glycine of 2,2 mM and a Hill slope of 1,18.

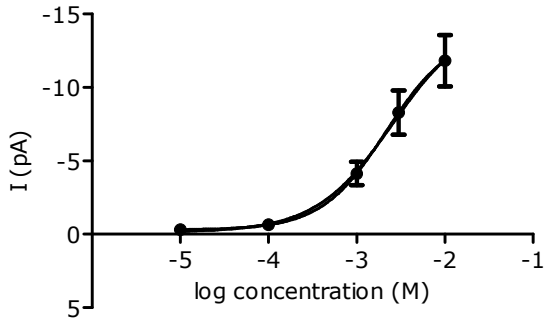


Figure 14. *Dose-response curve for glycine in the MO3.13 cells.* The curve reveals an EC50 value for glycine of 2,2 mM in the MO3.13 cells and a Hill slope of 1,18.

To see if there are functional GlyRs present, the GlyR antagonist strychnine was applied at 10 µM and 1 µM. At a concentration of 10 µM, strychnine significantly reduced (t-test, p = 0,0177) the glycine-induced currents to only 89,7 ± 3,7% of control (N =13) (Fig 15 A, C); 1 µM however had no significant effect (t-test, p > 0,05) on the currents as they were still 99,4 ± 1,4% (N = 4) (Fig 15 B, C).

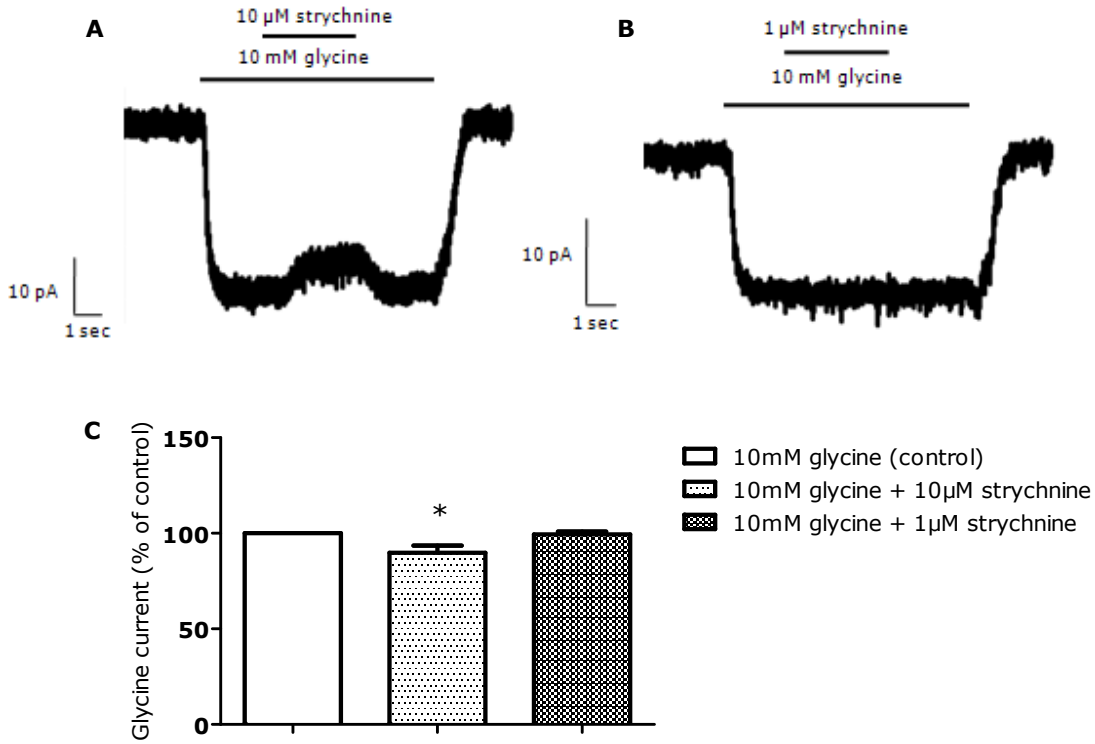


Figure 15. *Effect of strychnine, a GlyR antagonist, on the glycine-evoked currents in MO3.13 cells*. A. A representative current induced by 10 mM of glycine in the MO3.13 cells, which is sensitive to 10 µM strychnine. B. The glycine-evoked current in the MO3.13 cells is insensitive to 1 µM strychnine. C. At a concentration of 10 µM strychnine significantly reduced the glycine-induced current, whereas 1 µM of strychnine has no effect. * $p < 0,05$; ** $p < 0,01$ and *** $p < 0,001$.

The possibility that the glycine-evoked currents are mediated by GlyRs is ruled out by the fact that the GlyR specific concentration of 1 µM of strychnine has no effect. This suggests that another protein must be responsible for the glycine-induced current in the MO3.13 cells.

To investigate the presence of functional GlyTs a Na^+-free SES was applied. Substituting NaCl by choline chloride had a significant effect (t-test, $p < 0,0001$), the glycine-induced currents were inhibited to only 0,9 ± 0,1% of the control (N = 7) (Fig 16 A, D). Since removal of the extracellular Na^+ virtually completely blocked the glycine-evoked currents the involvement of GlyT1 and/or GlyT2 to the currents was investigated by applying specific blockers. The GlyT1 blocker ALX-5407 had no effect (t-test, $p > 0,05$) on the glycine-evoked currents, as the currents were 99 ± 0,9% of the control (N =9) (Fig 16 B, D). Next the effect of the GlyT2a blocker amoxapine was tested. This blocker significantly inhibited (t-test, $p = 0,0005$) the glycine-induced currents to only 61 ± 5,7% of control (N = 7) (Fig 16 C, D).

