"Research is the process of going up alleys to see if they are blind."

Marston Bates

American zoologist, 1906-1974

Preface

The brain, and by extension the nervous system, is the centre of our being. It is the place of our emotions and our thoughts, of coordination and fine tuning. Although science now acknowledges its importance, it has not always been appreciated to the extent it deserves. The Egyptians discarded the brain during mummification, whilst the other organs were preserved carefully. Aristotle (384-322 BC) saw the brain as a blood cooler and the heart as the centre of intelligence. Aside from the fact that the heart is a fascinating organ, certainly for an electrophysiologist, this statement gives our blood pump too much credit. Nevertheless still today, there is this tight link between emotions and the heart, or did you ever say "I love you from the bottom of my brain"?
Hippocrates (460-370 BC) did believe the brain was involved in sensation and intelligence. However, this view was not generally accepted until Galen (129-200 AD), a Greek physician who moved to Rome and a follower of Hippocrates, observed a link between brain damage and mental problems in the gladiators he treated. Galen, for his part, was a great influence for Andreas Vesalius (1514-1564), who is considered a founding father of modern anatomy thanks to his work "*De homani corporis fabrica*".
And so we take a leap into modern times, where the study of the brain, and by extension the whole nervous system, is a discipline on its own, ranging from behavioural studies, over molecular studies to even computational studies. This broadness of neuroscience reflects the complexity of the nervous system. Except from neurons, the nervous system is home to the glial cells such as microglia, astrocytes, oligodendrocytes and their peripheral counterpart Schwann cells. And again history repeats itself, this time on a cellular level. Other than neurons, glial cells have long been seen as some sort of connective cells without any special function. They were not excitable and therefore not interesting. Evidence for that view is already found in the name of these cells, since "glia" is Greek for "glue" and derived from the term "Nervenkitt" or "nerve glue" as proposed by Rudolf Virchow (1821-1902). In other words, their function was to hold the neurons together. Again, this view has now changed as there is growing evidence of the influence of glial cells on neuronal development, synaptic transmission, regulation of the extracellular environment, etc.
It is hard not to be fascinated by the nervous system, especially from an electrophysiological point of view. The delicate balance of neurotransmitters and ions, controlled by ion channels, receptors and transporters is the basis of the functioning of the nervous system and is a challenge to dive into for a biomedical researcher. This work describes electrophysiological properties of oligodendroglial cells and has special attention for glycine activated molecules such as the glycine receptors and transporters. It is a small contribution to the ever extending wondrous world of neuroscience and hopefully the start of an exploratory journey that will result in some answers, but above all a lot of new interesting questions.

Roeland Buckinx

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Abbreviations & Symbols

(b)FGF	(basic) fibroblast growth factor
$[X]_{o/i}$	extracellular (o) or intracellular (i) concentration of substance X
5-HT	5-hydroxytryptamine = serotonin
A	agonist (in receptor kinetics)
AC	adenylate cyclase
ACh	acetyl choline
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
BMP	bone morphogenic protein
bp	base pairs
C	resting (closed) state of the receptor (in receptor kinetics)
CAK	CDK activating kinase
CaM-K	Ca^{2+}/calmoduline dependent kinase
cAMP	cyclic adenosine monophosphate
CDK	cyclin dependent kinase
CGE	caudal ganglionic eminence of the developing forebrain
CHO	Chinese hamster ovarian cells
CHO-SES	standard extracellular solution in experiments with CHO cells
CHO-SIS	standard intracellular solution in experiments with CHO cells
CKI	cycline/CDK inhibitors
C_m	membrane capacitance
CNP	2',3'-cyclic nucleotide-3'-phosphodiesterase
CNS	central nervous system
D	desensitized state of the receptor (in receptor kinetics)
DAG	diacylglycerol
DIV	days in vitro
DMEM	Dulbecco's modified eagle medium
E	efficacy (in receptor kinetics)
E	embryonic day
E2F	family of transcription factors
ERK	extracellular signal regulated kinase
E_X	equilibrium potential of ion X
FCS	fetal calf serum
FP	forward primer
GABA	γ-amino butyric acid
$GABA_{A/B/C}R$	GABA receptor A, B or C
GalC	galactocerebroside
GFAP	glial fibrillary acidic protein
GluR	glutamate receptor
GluT	glutamate transporter
GlyR	glycine receptor
GlyT	glycine transporter
GRP	glial restricted precursor
Hes	Mammalian homolog of *hairy* and *Enhancer of split*
Hox	Homeobox
IC_{50}	antagonist concentration at which inhibition is half-maximal
Id	inhibitor of DNA binding
IP_3	inositol phosphate

Irx3	Iroquois 3
K2P	two pore potassium channels
K_A	binding affinity
KA	kainate
KCC2	K^+-Cl^- cotransporter
k_{off}	dissociation rate constant (s^{-1})
k_{on}	association rate constant ($M^{-1}s^{-1}$)
K_v	voltage-gated potassium channel
LGE	lateral ganglionic eminence of the developing forebrain
MAG	myelin associated glycoprotein
MapK	mitogen activated protein kinase
MBP	myelin basic protein
MEM	minimum essential medium
MGE	medial ganglionic eminence of the developing forebrain
MOG	myelin oligodendrocyte glycoprotein
n/mAChR	nicotinic/muscarinic actetyl choline receptor
N_c	number of channel closures
Ngn	neurogenin
NKCC	$Na^+/K^+/2Cl^-$ cotransporter
Nkx2.2	NK2 transcription factor related, locus 2
NMDA	N-methyl-D-aspartate
N_o	number of channel reopenings
NSF	N-ethyl maleimide sensitive factor
O	open state of the receptor (in receptor kinetics)
OLG	oligodendrocyte
Olig	oligodendrocyte lineage gene 2
OLN-93	oligodendroglial cell line derived from rat brain glial culture
OLN-SES	standard extracellular solution in experiments with oligodendroglial cells
OLN-SIS	standard intracellular solution in experiments with oligodendroglial cells
OLP	oligodendrocyte precursor cell
P	picrotin or picrotoxinin (in receptor kinetics)
P/S	100 U/ml penicillin and 100 µg/ml streptomycin in culture medium
Pax6	paired box gene 6
PBGD	porphobilinogen deaminase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	plateled derived growth factor
PDGFRα	plateled derived growth factor receptor alpha
PFA	paraformaldehyde
PGA	prolong gold antifade
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PLL	poly-L-lysine
PLP	proteolipid protein
pMN	motor neuron progenitor domain in the developing spinal cord
PTK	protein tyrosine kinase

PTN	picrotin
PTX	picrotoxin
PXN	picrotoxinin
RA	retinoic acid
Rb	retinoblastoma protein
rcf	relative centrifugal force
RMP	resting membrane potential
RP	reverse primer
rpm	rounds per minute
RT	reverse transcription
Shh	sonic hedgehog protein
SNAP	NSF attachment protein
SNARE	soluble NSF attachment protein receptor
Sox	SRY-box containing gene
SUR	sulphonyl urea receptor
Sx	transmembrane segment x
TEA	tetraethylammonium
TM	transmembrane domain
t-SNARE	target SNARE
V_{50}	potential at which activation is half-maximal
VGCC	voltage gated calcium channel
V_h	holding (clamp) potential
VIAAT	vesicular inhibitory amino acid transporter
V_m	membrane potential
v-SNARE	vesiculare SNARE
α	closing rate constant (s^{-1}) (in receptor kinetics)
β	opening rate constant (s^{-1}) (in receptor kinetics)
τ_b	mean burst duration
τ_c	mean closed time
τ_{decay}	decay time constant
τ_{fast}	time constant of fast phase
τ_o	mean open time
τ_{slow}	time constant of slow phase

Aims of the study

This study is the start of a new research line investigating the role of neurotransmitters in neuroglial cell functions, with specific attention for extrasynaptic function of neurotransmitters.