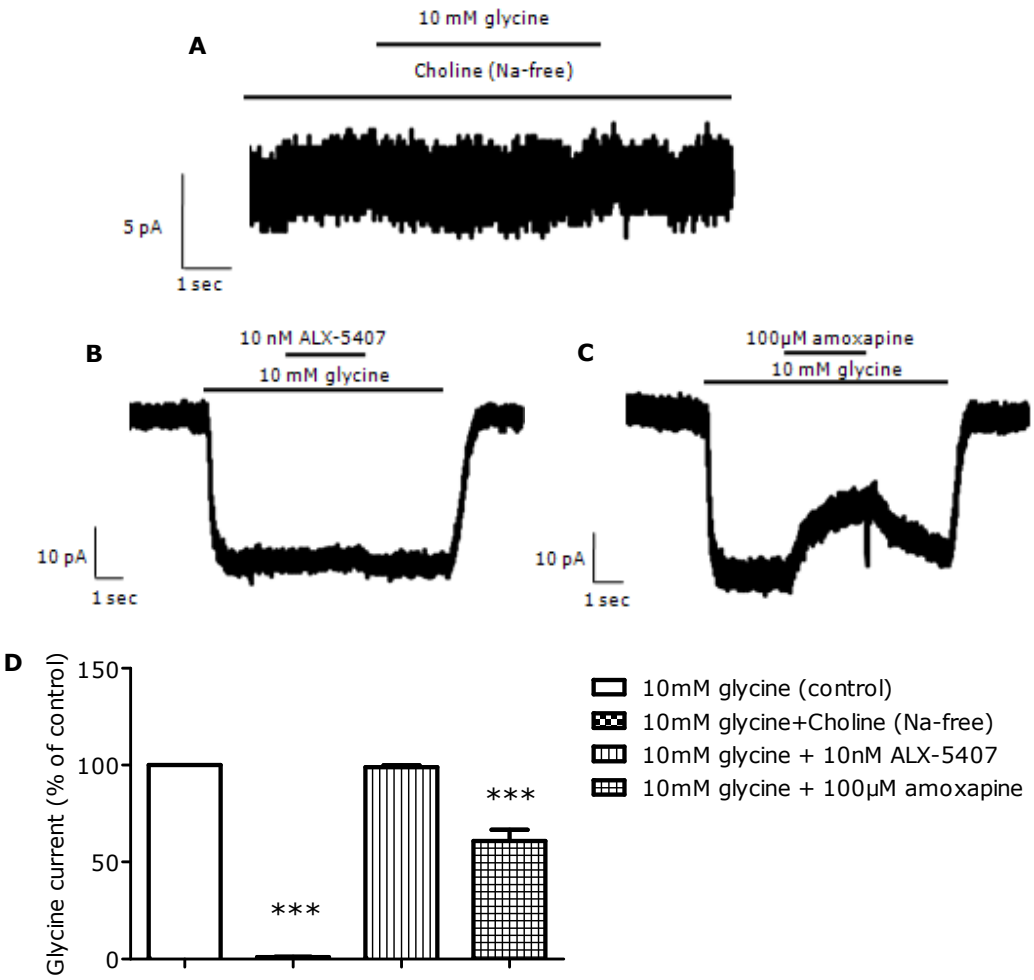


Figure 16. *Involvement of GlyTs in evoking the glycine currents in MO3.13 cells*. A. Replacing NaCl by choline chloride significantly reduced the glycine-induced current in the MO3.13 cells. B. The specific GlyT1 blocker ALX-5407 has no significant effect on the currents. C. The GlyT2 blocker amoxapine significantly inhibits the glycine current. D. Choline chloride and the GlyT2 blocker both significantly block the current; the specific GlyT1 blocker has no significant effect.* $p < 0,05$; ** $p < 0,01$ and *** $p < 0,001$.

There is no evidence for the contribution of GlyT1s to the glycine-evoked currents in the MO3.13 cells since they are insensitive to ALX-5407. The sensitivity of the glycine-induced currents to 100 µM amoxapine suggests the presence of functional GlyT2a's. A study of Núñez et al. showed that 1 M of Methanol (MeOH) can significantly decrease the GlyT2 activity (51). Since the stock solution of amoxapine was dissolved in MeOH it could be possible that the dilution was not strong enough to eliminate the effect of MeOH on the GlyT2 completely. After dilution the final concentration of MeOH was 0,5 M, at this concentration MeOH did not significantly reduce (t-test, $p > 0,05$) the current (Fig 17 A, B) as it was 101 ± 3,9% of the control (N = 5).

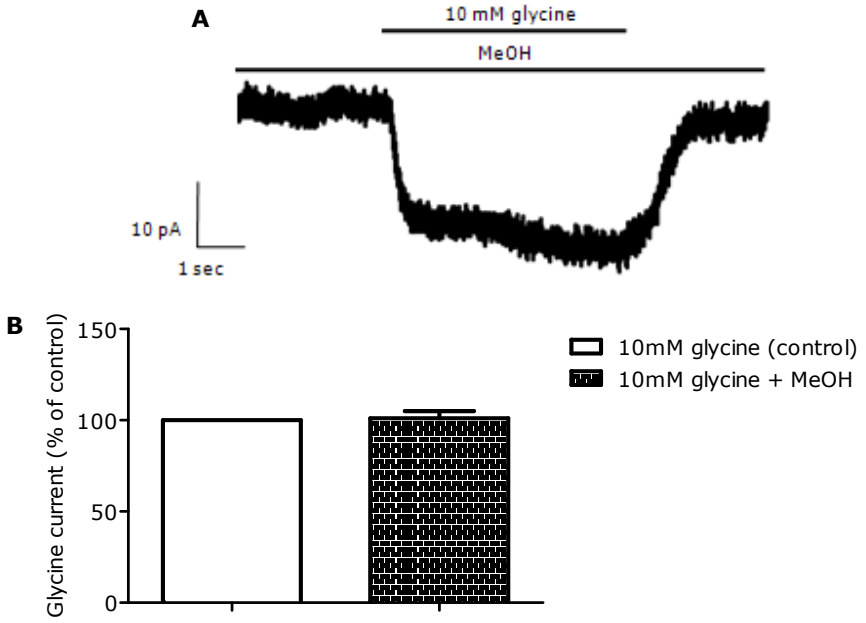
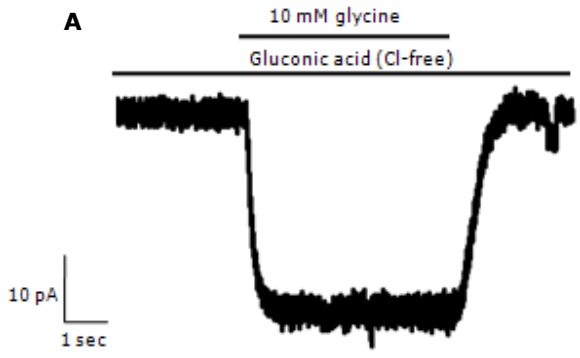


Figure 17. *Effect of MeOH on the glycine-induced currents in MO3.13 cells.* A. A representative current induced by 10 mM of glycine in the MO3.13 cells, which is insensitive to 0,5 M MeOH. B. MeOH has no significant effect on the glycine-induced current. MeOH = methanol. * $p < 0,05$; ** $p < 0,01$ and *** $p < 0,001$.

Thus, the observed inhibition of the glycine-evoked current is solely due to amoxapine, this suggests that there are functional GlyT2a's present. This finding is opposite to previous results, since the GlyT2 mRNA transcript could not be detected. Besides the partial effect of amoxapine, the glycine-evoked currents were shown to be completely Na^+ -dependent. This indicates the presence of a different transporter that also couples the transport of glycine to Na^+ and that can also induce an inward current, like for example Na^+–dependent neutral amino-acid transporters (49).

To allow discriminating between GlyTs and other types of transporters and to confirm the previous result obtained with amoxapine, the effect of Cl^- substitution by gluconic acid was studied. Applying a Cl^- free SES significantly inhibited (t-test, $p < 0,0001$) the glycine-evoked currents (Fig. 18 A, B) to 87,6 ± 0,9% of the control (N =7).



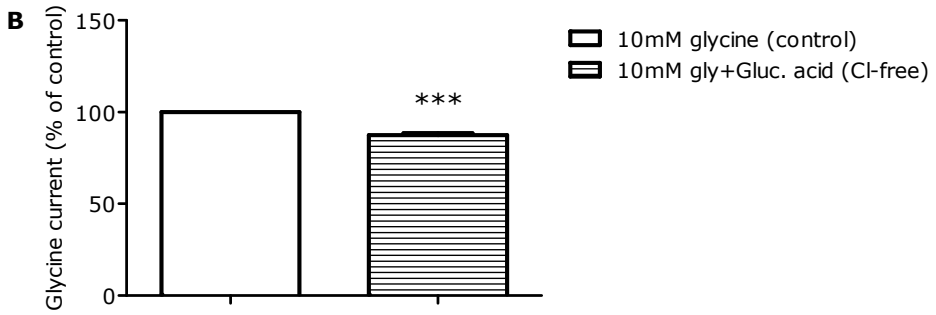


Figure 18. *Involvement of GlyTs in evoking the glycine currents in MO3.13 cells*. A. A representative current induced by 10 mM of glycine in the MO3.13 cells, which is blocked by Cl^- substitution B. Replacing Cl by gluconic acid significantly blocks the glycine-induced current. Gluc. acid = gluconic aid; gly = glycine. * $p < 0,05$; ** $p < 0,01$ and *** $p < 0,001$.

This partial Cl^-–dependency confirms the patch-clamp results of the amoxapine, there is a contribution of GlyT2a's to the glycine-induced currents. The complete Na^+ dependency suggests that the main part of the glycine-evoked currents are mediated by Na^+-dependent-like transporters. There is no contribution of GlyRs or GlyT1s to the glycine-evoked current since 1 µM of the GlyR antagonist strychnine and the GlyT1 blocker ALX-5407 have no effect.

To gain more details about the identity of the protein(s) responsible for the glycine currents, the IV relationship of whole-cell glycine currents was investigated. SIS 3 was used for the IV protocol, this solution contained CsCl instead of KCl to eliminate K^+ rectifiers. The IV relationship of the glycine-evoked currents was roughly linear between -100 and +80 mV (Fig. 19) and did not reverse at test potentials of up to +80 mV (N = 4). This suggests that the glycine-evoked current is mainly the result of a transporter.

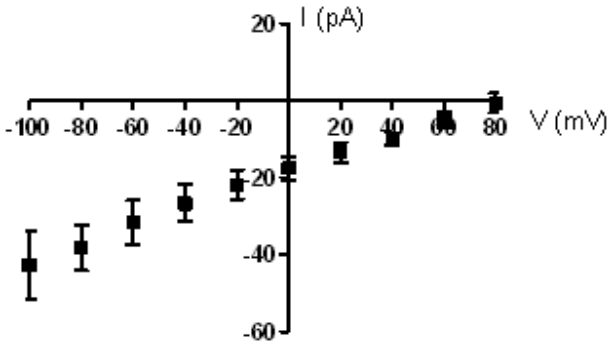


Figure 19. *Current-voltage relationship of the glycine-evoked currents in the MO3.13 cells*. The IV relationship is roughly linear and no outward currents are observed.

All together these results suggest that the glycine-induced currents in the MO3.13 cells are transporter-sustained. They are mainly the result of Na^+-dependent-like transporters but a small part is contributed by GlyT2a's.

3.3.3 OLN-93 cell line

Applying glycine at a concentration of 10 mM induced an inward current of -3,78 ± 0,52 pA (or -0,26 ± 0,16 pA/pF) (N = 17) at a holding potential of -60 mV in the OLN-93 cells (Fig. 20).

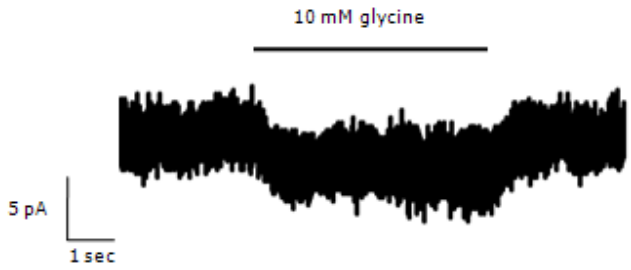


Figure 20. *Glycine-induced current in OLN-93 cells*. A representative trace of a sustained inward current in OLN-93 cells, evoked by 10mM glycine.

To see if the currents are GlyR-sustained, the GlyR antagonist strychnine was used. At a concentration of 10 µM strychnine could not significantly reduce (t-test, p > 0,05) the glycine-induced currents, they were still 95 ± 3,6% of control (N =10) (Fig 21 A, C); 1 µM also had no significant effect (t-test, p > 0,05) as the currents were 97,7 ± 5,1% of control (N = 10) (Fig 21 B, C).