The first part of this thesis will focus on the function of glycine receptors themselves, with a special interest in the functioning of glycine receptors containing α2 subunits. This subunit is mainly expressed during development and is an interesting candidate for transducing extrasynaptic signals of glycine during the development of different neuronal and glial cell types. Inhibitory neurotransmitter signalling, and glycine signalling in particular, will be introduced in chapter 2. In chapter 3 the properties of the different components of picrotoxin, a widely used pharmacological tool in glycine receptor research, will be investigated. This results in a proposed model for blocking mechanisms by picrotoxinin and picrotin, the picrotoxin components, on α2 glycine receptors. The results provide further insight in glycine receptor kinetics and pharmacology. In chapter 4 data will be presented which indicate how the functional expression of the β subunit influences α2 glycine receptor properties. The expression of the β subunit increases during development and the hypothesis is that its incorporation into heteromeric α2β glycine receptors is necessary for the α2 glycine receptor to function in a synaptic context.

The second part of the thesis investigates an oligodendroglial cell line (OLN-93) in light of the study of influence of neurotransmitter receptors on oligodendrocyte cell function. In chapter 5, the oligodendroglial development will be introduced, with special attention for the role of electrophysiological properties herein. Chapter 6 looks at neurotransmitter receptor expression by this cell line and correlates it to the expression of neurotransmitters in a primary oligodendroglial culture derived from rat. Given the marked differences between primary cells and OLN-93, chapter 7 characterizes the developmental stage of the OLN-93 cell line using functional and biochemical tools. This insight is necessary to correctly use the cell line in future studies and to be able to interpret results from it in light of developmental studies of oligodendroglial cells.

Altogether the work presented provides tools for further research in the domain of extrasynaptic neurotransmitter roles, especially in the field of glial cell biology. Eventually it is our hope that insight into these mechanisms will subsequently lead to insight in disease mechanisms such as in Multiple Sclerosis, where oligodendrocytes are damaged and fail to repair sufficiently.

Chapter 1 | Materials & Methods

1.1 Cell culture

1.1.1 Cell lines

1.1.1.1 OLN-93 cell line

The OLN-93 cell line, derived from spontaneously transformed cells in primary rat brain glial cultures (see chapter 5), was a kind gift of Prof. Dr. Richter-Landsberg (Department of Biology, Molecular Neurobiology, University of Oldenburg, Germany). Cells were grown in a 90% air-10% CO_2 humidified incubator at 37 °C on glass coverslips (Ø12 mm) or in 25 cm^2 flasks (Thermo Fisher Scientific/Nunc, Waltham, MA, USA) in Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine, 4500 mg/L D-glucose and without sodium pyruvate (Invitrogen/Gibco, Carlsbad, CA, USA). This medium was supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen/Life Technologies, Carslbad, CA, USA) and 10 % (v/v) heat-inactivated fetal calf serum (FCS). To promote differentiation-like arborisation of the cells, culture surfaces were coated with 50 µg/ml poly-L-lysine (PLL) and cells were switched from 10% FCS to 0.5% (v/v) FCS after overnight attachment. Experiments were conducted on cells of 1 to 2 days in vitro (DIV) in case of 10% FCS and 2 to 3 DIV in case of 0.5% FCS. For experiments investigating the response to glycine, cells were cultured in the glycine-free minimum essential medium (MEM) with 25 mM HEPES (Invitrogen/Life Technologies, Carslbad, CA, USA). Cells were seeded at a density of 5000 cells/coverslip for patch-clamp experiments. The OLN-93 cell line was 20 passages old when received, stored in liquid nitrogen, and was used for 10 passages.

1.1.1.2 CHO cells transfected with α2 glycine receptor subunits

Chinese hamster ovarian cells (CHO-K1, ATCC no. CCL61) were cultured in a 95% air – 5% CO_2 humidified incubator at 37 °C in DMEM supplemented with 0.11 g/l sodium pyruvate, 4.5 g/l D-glucose (Invitrogen/Life Technologies, Carslbad, CA, USA) and 10% (v/v) heat-inactivated FCS. Glycine receptor α2 subunit cloning and transfection were performed as described by Mangin et al. (Mangin et al. 2003; Mangin et al. 2005). Cells were grown on glass coverslips (Ø 12 mm) or 25 mm^2 flasks (Thermo Fisher Scientific/Nunc, Waltham, MA, USA) coated with 50 µg/ml PLL. For patch-clamp experiments, cells were seeded at a density of 8000 cells/coverslip.

1.1.1.3 Transient transfection of the β subunit in α2 expressing CHO cells

CHO cells permanently transfected with the α2 subunit (Mangin et al. 2003) were plated on Ø 12 mm coverslips with a density of 2500 cells/well. The plasmid with β subunit was kindly provided by Bernard Rogister (Centre de Neurobiologie Cellulaire et Moléculaire, Université de Liège) and is constructed as described by Mangin *et al.* (Mangin et al. 2005). Per well, 3 µl lipofectamine 2000 was diluted in 50 µl OPTIMEM (mix 1) and allowed to incubate for 5 minutes. Again per well, 2 µg cDNA (of both the β subunit and the GFP reporter protein) was also diluted in 50 µl OPTIMEM (mix 2). Lipofectamine 2000 and OPTIMEM were from Invitrogen/Life Technologies (Carlsbad, CA, USA). After incubation of mix 1, mix 1 and 2 were combined and allowed to incubate for exactly 20 minutes. Culture medium was exchanged for 400 µl serum-free DMEM (Invitrogen/Gibco, Carlsbad, CA, USA) per well. After incubation, 100 µl of the transfection mix was added per well. Cells were allowed to incubate for 3-4 hours at 37 °C. Experiments were performed 24-48 hours after transfection procedure.

1.1.1.4 CHO cells permanently transfected with α2 and β glycine receptor subunits

Chinese hamster ovarian cells (CHO-K1, ATCC no. CCL61) were cultured in a 95% air – 5% CO_2 humidified incubator at 37 °C in DMEM supplemented with 0.11 g/l sodium pyruvate (Invitrogen/Life Technologies, Carslbad, CA, USA), 6 g/l D-glucose and 10% (v/v) heat-inactivated FCS. Glycine receptor α2 and β subunit cloning and transfection were performed as described by Mangin *et al.* (Mangin et al. 2005). To select for cells expressing both α2 and β subunits, 0.5% (v/v) zeocine and 1% (v/v) geneticine (G148) were added to the culture medium. Cells were grown on glass coverslips (Ø 12 mm) or 25 cm^2 flasks (Thermo Fisher Scientific/Nunc, Waltham, MA, USA) coated with 100 µg/ml poly-L-ornithine (PLO). PLO is stored in a stock solution of 1 mg/ml borate solution at pH 8.4. For patch-clamp experiments, cells were seeded at a density of 8000 cells/coverslip.

1.1.2 Primary cultures

1.1.2.1 Isolation of primary oligodendrocytes using the "shake-off" method

Brains were isolated from Wistar rats (Harlan, Indianapolis, IN, USA) at postnatal day 1 or 2 and placed in MEM with 100 U/ml penicillin and 100 µg/ml streptomycin (P/S). Brain stem, cerebellum and meninges were removed and the brain lobes were transferred to a sterile polystyrene container (Greiner Bio-