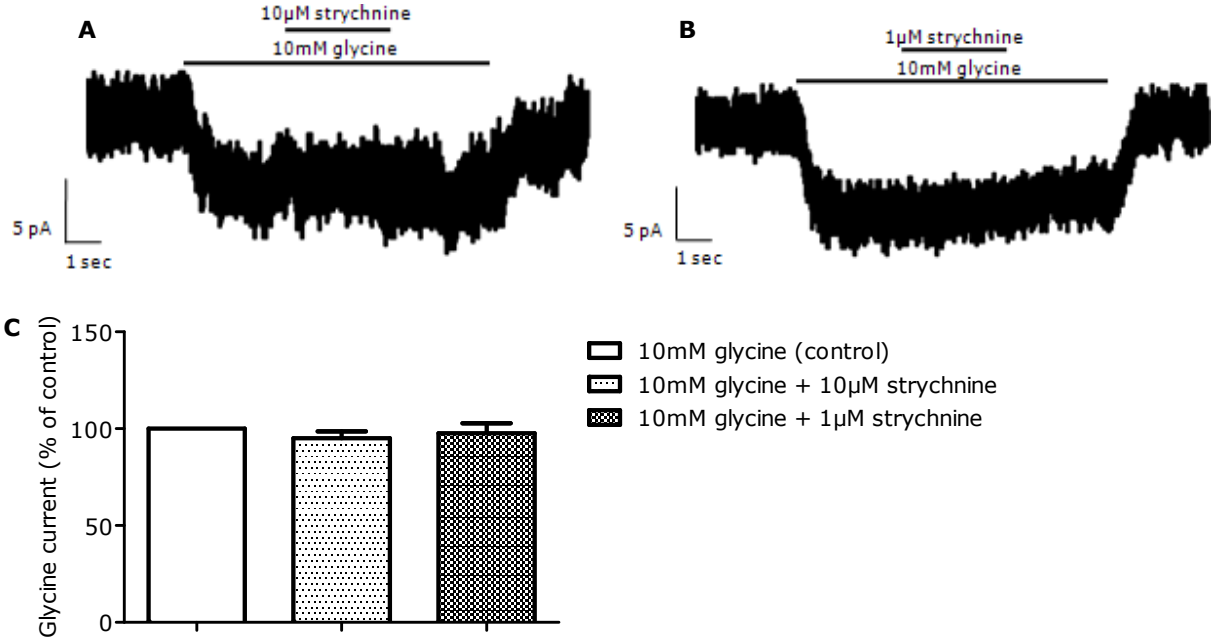


Figure 21. *Effect of strychnine, a GlyR antagonist, on the glycine currents in OLN-93 cells*. A. A representative current induced by 10 mM of glycine in the OLN-93 cells, which is insensitive to 10 µM strychnine. B. The glycine-evoked current in the OLN-93 cells is insensitive to 1 µM strychnine. C. Both, 10 µM and 1 µM of strychnine could not significantly reduce the glycine-induced current. * p < 0,05; ** p < 0,01 and *** p < 0,001.

The glycine-evoked inward current is a non-receptor like sustained current since strychnine can not significantly reduce the currents. Another glycine-associating protein is thus responsible for the induced currents.

To test if there are functional GlyTs present, Na^+-free SES was applied. Substituting NaCl by choline chloride reduced the glycine-evoked currents significantly (t-test, $p < 0,0001$) to 20,2 ± 3,8% of the control (N = 6) (Fig 22 A, D). Since removal of the extracellular Na^+ significantly inhibited the currents, the contribution of GlyTs was investigated by use of specific blockers. The GlyT1 blocker ALX-5407 had no effect (t-test, $p > 0,05$) on the glycine-evoked currents, as they were 98,1 ± 3% of the control (N =13) (Fig 22 B, D). Next the effect of the GlyT2a blocker amoxapine was tested. This blocker significantly inhibited (t-test, $p = 0,0075$) the glycine-induced currents, as they were reduced to only 64,8 ± 10,3% of control (N = 10) (Fig 22 C, D).