One, Kremsmünster, Austria) with 1 ml MEM, P/S. Next, brain lobes were cut with small scissors and passed trough an 18, 21 and 23 gauche needle sequentially to mechanically homogenise the tissue. The homogenised tissue was transferred to a falcon tube with culture medium being DMEM with L-glutamine, 4500 mg/L D-glucose and without sodium pyruvate (Invitrogen/Gibco, Carlsbad, CA, USA) with 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen/Life Technologies, Carslbad, CA, USA) and 10 % (v/v) non-inactivated fetal calf serum (FCS). This culture medium was added to a volume of 10 ml. By centrifugating at relative centrifugal force (rcf) of 140 g a soft pellet was obtained. The supernatans was discarded and the pellet was resuspended in culture medium to a volume of 2 ml per 1.5 isolated brain. Of this single cell suspension, 2 ml was added per 80 cm^2 culture flask (Thermo Fisher Scientific/Nunc, Waltham, MA, USA).
Cells were allowed to grow for 10 days after isolation, with medium renewal every 5 days. At day 11 after isolation, the culture flaskes were placed in a shake incubator, shaking them for 1 hour in 37 °C at 180 rounds per minute (rpm) to shake off the microglia. After this "pre-shake", supernatans was discarded, new culture medium was added to the flasks and the cells were shaken again overnight for 18-24 hours in 37 °C at 240 rpm to shake off oligodendrocytes.
After this overnight shake the supernatans was transferred to a sterile Petri dish (one dish per flask) (Greiner Bio-One, Kremsmünster, Austria) which was placed in an incubator for 30 minutes at 37 °C. Remaining astrocytes and microglia will differentially adhere to the surface of the culture dish. Culture medium could be added to the flasks again to use them for one more shake-off protocol a week later. After 30 minutes, the supernatans of the Petri dish was transferred to a falcon tube. When aggregates were still present in the supernatans, these were allowed to precipitate to the bottom of the falcon and the supernatans was again transferred to a new falcon tube, since these aggregates are mainly astrocytes. The falcon tube was then centrifuged at 85 g, the supernatans was discarded, cells were resuspended in Sato's medium (see table 1.1) and were counted before being seeded on appropriate culture surfaces.

1.1.2.2 Culture procedure for primary oligodendrocytes

Primary oligodendrocytes were cultured in Sato's medium (see table 1.1). Cells were grown in a 95% air- 5% CO_2 humidified incubator at 37 °C on glass coverslips (Ø12 mm) or in 25 cm^2 flasks (Thermo Fisher Scientific/Nunc, Waltham, MA, USA) coated with 10 µg/ml PLL. Cells were seeded at a density of 25000 cells/coverslip for patch-clamp experiments. To prevent cells from differentiating and to promote cell division PDGF and FGF were added to the culture wells to a final concentration 10 ng/ml. Stock solutions of 10 µg/ml were

prepared in 10 mM acetic acid for PDGF and in PBS for FGF and stored at -80 °C. Because of degradation of these growth factors, PDGF was added every 2 days and FGF every day the cells were in culture. Primary oligodendrocytes were used in experiments until 3 days after seeding. Cultured in this fashion cells primarily have few processes and are A2B5+/GFAP-, indicating they are in an oligodendrocyte progenitor stage (Baron et al. 2000).

Table 1.1 Sato's medium

Product	*Concentration in DMEM*
Bovine Serum Albumine fraction V*	0.1 mg/ml
Progesteron*	0.06 µg/ml
Putrescin*	16 µg/ml
Na-selenite*	0.005 µg/ml
T3*	0.4 µg/ml
T4*	0.4 µg/ml
Transferrin*	0.05 mg/ml
Insulin*	0.01 mg/ml
Penicillin	100 U/ml
Streptomycin	0.1 mg/ml

**These components were mixed in 100x stock (sato's mix in DMEM) and stored in 5 ml aliquots at -80 °C.*

1.2 Immunocytochemistry

For immunocytochemical characterisation, cells were washed extensively in phosphate buffered saline at pH 7.4 (PBS), fixed with 4% paraformaldehyde (PFA) for 12 minutes and permeabilized with 0.2% triton X-100 in PBS for 5 minutes. Following permeabilisation, 200 µl/well Image-iT FX signal enhancer (Invitrogen/Molecular Probes, Carlsbad, CA, USA) was applied for 30 minutes to block aspecific interaction of fluorescent dyes with cellular components. To prevent aspecific binding from secondary antibodies, 3% goat serum in PBS was used next as a blocking agent. Primary antibodies were then applied for 1 hour. Used primary antibodies include rabbit polyclonal anti-NG2 (1:200) (Millipore/Chemicon, Temecula, CA, USA), anti-Kv1.1 (1:200) and anti-Kv1.3 (1:50) (Alomone Labs, Jerusalem, Israel), mouse monoclonals anti-CNP (1:200) (Covance/SMI, Princeton, NJ, USA), anti-MAG (1:250), anti-GFAP (1:500) (Millipore/Chemicon, Temecula, CA, USA) and anti-MOG (a gift from Prof. C.C.A. Bernard, Monash University, Clayton Victoria, Australia). To conclude, cultures were incubated with the goat anti-mouse or anti-rabbit secondary antibodies conjugated to Alexa 555 or 647 (1:200) (Invitrogen/Molecular probes, Carlsbad, CA, USA) for 1 hour. The wells were thoroughly washed with PBS three times after every step. Omission of the primary antibodies served as negative control. Slides were mounted using Prolong Gold Antifade (PGA) (Invitrogen/Molecular probes, Carlsbad, CA, USA) and stains were visualized using confocal microscopy

(Zeiss LSM 510 META) and LSM browser imaging software (Zeiss, Jena, Germany).

1.3 Reverse transcriptase polymerase chain reaction

RNA was isolated with an RNeasy Mini Plus kit following the manufacturer's protocol (Qiagen, Venlo, The Netherlands). This kit ensured additional elimination of genomic DNA. cDNA was then prepared from the collected RNA using the Reverse Transcription system (Promega, Madison, WI, USA). Reverse transcription (RT) was allowed for 1 hour at 42 °C followed by a denaturation step at 95 °C for 5 minutes. After this RT, DNA was extracted using phenol/(chloroform:isoamyl alcohol) (1/1) (Sigma-aldrich, St. Louis, MO, USA). To remove traces of phenol, extraction was repeated with chloroform:isoamyl alcohol (24:1). DNA was then precipitated overnight at -20°C or 1 hour at -80 °C using 100% ethanol (200 µl) and 3 M sodium acetate (10 µl) (Sigma-aldrich, St. Louis, MO, USA). The precipitate was then centrifuged (20 min / 13200 rpm / 4°C), supernatans discarded, and 200 µl of cold 70% ethanol was added before again centrifuging (15 min / 13200 rpm / 4 °C). Finally, the supernatans was discarded, the sample was allowed to dry before being resuspended in autoclaved water and stored at -20 °C until analysis. After obtaining RNA as well as after obtaining DNA, the nucleic acid purity was determined by measuring the A260/A280 and A260/230 absorption ratios using a GeneQuant pro spectrophotometer (GE Healthcare, Munich, Germany).

The PCR mix consisted of 0.75 µl Taq polymerase (1 U/µl), 2.5 µl PCR buffer, 0.25 µl dNTP's (20 mM), 1 µl of both primers (10 µM) and cDNA and destilled water to obtain a total volume of 25 µl. All components were from Roche (Roche Applied Science, Basel, Switserland). In the PCR reaction, PBGD (Porphobilinogen deaminase) was used as housekeeping gene. The PCR reaction was performed by a BioRad Thermal cycler (BioRad, Hercules, CA, USA). A denaturation step (95 °C for 20 min) was followed by amplification over 40 cycles of denaturation (94 °C for 20 s), annealing (57.3-63.3 °C for 60 s), dependent on the primers, and elongation (72 °C for 40 s). Primers were designed using Oligo6 software (Molecular Biology Insights, Cascade, CO, USA), except when mentioned otherwise (table 1.2) and synthesized by Eurogentec (Seraing, Belgium). See table 2.2 for exact primer sequences.

When the reaction was completed, the PCR product was placed on a 1% agarose gel after adding 9 µl of the color marker Orange G (Sigma-aldrich, St. Louis, MO, USA). After electrophoresis gels were stained with ethidium bromide (Invitrogen/Gibco, , Carlsbad, CA, USA) in TEA buffer (Roche Applied Science, Basel, Switserland) and visualised using a Gel doc system controlled by Quantity one software (Bio-rad, Hercules, CA, USA).