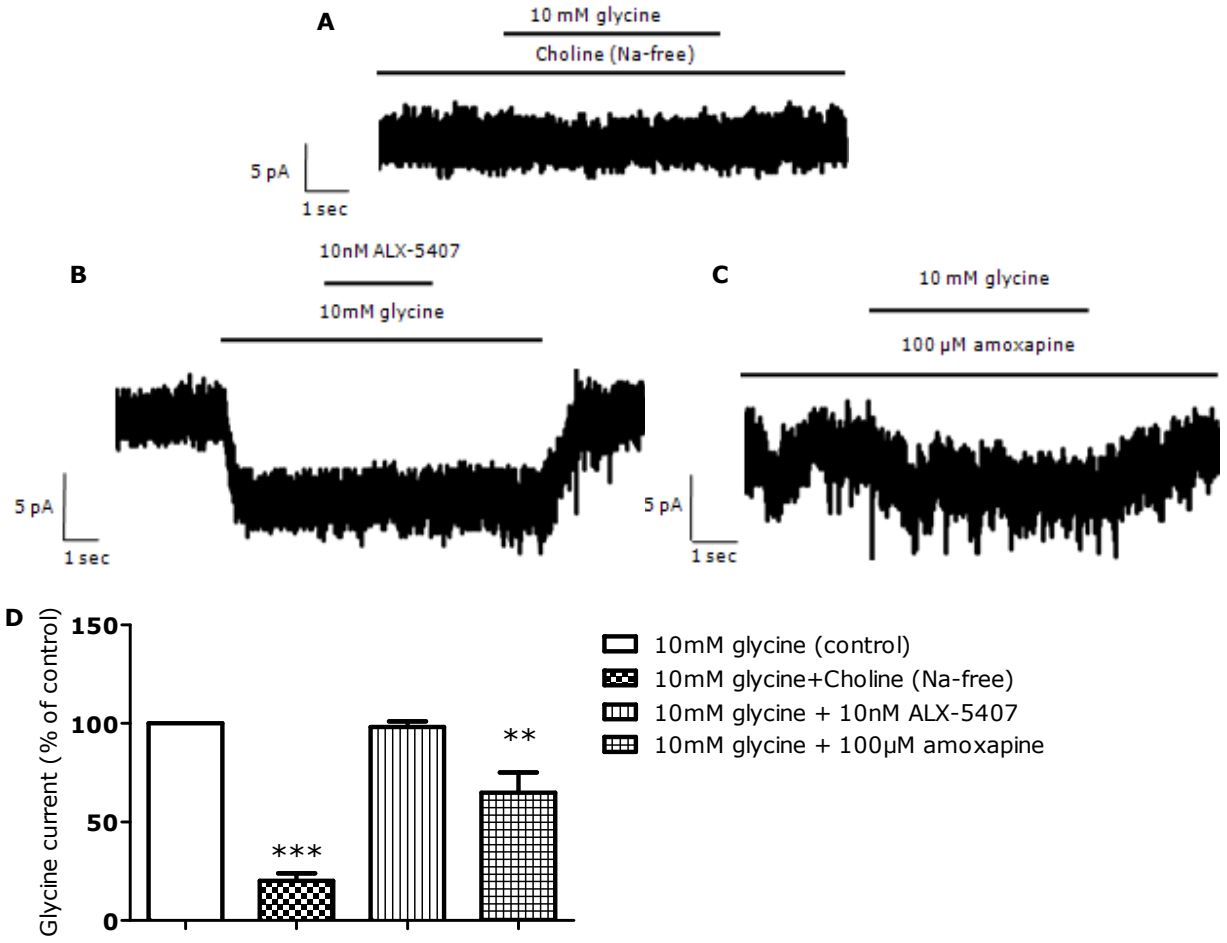


Figure 22. *Involvement of GlyTs in evoking the glycine currents in the OLN-93 cells*. A. Replacing NaCl by choline chloride significantly reduced the glycine-induced current. B. The specific GlyT1 blocker ALX-5407 has no significant effect on the current. C. The GlyT2 blocker amoxapine significantly blocks the current. D. Choline chloride and the GlyT2 blocker both significantly reduce the current; whereas the specific GlyT1 blocker has no significant effect. * $p < 0,05$; ** $p < 0,01$ and *** $p < 0,001$.

Since the glycine-induced currents are insensitive to ALX-5407, there is no indication for the presence of functional GlyT1s. The sensitivity of the currents to 100µM amoxapine suggests that functional GlyT2a's are expressed by the OLN-93 cells. The contribution of MeOH was investigated since the stock solution of amoxapine was dissolved in MeOH (51). At a final concentration of 0,5 M, MeOH did not significantly reduce (t-test, $p > 0,05$) the glycine-evoked currents (Fig 23 A, B) as they were 99,9 ± 8,8% of control (N = 4).

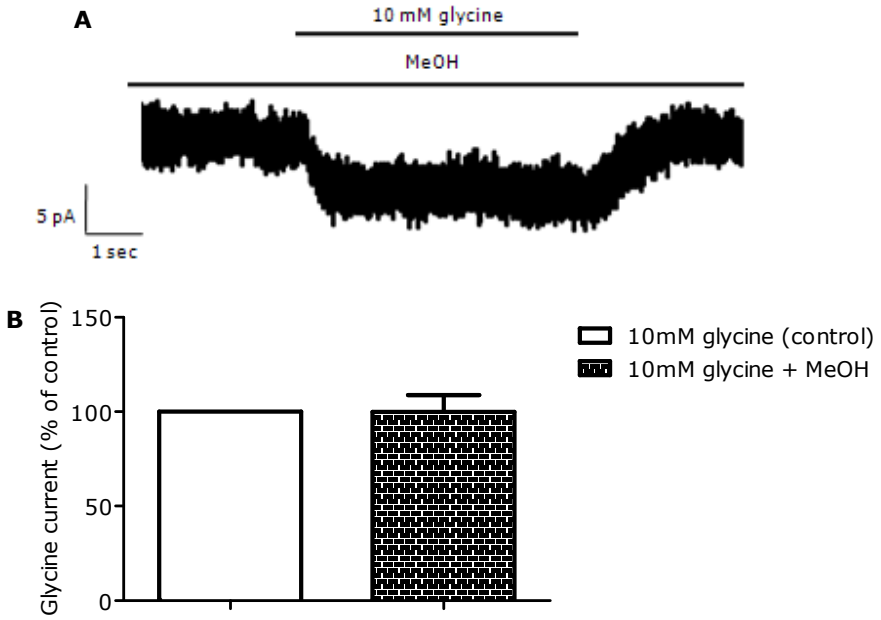


Figure 23. *Effect of MeOH on the glycine-induced currents in the OLN-93 cells.* A. A representative current induced by 10 mM of glycine in the OLN-93 cells, which is insensitive to 0,5 M MeOH. B. MeOH has no significant effect on the glycine-induced current. MeOH = methanol. * $p < 0,05$; ** $p < 0,01$ and *** $p < 0,001$.

The observed effect on the glycine-induced currents is therefore only due to the amoxapine and suggests the presence of functional GlyT2a's. This finding is contrary with the previous results, since the GlyT2 mRNA transcript could not be detected by means of PCR. Besides the contribution of GlyT2a's to the glycine-evoked currents, a small fraction (20%) was shown to be Na^+-independent and the main part (80%) was Na^+-dependent. This implies that, in addition to the GlyT2s, there are several other electrogenic transporters present, like for example different types of neutral amino acid transporters (49).

The effect of Cl^- substitution by gluconic acid was studied next. Elimination of extracellular Cl^- allows discriminating between the Na^+/Cl^--coupled GlyTs and other types of transporters, for instance neutral amino acid transporters. Application of the Cl^- free SES significantly inhibited (t-test, $p = 0,0013$) the glycine-evoked currents (Fig. 24 A, B), they were 68,8 ± 6,8% of control (N =10).

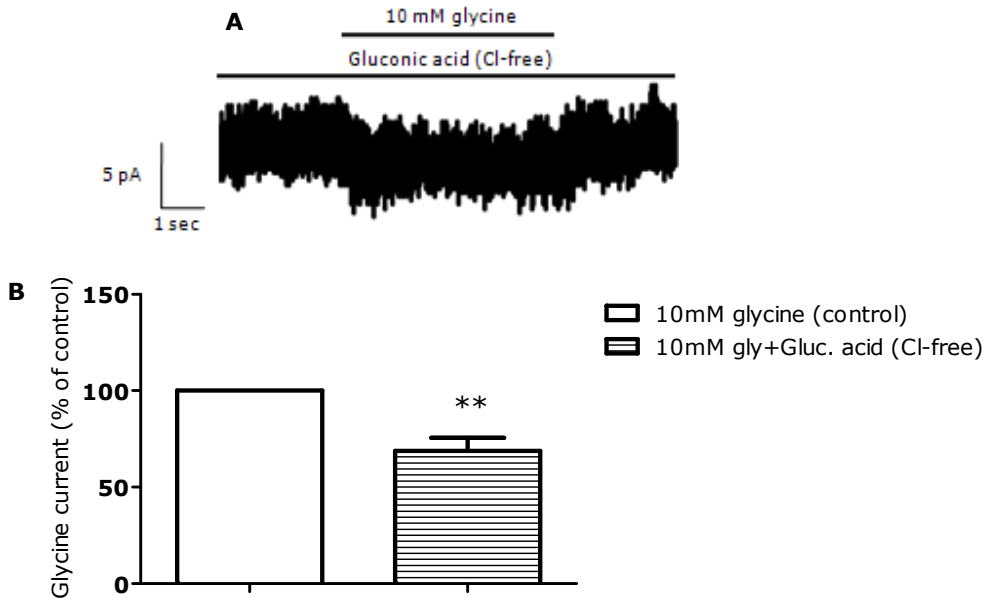


Figure 24. *Involvement of GlyTs in evoking the glycine currents in the OLN-93 cells*. A. A representative current induced by 10 mM of glycine in the OLN-93 cells, which is blocked by Cl^- substitution B. Replacing Cl by gluconic acid significantly blocks the glycine-induced current. * $p < 0,05$; ** $p < 0,01$ and *** $p < 0,001$.