Table 1.2 Primer sequences

mRNA		Primer sequence	Product length (base pairs)
PBGD[1]	FP	5'ACCTAGTGGGTGTGTT 3'	525
	RP	5'CAAGGCCGAAGTCTCA 3'	
NG2	FP	5'CTGAGGTGAATGCTGGGAAT 3'	190
	RP	5' CC TTTGTTCCTCCAGAGACG 3'	
CNP	FP	5' CCCAGGTGGTGCTTAATGAG 3'	272
	RP	5' CATCTTCTTCGCCAAGCTC 3'	
MBP	FP	5' CATCACAGAAGAGACC CT 3'	145
	RP	5' CCTGTCACCGCTAAAGA 3'	
MAG	FP	5' GGTGCTGTGGTCGCCTTT 3'	365/320
	RP	5'CGGATTTCTGCGTACTCAGCC 3'	
PLP/DM20	FP	5' TGCTGGCCGAGGGCTTCTACAC 3'	791/756
	RP	5' CGGCCCATGAGTTTAAGGAC 3'	
MOG	FP	5' CAGTTGTCAC GCAGCTACGC 3'	349
	RP	5' CGGCTTCTTCTTGGTAGGAG 3'	
GFAP	FP	5' TATGCCTCCTCCGAGACGAT 3'	545
	RP	5' TCCTCCTCCAGCGACTCAAC 3'	
Kv1.1	FP	5' GTCAGGGGAGAATGCAGACGA 3'	73
	RP	5' CGCCTGCCTTGGGTAGC 3'	
Kv1.2[2]	FP	5' CACCGGGAGACAGAGGGA 3'	248
	RP	5' TCAGACATCAGTTAACAT 3'	
Kv1.3	FP	5' GCAGAGGAGCTCCGAAAAG 3'	171
	RP	5' GATGTTGACACAGGAGTTGGG 3'	
Kv1.4	FP	5' GAAATTTCGGAGCTCTACTTC 3'	191
	RP	5' TGGGGAAAGAGATTCACA 3'	
Kv1.5	FP	5' CGACGTCTGGACTCAATAATC 3'	142
	RP	5' TCATCCTCAGCAGATAGCCT 3'	
Kv1.6[2]	FP	5' ATCGGAGAAATCCCTGACGCT 3'	206
	RP	5' CGGCCTCCTGGAACTCAC 3'	
GlyR α1	FP	5' GTCCCAACAACAACAACACC 3'	211
	RP	5' TCCCAGAGCCTTCACTTGTT 3'	
GlyR α2	FP	5' CTACACCTGCCAACCCAC 3'	182
	RP	5' CTTGTGGACATCTTCATGCC 3'	
GlyR α3	FP	5' GCACTGGAGAAGTTTTACCG 3'	309
	RP	5' AATCTTGCTGATGATTGAATGTC 3'	
GlyR β	FP	5' GAAGAACACTGTGAACGGCA 3'	228
	RP	5' GGCTTCTTGTTCTTTGCCTG 3'	
GlyT1	FP	5' CTGGAGGCTGTATGTGCTGA 3'	157
	RP	5' GGCCGTGAAGTACACCACTT 3'	
GlyT2	FP	5' TATGAGCCAGAGCACAGTGG 3'	239
	RP	5' AAGGGATGAGGAAAGCACCT 3'	
Gephyrin	FP	5' CCATGGGGGAAAAGGACTAT 3'	160
	RP	5' GGATTCCCTGGTAGTGCAAA 3'	

[1] *Positive control*

[2] *Primers references: Kv1.2 and Kv1.6 (Attali et al. 1997), GlyR α1, α2 and β (Nguyen et al. 2002), GlyR α3 (Thio et al. 2003)*

PBDG: porphobilinogen deaminase, CNP: 2',3'-cyclic nucleotide-3'-phosphodiesterase, MBP: myelin basic protein, MAG: myelin associated glycoprotein, PLP: proteolipid protein, MOG: myelin oligodendrocyte glycoprotein, GFAP: glial fibrillary acidic protein, Kv: voltage gated potassium channel, GlyR/T: glycine receptor/transporter, FP: forward primer, RP: reverse primer

1.4 Patch-clamp recording

1.4.1 Patch-clamp setup

1.4.1.1 Whole-cell recordings

For whole-cell patch-clamp recordings, cells were grown on glass coverslips with 12 mm diameter. The coverslips were mounted on a RC-25 perfusion chamber (Warner Instruments/Harvard Apparatus, Holliston, MA, USA) and placed on the stage of a Diaphod-TMD inverted phase contrast microscope (Nikon, Tokyo, Japan). The bathing solution was gravity fed in the perfusion chamber and total bath volume was kept constant by a collection needle connected to a vacuum system. The reference electrode was placed in a separate bath filled with extracellular solution, connected to the recording bath using an agar bridge. Both recording and reference electrodes were made of chlorated silver wire. The microscope was placed on a home made vibration free table and in a Faraday cage, wherein all electrically isolated metal parts were grounded.
Patch electrodes were made from borosilicate glass with an outer diameter of 1.5 mm and a wall thickness of 0.315 mm (Hilgenberg, Maslfeld, Germany). To facilitate filling, the electrodes had a filament. Electrodes were pulled using a horizontal programmable DMZ-universal puller (Werner Zeitz, Augsberg, Germany). Resistance of the electrodes was between 3 and 6 MΩ. Electrodes were mounted on the headstage of the patch-clamp amplifier, of which movement was controlled by a WR-98 hydraulic micromanipulator (Narishige Scientific Instruments, Tokyo, Japan).

1.4.1.2 Outside-out recordings

For outside-out recordings, the setup was equivalent except for the following aspects. Cells were mounted on a home-made perfusion chamber. This was placed on the stage of a Optiphod upright microscope (Nikon, Tokyo, Japan).
Patch electrodes were made from borosilicate glass with an outer diameter of 1.5 mm and an inner diameter of 0.86 mm (Harvard Apparatus, Kent, UK). Electrodes were pulled using a horizontal programmable P-97 puller (Sutter Instruments, Novato, CA, USA) and were fire polished using a MF-830 microforge (Narishige Scientific Instruments, Tokyo, Japan). Resistance of the electrodes was between 8 and 10 MΩ.

1.4.2 Perfusion systems

For experiments requiring fast solution changes, such as application of neurotransmitters, a fast application perfusion system was used. In the whole-cell experiments, this system was based on a SF-77B perfusion step system (Warner Instruments, Holliston, MA, USA) of which the standard 3-barrel glass

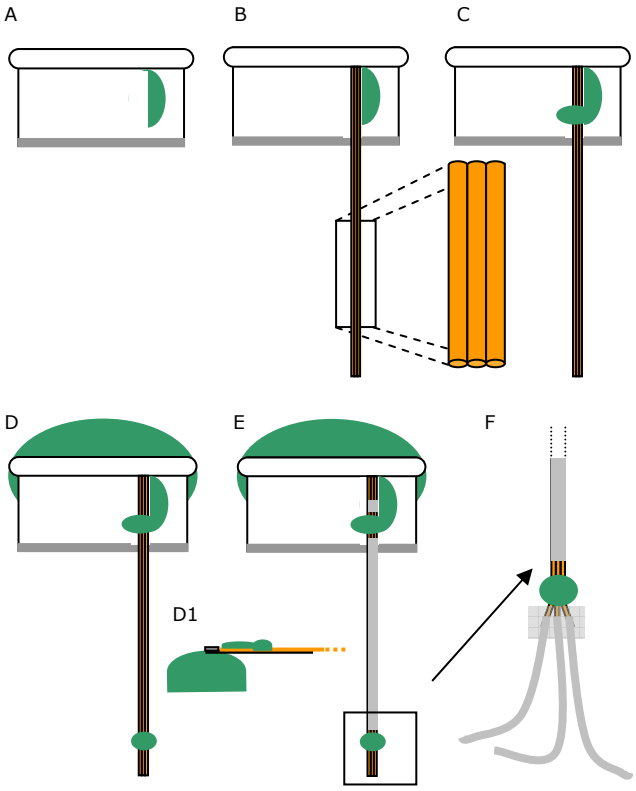

Figure 1.1 *Construction of a 3-barrel system for fast solution exchange using quartz glass.* A straight cut piece of clay was placed against the upper edge of a razor blade in a 90° angle (A). 3 pieces of quartz glass of about 8 cm were placed against the clay (B) using a fine pincer and fixed with another piece of clay (C). The alignment of the tubes was checked under the microscope before placing the razor blade on a large ball of clay to lift it from the surface and fixing the end of the tubes with another bit of clay (D, D1). A layer of two component glue was applied on the beginning and the middle of the tubes but care was taken to keep the ends of the capillaries free of glue to avoid clogging of the tubes (E). The glue is then allowed to dry for 1 hour. Next, tubing is fitted on the end of the capillaries and glue is applied to fix the tubing (F). Finally, the glue is again allowed to dry for 1 hour. The barrels could then be mounted on the SF-77B and fixed using wax. The front of the capillaries was cut to the needed length. Further drying of the glue was allowed for 24 hours before use.

was substituted by a home-made 3-barrel quartz glass system. The construction of this system is schematised in figure 1.1.

In outside-out experiments mimicking synaptic transmission, a custom made ultrafast perfusion system was used. These experiments were conducted in the lab of Dr. Legendre at the Université Pierre & Marie Curie in Paris (Paris VI). This system was driven by a P245.30 piezoelectric translator (Physik Instrumente, Karlsruhe, Germany). Solutions were gravity-fed to the patch via the two channels of a thin-walled glass theta tube of 2 mm outer diameter (Hilgenberg, Maslfeld, Germany) which was pulled and broken so it had a tip diameter of 200 µm. The patch was place close to the interface of the control and drug solution at about 100 µm away from the perfusion tip.

In both perfusion systems, solution exchange was achieved by rapidly moving the solution interface across the cell (whole-cell) or patch (outside-out). Exchange times were determined after each experiment by rupturing the seal and monitoring the change in liquid junction potential resulting from application of control solution diluted with 20% water on the open tip of the patch pipette. This also allows detection of rebounds or other perfusion problems, which could

bias the kinetics of currents measured during the experiment. Figure 1.2 shows an exemplary result of such a perfusion test of the SF-77B system and shows that solution exchange with this system could be obtained within less than 50 msec. The ultrafast system reaches an exchange speed of less then 0.1 msec, allowing mimicking of synaptic transmission. The kinetics of this system are discussed more thoroughly by Legendre (Legendre 1998).

1.4.3 Solutions

For experiments on CHO cells, bathing solution (CHO-SES) had a composition of (in mM) 124 NaCl, 2 KCl, 2 $MgSO_4$, 1.15 KH_2PO_4, 2 $CaCl_2$, 16.7 Glucose, 26 $NaHCO_3$. Osmolality was adjusted to 325 mOsm/kg H_2O with dextrose and pH was adjusted to 7.25 with NaOH if needed. Glycine was diluted to the desired amount in CHO-SES. Because of the HCO_3^-/CO_2 buffer, CHO-SES was freshly made before the experiment. The intracellular solution (CHO-SIS) contained (in mM): 130 CsCl, 4 MgCl, 10 HEPES, 10 EGTA and 4 Na_2ATP. All products, unless mentioned otherwise, were from Sigma (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

Oligodendroglial cells were perfused at room temperature with an extracellular solution (OLN-SES) containing (in mM): 145 NaCl, 5 KCl, 1.5 $MgCl_2$, 2 $CaCl_2$, 10 HEPES. Osmolality was adjusted to 310 mOsm/kg H_2O with dextrose and pH was adjusted to 7.4 with NaOH. Margatoxin (MgTx) (Alomone labs, Jerusalem, Israel) was diluted in extracellular solution and stored in aliquots of 1 µM concentration. The toxin was further diluted to 10 nM in extracellular solution just before use. When tetraethylammonium (TEA) was used, TEACl substituted NaCl in equimolar amount. For experiments with neurotransmitters stock solutions of 1M glycine, 100 mM glutamate, 10 mM γ-amino butyric acid (GABA) and 10 mM acetyl choline (ACh) in OLN-SES were made and stored at 4 °C. ACh stock was kept for maximum 14 days (Sletten et al. 2005). Solutions containing 1 mM serotonine or 1 mM Na_2ATP in OLN-SES were freshly prepared before use because of the limited stability of these

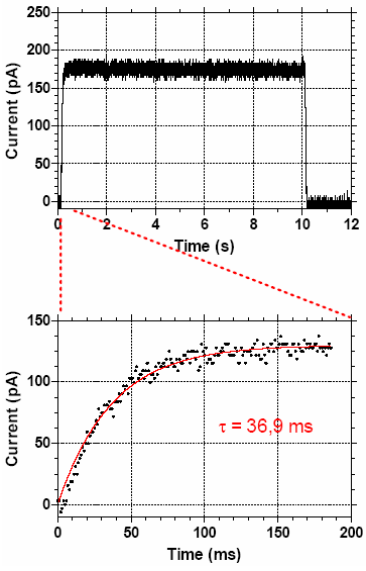

Figure 1.2 *Example of a perfusion test of the SF-77B perfusion system.* The solution flowing over an open patch pipette was changed from control to control diluted with 20% water. This resulted in a shift in liquid junction potential. This shift could then be used to evaluate exchange speed of the system. Unwanted rebounds would also be visible as recurring peaks right after solution exchange.

neurotransmitters. Serotonine was protected from light during the experiments. The intracellular solution in experiments with oligodendroglial cells (SIS-OLN) contained (in mM): 125 KCl, 5 NaCl, 2 $CaCl_2$, 2.5 MgATP, 2.5 Na_2ATP, 10 HEPES, 10 EGTA. Osmolality was adjusted to 290 mOsm/kg H_2O with dextrose and pH was adjusted to 7.4 with KOH.
Because of limited stability of ATP salts in solution, 100 ml of the intracellular solutions was prepared without ATP salts and divided in 10 ml aliquots before being stored at -20 °C. ATP salts were added to a 10 ml aliquot, which was then divided again in 1 ml aliquots. These ready-for-use 1 ml aliquots were stored at -20 °C for 2 weeks maximum. During patch-clamp experiments intracellular solution was always kept on ice. This ensured maximal ATP content in the intracellular solution, which facilitated gigaseal formation.
Liquid junction potential was calculated with Junction Potential Calculator for Windows (Peter H. Barry, University of New South Wales, Sidney, Australia), and was compensated for at the start of each experiment.

1.4.4 Patch-clamp procedures

Patch-clamp experiments were conducted in the whole-cell and outside-out configurations (Hamill et al. 1981) at room temperature. The patch pipette was kept under constant positive pressure while approaching the cell. Cells were approached from the top, whilst avoiding to place the pipette on the nucleus of the cell. When the pipette was close enough to the cell membrane, a dent in the cell membrane could be clearly seen on an upright microscope. On the phase contrast inverted microscope, this was not always clear to see, so alternatively a rise in the pipette resistance of about 0.5 MΩ was used as an indication that the positive pressure in the pipette could be released. This resulted in the cell membrane contacting the pipette and gentle suction was applied, whilst lowering

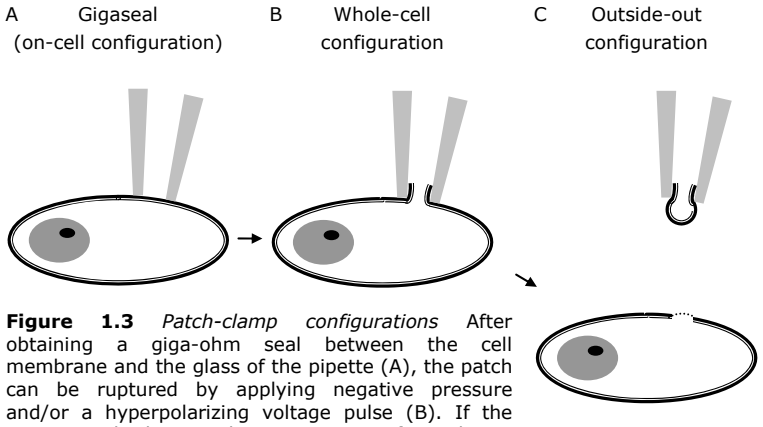

Figure 1.3 *Patch-clamp configurations* After obtaining a giga-ohm seal between the cell membrane and the glass of the pipette (A), the patch can be ruptured by applying negative pressure and/or a hyperpolarizing voltage pulse (B). If the pipette is slowly moved away, a piece of membrane fuses to a vesicle at the tip of the patch pipette (C).