This partial Cl^-–dependency confirms the patch-clamp results of the amoxapine, there is a contribution of GlyT2a's to the glycine-induced current. The currents were shown to be mainly (80%) Na^+ -dependent. This implies that the glycine-evoked currents are principally the result of Na^+-dependent-like transporters. However, a little fraction of the glycine-evoked currents was Na^+-independent which suggests the involvement of other electrogenic transporters that use the electrochemical driving force of another ion then Na^+ for the glycine transfer. It is thus possible that, besides the GlyT2a's, different types of neutral amino acid transporters are responsible for the glycine-induced currents in the OLN-93 cells (49, 50).

The results obtained with the patch-clamp experiments suggest that, in all three cell lines, glycine can induce an inward current, which is a transporter like sustained current. In the HOG cells the glycine-evoked currents are the result of Na^+-dependent-like transporters. In the MO3.13 and OLN-93 cells the main part of the glycine-evoked current is the result of Na^+-dependent-like transporters, but there is also a small contribution of GlyT2a's. There is no involvement of GlyRs or GlyT1s to the glycine-evoked current in the three cell lines since the GlyR antagonist strychnine (1 µM) and the GlyT1 blocker ALX-5407 can not block the inward currents. However, the Western blotting and immunostainings suggested the presence of GlyRs and GlyT1s. Since these techniques could not show clear membrane localization it could be possible that these proteins are only present at the intracellular compartment. Therefore the localization of these proteins was studied next.

3.4 Biotinylation reveals the localization of the GlyR and GlyT1

As the immunocytochemical staining showed no clear membrane localization of the glycine-associating proteins and the patch clamp results failed to prove the presence of functional GlyT1s and GlyRs on the three cell lines, the localization of the different proteins was investigated next. Membrane bound proteins were biotinylated, which allowed them to be separated from the intracellular proteins. Western blotting was performed on the different fractions obtained with the biotinylation kit to analyze where the glycine-associated proteins are located. Actin, a cytoskeletal filament, was used as a control for the biotinylation protocol. Since this protein is located in the cell, it should only be present in the intracellular fraction. No membrane fraction was positive for actin; this suggests that the biotinylation protocol was successful. The membrane fraction of all three cell lines appeared positive for the GlyT1 protein (Fig 25). Gephyrin, the intracellular GlyR anchoring protein, could not be detected in the membrane fraction of the HOG and OLN-93 cell line. There was a light band present in the membrane fraction of the MO3.13 cells. As gephyrin is directly linked to the GlyR via the β subunit it is very likely that a small fraction was still coupled after the biotinylation and was dragged into the membrane fraction. Only the membrane fractions of the HOG and MO3.13 cells were positive for the α and β subunit of the GlyR.

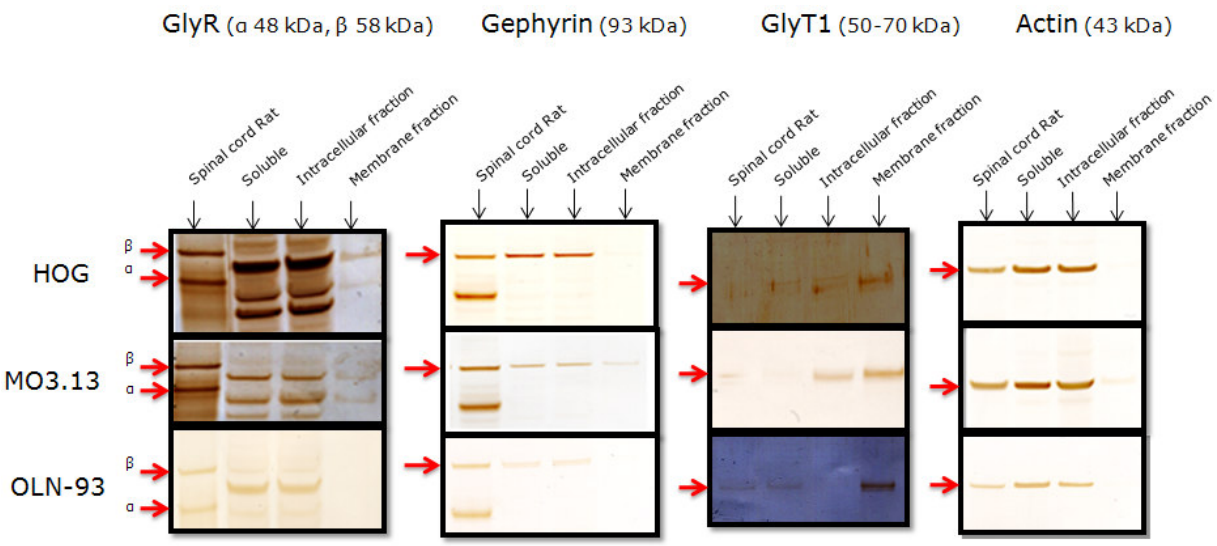


Figure 25. *Membrane protein biotinylation and Western blotting*. The membrane fractions of HOG and MO3.13 are positive for the GlyR and GlyT1. The membrane fraction of the OLN-93 cells is positive for the GlyT1. No membrane fraction is positive for actin. Rat spinal cord was used as a positive control; actin was used as a control for the biotinylation.

The bands detected with the antibody for the GlyR α and β subunit in the membrane fraction of the HOG and MO3.13 cells have a slightly lower molecular weight as they lie lower than the bands of the positive control. It is possible that there had occurred a modification of both subunits that resulted in shorter proteins, which altered their functionality and/or the sensitivity to strychnine. The main part of

the GlyR subunits appears to be located in the intracellular fraction; this can also explain why no GlyR-mediated currents could be detected. All three cell lines express the GlyT1 on their membrane however the patch-clamp experiments failed to prove their functionality. It could be that this GlyT1 is an isoform which is unaffected by the tested blocker, ALX-5407. Since there was no transfected cell line available there was no possibility to test the functionality of the blocker itself.