holding potential to -60 mV, to enhance gigaseal formation. Negative pressure was released if the pipette resistance raised above 100 MΩ. Giga-Ohm seals could generally be reached within 30 seconds, indicating a strong fusion between the cell membrane and the glass of the patch pipette (figure 1.3A). The seal was allowed to rest for about 1 minute and pipette capacitance was compensated before applying slowly rising negative pressure to break the seal and achieve whole-cell configuration (figure 1.3B), hallmarked by the appearance of the capacitative currents arising from the membrane (C_{slow}). When using smaller pipettes, like in outside-out experiments, a short hyperpolarizing voltage pulse ("zap") could be necessary to rupture the patch by initiating dielectric breakdown of the membrane patch.
In whole-cell experiments, the intracellular solution of the pipette was allowed to equilibrate with the cell interior for 2-3 minutes and C_{slow} was compensated for. Series resistance (see below) was also compensated for in order for it to be lower than the double of the tip resistance, with a maximum of 80% compensation. In outside-out experiments, the pipette was slowly moved away from the cell after obtaining whole-cell configuration, moving it diagonally upwards. During this movement, the membrane capacitance peaks would disappear, whilst pipette resistance would become in the giga-Ohm range again, indicating a successful outside-out patch configuration (figure 1.3C).

1.4.5 Confounding electrical phenomena

A patch-clamp setup measures potential differences between the recording electrode and the grounding electrode and correlates this with membrane potentials. The recording electrode in a patch-clamp setup is located in the glass pipet. Changes in membrane potential thus are measured or applied through this electrode. However, it cannot simply be assumed that the potential difference between the recording and grouding electrode is exactly the same as the potential across the membrane. Therefore a series of compensations are needed to make the measurements as accurate as possible (Sakmann and Neher 1995; Walz et al. 2002).

1.4.5.1 Liquid junction potential

Before contacting the cell membrane, the fluid in the glass pipet (intracellular fluid) is in direct contact with the extracellular fluid. The ionic composition of both fluids is not the same. This difference causes diffusion of ions along their concentration gradient. For example, in most standard solutions [Na^+] is higher in the extracellular fluid and [K^+] is higher in the intracellular fluid. This will cause diffusion of Na^+ into the pipet and diffusion of K^+ out of the pipet. Because of electroneutrality, dipoles (e.g. Na^+ and Cl^- or K^+ and Cl^-) arise. Since Na^+ and K^+ have different mobilities, the opposite dipoles will cause a potential difference

across the junction between intra- and extracellular fluid. This is the "liquid junction potential". This effect is enlarged if other anions, with lower mobility than Cl^-, are present, because of the existence of larger dipoles. The liquid junction potential has to be compensated for, or it will bias measurement of the membrane potential.

1.4.5.2 Series resistance compensation

Ohm's law states that a potential changes when current flows over a resistance. The current applied through the recording electrode in fact crosses three resistances, being (1) the resistance of the glass tip, (2) the resistance of the membrane patch and (3) the resistance of the cytosol. These three resistances are in series and form the series resistance (R_s) (fig. 1.4). The R_s has to be compensated to prevent an unwanted potential difference. The goal is to keep R_s as small as possible compared to the membrane resistance (R_m). To achieve this, firstly the diameter of the glass tips has to be as large as is possible for the intented experiment. Secondly, R_s should be compensated. Most patch-clamp setups have a positive feedback circuit which adds a signal to the clamped potential to compensate for the potential decay over R_s. Thirdly, the R_s compensation should be checked regularly during the experiment since R_s can increase because of for example clogging of the pipette by membrane fragments. In practice, one shall try to keep the R_s less than twice the R_m.

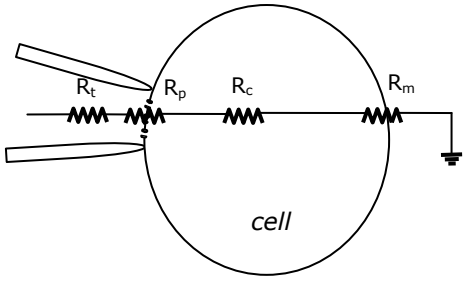

Figure 1.4 *Series resistance and membrane resistance in whole cell measurements.* The series resistance (R_s) consists of the tip resistance (R_t), the patch resistance (R_p) and the resistance of the cytosol (R_c).

If R_s is not or insufficiently compensated, it will slow down the response to an applied change in potential, since the time constant of a change in potential is described by:

$$\tau = R.C$$

With R being the resistance and C the capacitance (see below).

1.4.5.3 Capacitance compensation

When performing whole-cell experiments, there are two capacitances that can confound the measurements, being the pipet capacitance (C_p) and the membrane capacitance (C_m). In outside-out experiments, only C_p is of concern. Charging of capacitances demands current, which can cause a delay in clamping the membrane potential because current is "lost" is charging of capacitances. This is of extra importance when measuring quick responses to different steps in membrane potentials, since capacitative currents will overlap fast voltage-

dependent currents. Patch-clamp amplifiers are capable of compensating for these capacitances.
Membrane capacitance is often used as a measurement for membrane surface area. The larger C_m, the larger the membrane surface area. When analyzing measurements, normalisation for cell size thus can be easily done by dividing the measured currents by C_m.

1.4.6 Data acquisition and analysis

Whole-cell data were recorded using a EPC9 or EPC10 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) controlled by PULSE or TIDA software (HEKA Elektronik, Lambrecht, Germany) on a windows based personal computer. Data were filtered at 2.9 kHz (low pass filter), sampled at 20 kHz and stored for offline analysis. Outside-out data were recorded using a Axopatch 1D amplifier controlled by pClamp software, both from Axon instruments (Foster City, CA, USA), on a Windows personal computer. Data were filtered at 5 kHz, using an eight-pole bessel filter (Frequency Devices, Ottawa, IL, USA), sampled at 50 kHz and stored for offline analysis.
Offline analysis was performed with PULSEfit (HEKA Elektronic, Lambrecht, Germany), Graphpad Prism 4 (Graphpad Software, San Diego, USA) and Axograph software (Axon Instruments, Foster City, CA, USA). All values in the text and the figures are expressed as means ± s.e.m. with *n* being the number of experiments. When averages from repeated measurements one a patch were calculated, this was regarded as one experiment. Statistical tests were considered significant for $p \leq 0.05$.

Chapter 2 | An introduction into glycine signaling

The central nervous system (CNS), consisting of the brain and the spinal cord, integrates signals coming from and going to the periphery. This requires fast signal propagation within and between neurons. Myelin sheats, formed by oligodendrocytes in the CNS, improve the speed of propagation of an action potential. The importance of this myelin sheath is evident in demyelinating diseases, such as multiple sclerosis, where symptoms include motor, vision and coordination problems, amongst others. The signal between neurons is transmitted through synapses by soluble factors called neurotransmitters.
In recent years, it has become evident that the function of neurotransmitters goes beyond synaptic transmission. Several neurotransmitters have become implicated in regulation of developmental processes. In this work we will investigate the properties of the α2 glycine receptor, which is mainly expressed prenatally and thus is a good candidate for future study of its influence on developmental processes. Thereby the main research focus will be on its role in the development of oligodendroglial cells. For this purpose, the glycine receptor expression by an oligodendroglial model, OLN-93, was investigated, and its developmental stage was characterized.
In this chapter, an introduction into the synaptic functions of inhibitory neurotransmitters, such as glycine, will be given. We will then focus on the properties of the glycine receptor in particular. Next, the involvement of inhibitory neurotransmitters in the developmental processes of neurons will be overviewed. In chapter 5, the second part of the thesis will be introduced, with a focus on the development of oligodendroglial cells and the roles of electrophysiological properties herein.

2.1 Inhibitory neurotransmission in the central nervous system

Communication between cells is a key feature in the functioning of the nervous system. The interplay of inhibitory and excitatory signalling between neurons results in a wealth of responses crucial to the functioning of the organism. The fast intercellular communication required for neuronal signalling occurs in specialised regions called synapses. In synapses of the central nervous system (CNS), specialized membrane regions of neurons come closely together, separated by a synaptic cleft, which is about 30 nm wide. The presynaptic terminals are located at the end of the axons, and they form synapses with postsynaptic membrane regions mainly located on dendrites and cell bodies. Since pre- and postsynaptic membranes thus are separated by this cleft filled with extracellular fluid, communication between them has to be mediated by soluble factors. These substances are called neurotransmitters. Hereafter an overview of inhibitory neurotransmission in the central nervous system will be provided, after which the focus will shift to neurotransmitter receptors mainly

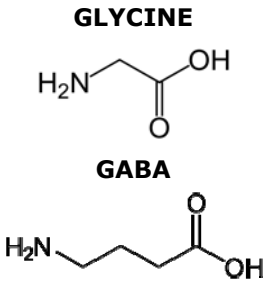

Figure 2.1 *Structure of glycine and GABA.* Note that GABA is not a classical proteinogenic amino acid.

involved in inhibitory neurotransmission, namely the members of the cys-loop family of receptors. Special attention will be given to the glycine receptors, which are an important research subject of the work presented in this thesis. Lastly, this part of the introduction will be concluded by having a look at possible extrasynaptic functions of neurotransmitters, especially in development.

2.1.1 The synaptic machinery

Synaptic communication can be excitatory, inhibitory or modulatory. This means that the neurotransmitter released in a synapse can respectively cause a depolarization or a hyperpolarization of the postsynaptic membrane or can have a modulatory effect through G protein coupled mechanisms such as phosphorylation. If a depolarisation reaches a certain threshold, this will evoke an action potential in the postsynaptic neuron, while hyperpolarization inhibits generation of action potentials. Whether synaptic communication is excitatory, inhibitory or modulatory depends on the neurotransmitter and its receptors involved, and on the ion concentrations in the intra- and extracellular environment. The main excitatory neurotransmitter of the central nervous system is glutamate, while its main inhibitory counterparts are γ-amino buteric acid (GABA) and glycine. Going into detail on the excitatory neurotransmitters would be beyond the scope of this thesis, but the general principles of the synaptic machinery are alike.

2.1.1.1 Presynaptic filling of vesicles

GABA and glycine both belong to the chemical class of amino acids, but only glycine is proteinogenic (fig. 2.1). GABA is synthesized from glutamic acid (glutamate) by the action of glutamic acid decarboxylase (GAD). Glycine is produced from serine by serine-hydroxymethyltransferase (Legendre 2001). GABAergic transmission mainly occurs in the brain, while glycinergic transmission mainly occurs in the spinal cord. In the presynaptic neuron, GABA and glycine share the same transporter which functions to concentrate the inhibitory neurotransmitters in presynaptic vesicles. This transporter is the vesicular inhibitory amino acid transporter (VIAAT), which is a H^+/neurotransmitter exchanger (Gasnier 2000). The H^+ gradient needed for this exchange is provided by an ATP-dependent proton pump (fig. 2.2). This results in an acidified intravesicular environment with pH 5.2-5.5 (Fuldner and Stadler 1982; Michaelson and Angel 1980). It is thought that in an inhibitory synapse vesicles are filled with both glycine and GABA, so postsynaptic effects mainly

depend on the receptors present. However, the mechanisms of uptake of inhibitory neurotransmitters are still under investigation and studies suggest presynaptic factors, such as plasmalemmal neurotransmitter transporters or VIAAT associated proteins, have a more important role in determining vesicle filling than originally thought (Aubrey et al. 2007; Gasnier 2000; Jin et al. 2003; Katsurabayashi et al. 2004).

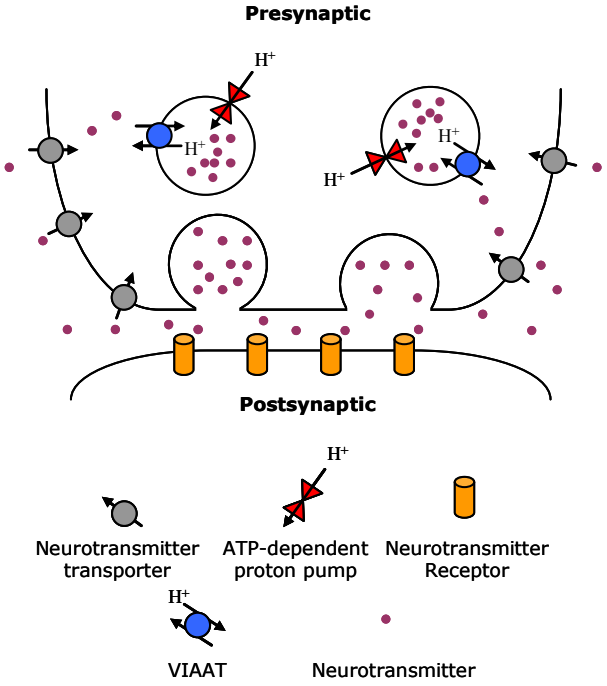

Figure 2.2 *The synaptic machinery of an inhibitory synaps.* Neurotransmitters are released from presynaptic vesicles into the synaptic cleft. Neurotransmitter-specific transporters serve to pump these neurotransmitters back into the presynaptic terminal. Using the H^+ gradient established by ATP-dependent proton pumps, VIAAT transports the neurotransmitters into presynaptic vesicles. VIAAT: Vesicular inhibitory amino acid transporter (H^+/neurotransmitter antiporter).

2.1.1.2 Vesicle release and clearance of neurotransmitters from synaptic cleft

Vesicles packed with neurotransmitters are localized in the presynaptic region and concentrated in so-called active zones. Some of the vesicles in the active zone are already in contact with the presynaptic membrane. Key players in this contact and in the later fusion of the vesicle with the membrane, and thus in neurotransmitter release, are the SNARE proteins (fig. 2.3). SNARE stands for soluble *N*-ethyl maleimide sensitive factor (NSF) attachment protein (SNAP)

receptor. Although the molecular nature of vesicle exocytosis is not yet fully elucidated, some key components in synaptic vesicle release have been identified (Moczydlowski 2003; Rizo et al. 2006; Schweizer and Ryan 2006). Synaptobrevin, a SNARE protein located on the vesicles (v-SNARE), forms a complex with syntaxin and SNAP-25 which are located on the presynaptic membrane and are target SNARE proteins (t-SNARE). Although this interaction brings the neurotransmitter-containing vesicle close to the presynaptic membrance, membrane fusion and neurotransmitter release are dependent on an increase of the Ca^{2+} concentration. This increase is caused by the activation of voltage gated Ca^{2+} channels by the action potential arriving through the axon.