3.5 Glycine effect on cell proliferation

Finally, the effect of glycine on cell proliferation was examined. Cells were stimulated with 10 mM of glycine and the amount of proliferation was measured by means of BrdU incorporation. A PI staining was used to correct for the number of cells in each well. The effect of 10 mM of glycine was compared to the control. There was no significant effect (t-test, $p > 0,05$) of glycine on the proliferation of the HOG (N = 3; n = 21) and OLN-93 (N = 2; n = 14) cells (Fig 26). The proliferation of the MO3.13 cells was significantly decreased (t-test, $p < 0,0001$) to 61,3 ± 5,5% of the control (N = 3; n = 21).

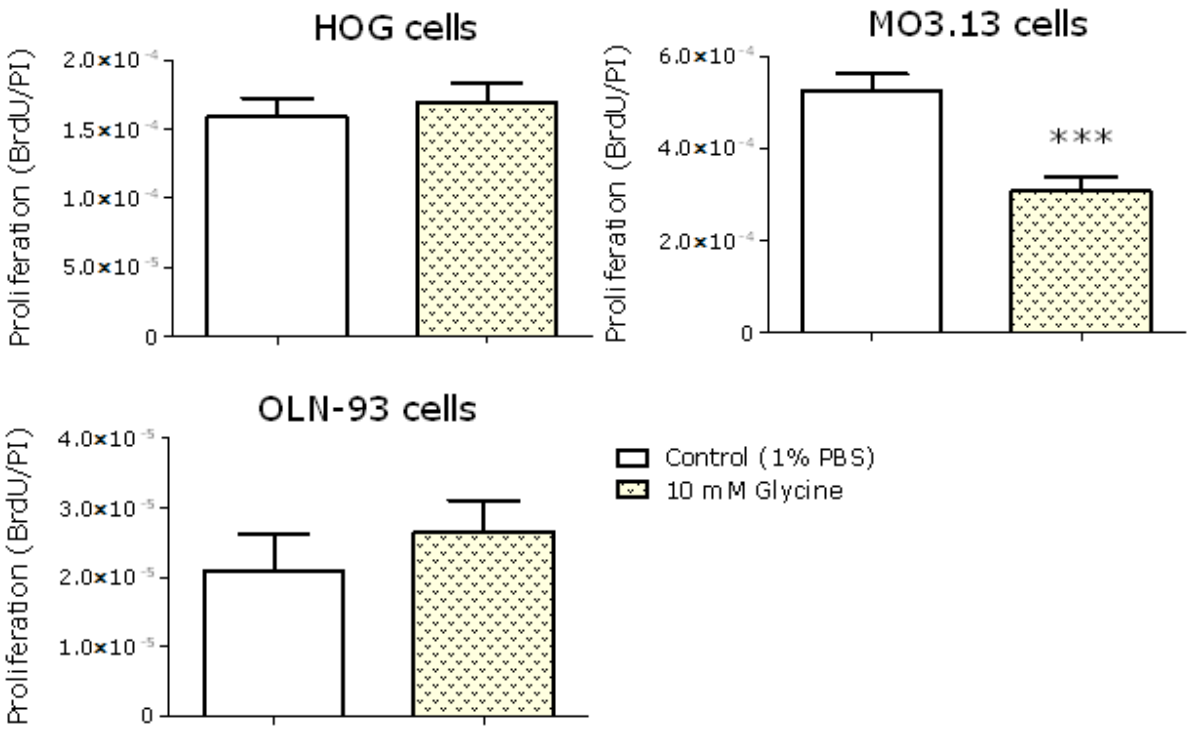


Figure 26. *Effect of glycine on cell proliferation.* Glycine had no effect on the proliferation of HOG and OLN-93 cells. The proliferation of MO3.13 cells was significantly decreased. * $p < 0,05$; ** $p < 0,01$ and *** $p < 0,001$.

These results suggest that glycine has a different effect on the used cell lines. Since the glycine-evoked currents in the MO3.13 cells are approximately six times larger than those induced in the HOG and OLN-93 cells. It could be possible that the cells respond different due to the presence of different glycine-associating proteins or a difference in their expression level. The density of the glycine-evoked

currents is four times lower in the HOG and OLN-93 cells, being -0,28 and -0,26 pA/100 nm² respectively, compared to the density in the MO3.13 cells, being -1,14 pA/100 nm². It could therefore be possible that the glycine-evoked currents in the HOG and OLN-93 two cell lines are too small to trigger any downstream events. The dissimilarity in density and current amplitude could be caused by the origin of the cell lines since the HOG and OLN-93 cell lines were derived from spontaneous mutated oligodendroglial cells and the MO3.13 cell line is derived from a fusion between a rhabdomyosarcoma RD with adult human oligodendrocytes.

3.6 Preliminary results of primary culture oligodendroglia

The primary cell culture was obtained from mice pups expressing eGFP under the CNP promoter. Western blotting with the mAb4a primary antibody was performed on cell lysates of the three cell lines and primary cells as described in materials and methods. All cell lysates were positive for the mAb4a antibody (Fig. 27).

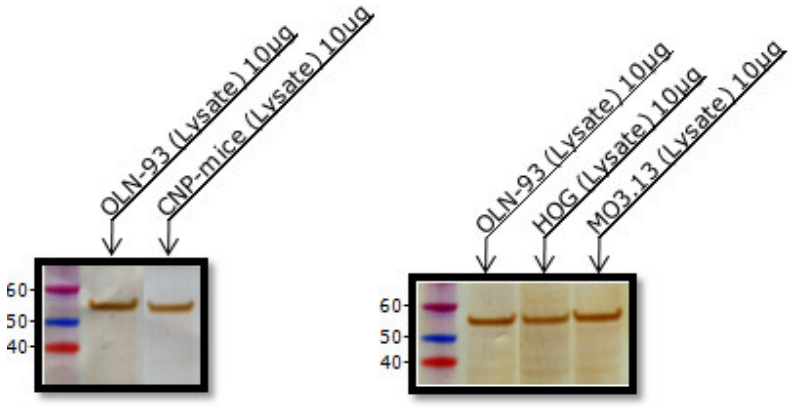


Figure 27. *Western blot of the cell lines and cell sorted primary culture.* All lysates are positive for the mAb4a antibody, directed against the GlyR subunits.

The molecular weight of the band present in the different fractions is ±55 kDa. This is too short for the GlyR β subunit and too long for the GlyR α subunit. Using the ExPASY tool for post-translational modification prediction revealed that the amino acid sequence of all GlyR subunits contains a possible signal peptide cleavage site. The shift in molecular weight of the GlyR positive band could be the result of whether or not the sequence is cleaved off. It could be possible that this sequence is involved in the trafficking of the GlyR to the cell membrane. Another possibility is that the sequence is engaged in determining the functionality of the GlyR. However these are just some speculations and further characterization of the bands that appeared positive for the mAb4a antibody is necessary to elucidate their identity. The unexpected alteration in size (molecular weight) of the mAb4a positive band is not confined to the cell lines; it is something characteristic to glial cells since it is present in the lysate of the cell lines and the primary culture cells.

4 Conclusion and synthesis

PCR results hinted the presence of the mRNA of different GlyR subunits, gephyrin and GlyT1. The results of the Western blot and immunocytochemical stainings showed that the used cell lines were immuno-positive for the GlyR, gephyrin and GlyT1 proteins. These results are indicative for the possible functional presence of the GlyR and GlyT1 on the cell lines.

The whole-cell patch-clamp technique was used to identify the functional glycine-associating proteins expressed by the different cell lines. This revealed that in the HOG cells, the currents are the result of Na^+-dependent-like transporters. In the MO3.13 cells Na^+-dependent-like transporters are responsible for the main part of the glycine-evoked currents but there is also a small contribution of GlyT2a's. Finally, the OLN-93 cells gave about the same results as the MO3.13 cells. Their glycine-evoked currents were mainly the result of Na^+-dependent-like transporters. There was however a small contribution of GlyT2a's and Na^+-independent transporters. These findings were contrary to the results derived from the molecular experiments (PCR, Western blot, immunocytochmistry) were the presence of GlyT1s and GlyRs was shown and absence of GlyT2s.

Biotinylation of the cell surface proteins was performed to differentiate between membrane bound and intracellular proteins. Subsequent Western blotting revealed that only the HOG and MO3.13 express GlyR subunits at their cell surface, however these subunits had a slightly deviating molecular weight compared to the control. It is possible that a modification occurred that affected the functionality of the GlyRs or their sensitivity to strychnine. The main part, and for the OLN-93 cells all, of the subunits was localized in the intracellular portion. This also explains why no GlyR-induced currents could be detected. The alteration in molecular weight of GlyR subunits was also observed in the primary cells. This suggests that this phenomenon is not something confined to cell lines but rather proper to glial cells. All three cell lines express the GlyT1 on their cell membrane; however patch-clamp failed to prove their functionality. It could be that this protein is an isoform, insensitive to the tested blocker (ALX-5407). Since there was no transfected cell line available it was not possible to test the functionality of the blocker, so it remains possible that the blocker did not function optimal. There was no molecular evidence for the presence of GlyT2s, however a part of the glycine currents induced in the MO3.13 and OLN-93 cells were Cl^--dependent and sensitive to 100 µM amoxapine. Núñez et al suggested that the inhibition of GlyT2a by amoxapine is ten times more efficient than that of GlyT1b, and that amoxapine behaves as a competitive inhibitor of glycine and Cl^- and a mixed-type inhibitor with respect to Na^+ (21). This all implies that the obtained patch results indicate the presence of GlyT2 proteins. It could be that this is a new, unknown isoform of the GlyT2, which could not be detected with the used molecular techniques, or that it is a GlyT1 isoform, with increased amoxapine sensitivity. However, this remains speculative and it is too early to draw conclusions about the identity of the protein(s) responsible for the glycine-evoked currents. Further investigations to characterize the glycine-evoked currents are necessary. In the future, other GlyT blockers could be tested to make sure whether or not GlyTs are present. Antidepressants, like doxepine, nortriptyline and amitriptyline, could be used to test for the presence of GlyTs and sarcosine, a GlyT1 blocker, could be tested instead

of ALX-5407 to check for GlyT1s (1, 21). To get more certainty about the identity of the other Na^+-dependent-like transporters, the ability of other amino-acids to induce a similar current could be investigated. It is very likely that the proteins responsible for the main part of the glycine-evoked currents are system A neutral amino-acid transporters. These transporters are Na^+-dependent and electrogenic, they are able to transport glycine, alanine, glutamine and other short-chained neutral amino-acids and α-(methylamino)isobutyricacid acts a specific substrate (48-50).
Examining the effect of glycine on the cell proliferation revealed that glycine has a different effect on the used oligodendroglial cell lines. There was no effect of glycine on the HOG and OLN-93 cells. However, the proliferation of the MO3.13 cells was significantly inhibited. This is probably the result of a difference in expression level or type of glycine-associating proteins. The density and the amplitude of the glycine-induced currents in the MO3.13 are several times larger than those recorded in the HOG and OLN-93 cells. It was therefore suggested that the magnitude of the glycine-induced currents was only sufficiently large enough in the MO3.13 cells to lead to a depolarization which in turn could trigger downstream events like an increase in intracellular Ca^{2+}.

The cell lines results do not concur with the results Belachew et al. acquired with oligosphere-derived oligodendroglial progenitor cells. At a concentration of 1 mM, glycine induced an inward current in these primary cells, 20 to 80 times larger as the one induced in the cell lines used in our study, and this current was sensitive to 1 µM of strychnine. The glycine-evoked currents in their primary culture model were able to induce an increase of intracellular Ca^{2+}, which was only completely blocked by simultaneous application of strychnine and a Na^+-free medium (34, 35). This suggests that their primary cells express functional GlyRs and Na^+-dependent transporters, probably GlyTs since immunocytochemical staining showed that the cells express GlyT1s and GlyT2s. In the three cell lines used in this study, the glycine-induced current was mainly the result of another type of Na^+-dependent-like transporter. In the future more patch-clamp and molecular experiments will have to be performed to give more exclusion about the identity of the responsible protein(s). After characterization of the glycine currents, their ability to induce a cell depolarization and an increase in intracellular Ca^{2+} will be further investigated. All these experiments will also have to be performed on primary cell cultures derived from transgenic mice that express eGFP under the CNP promoter. The results of the primary culture will be compared with those obtained from the cell lines. This will allow concluding whether or not one of the used cell lines could be used as a model for immature oligodendrocytes.

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