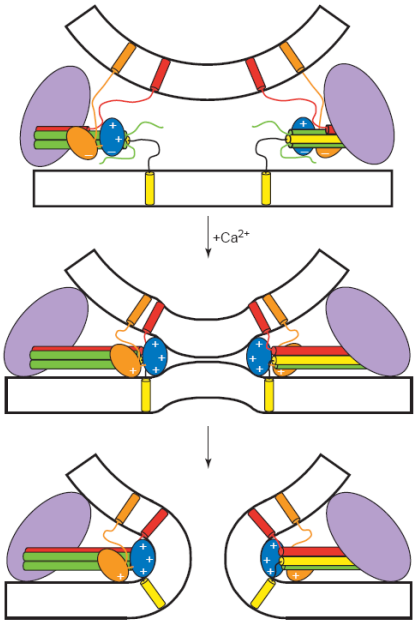

Figure 2.3 *Model for Ca^{2+} triggered vesicle release by cooperation between synaptotagmin, SNAREs and other proteins.* The v-SNARE synaptobrevin (*red*) forms a complex with the t-SNAREs syntaxin (*yellow*) and SNAP-25 (*green*). This complex associates with synaptotagmin, the Ca^{2+} sensor, located in the vesicle membrane. The different synaptotagmin-domains are depicted by *orange* and *blue* ellipses. Their charges might be important for the response to Ca^{2+} release, and are involved in interaction with the membranes. The *purple* ellipses indicate proteins of the Sec1-Munc18 family, of which the exact function is not known. Nevertheless, these proteins are essential for neurotransmitter release.
From Rizo et al. (2006), with permission.

The actual Ca^{2+} sensor which triggers the membrane fusion is believed to be synaptotagmin, a protein present in the vesicle membrane. Again, the exact mechanism is not fully understood (Moczydlowski 2003; Rizo et al. 2006). In addition to its role in exocytosis, Ca^{2+} has also been mentioned as playing a role in endocytosis and vesicle recycling at the synapse (Perissinotti et al. 2008; Sankaranarayanan and Ryan 2001).

Once released in the synaptic cleft neurotransmitters have to be cleared again and recycled. This is dependent on transporter mechanisms located in membranes of nearby glial cells and in the presynaptic terminal. The transporters involved in pumping the inhibitory neurotransmitters GABA and glycine belong to the same family, the SLC6 gene family, and are Na^+/Cl^- dependent. There are four GABA transporter subtypes, called GAT1-4 (Conti et al. 2004), and two glycine transporter subtypes, GlyT1 and GlyT2 (Eulenburg et al. 2005).

2.1.1.3 Postsynaptic receptors and response

To elicit a response in the postsynaptic cell, neurotransmitters bind to specific receptors. These receptors can be ionotropic or metabotropic. Ionotropic receptors are in fact ligand gated ion channels, which open after binding of the neurotransmitter. Metabotropic receptors are G-protein coupled receptors which initiate a signal transduction pathway after binding of the neurotransmitter. Ionotropic receptors for glycine and GABA belong to the same family, being the cys-loop receptor family. To date, only ionotropic glycine receptors have been described. The glycine receptor is either a homomer of five identical α subunits or a heteromer of two α subunits and three β subunits. Four different α subunits (α1-4) have been identified, which will be discussed in more detail further in this introduction. The molecular diversity of GABA receptors is much more complex. There are two ionotropic receptor subtypes, $GABA_A$ and $GABA_C$, and one metabotropic receptor, $GABA_B$. $GABA_A$ receptors are heteropentameric and can consist of a wide variety in subunits. Sixteen different subunits of $GABA_A$ have been described, being α1-6, β1-3, γ1-3, δ, ε, θ and π (Mohler 2006; Owens and Kriegstein 2002). The number of theoretical combinations from these subunits is immense. However, described native $GABA_A$ channels mostly contain α, β and γ subunits (Barnard et al. 1998). This still results in a large amount of theoretical possibilities, although there may be less than 20 widely occurring combinations (Mohler 2006; Owens and Kriegstein 2002). The $GABA_C$ receptors are homopentameric receptors composed of ρ subunits, of which three variant (ρ1-3) exist. In contrast to $GABA_A$ receptors, $GABA_C$ is not sensitive to bicuculline antagonism (Barnard et al. 1998; Mohler 2006). Moreover $GABA_C$ shows increased GABA sensitivity (Zhang et al. 2001). Despite these differences, some argue that the ρ subunit is too closely related to the subunits found in $GABA_A$ receptors to separate $GABA_C$ in a different subfamily. Therefore $GABA_C$ can also be classified as a homopentameric type of the $GABA_A$ receptor (Barnard et al. 1998; Mohler 2006; Owens and Kriegstein 2002). The $GABA_B$ receptor is a metabotropic receptor and not an ion channel like $GABA_{A/C}$. It is a heterodimer of subunits called R1 and R2. R1 exists in two isoforms R1a and R1b (Kornau 2006; Owens and Kriegstein 2002). Activation of the G-protein coupled $GABA_B$ receptor results in inhibition of Ca^{2+} channels and stimulation of K^+ channels (through the βγ complex) and in modulation of adenylate cyclase activity and thus cAMP levels (through the α complex) (Kornau 2006). $GABA_B$ receptor can be found both pre- and postsynaptically and can thus influence presynaptic neurotransmitter release as well as postsynaptic inhibitory responses (Kornau 2006; Owens and Kriegstein 2002).

Both ionotropic glycine receptors and GABA receptors are selective for Cl^- when activated. In the mature CNS the Cl^- equilibrium is such that Cl^- flows into the cell through the open channels of these receptors. This results in a

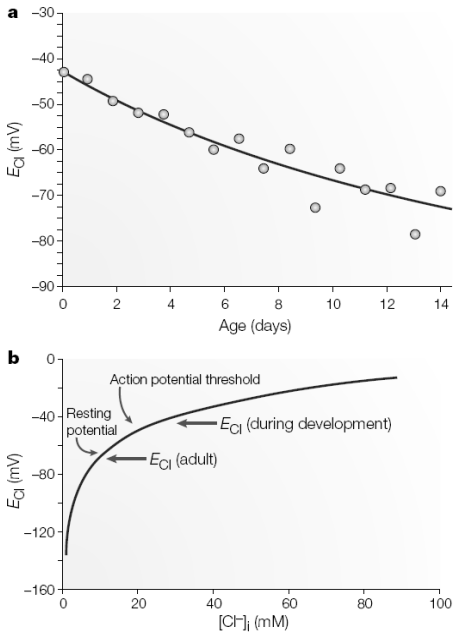

Figure 2.4 *Chloride equilibrium potential during maturation.* **a.** Data from the rodent hippocampus show that the reversal potential of Cl^- (E_{Cl}) decreases with age postnatally. **b.** The decrease of E_{Cl} is due to a lower intracellular chloride concentration ($[Cl^-]_i$).
From Ben-Ari (2002), with permission. Part b was adaptated by Ben-Ari from Staley & Smith (2001).

hyperpolarization of the postsynaptic membrane and thus an inhibitory postsynaptic potential (IPSP). The inward Cl^- gradient is the result from actions of the K^+-Cl^- cotransporter KCC2, which transports Cl^- out of the cell. However, during development, neurons exhibit a more depolarized equilibrium potential for Cl^- due to the dominant activity of $Na^+/K^+/2Cl^-$ cotransporter which increases intracellular Cl^- concentration (Ben-Ari 2002; Ouardouz and Sastry 2005) (fig. 2.4). This results in a depolarizing action of both glycine and GABA, which has effects on development, as will be discussed later.

2.2 Glycine receptors

As described in the previous section, glycine receptors are part of the inhibitory neurotransmission system in the adult CNS, along with GABA receptors. Although it was previously thought that GlyR were only expressed in the spinal cord and the brain stem, it is now clear that they are more widely expressed in the CNS, with different subunits being differently located and developmentally regulated. Knowledge of the GlyR structure and function is far from complete, but growing rapidly. Some excellent reviews on this receptor have appeared in recent years (Aguayo et al. 2004; Betz and Laube 2006; Legendre 2001; Lynch 2004). However, in the few years that have passed since their publication, interesting new insights, e.g. on receptor structure, have profoundly challenged some earlier views, demonstrating the dynamic nature of the GlyR research field. The physiological functions of glycine receptors, especially in extrasynaptic signalling, are not yet fully understood, but the glycine receptor diversity and developmental regulation opens up interesting possibilities and research opportunities.

2.2.1 Glycine receptors are part of the cys-loop receptor superfamily

The ionotropic receptors of the inhibitory neurotransmitters GABA and glycine belong to the same family, the cys-loop receptor superfamily. In vertebrates, the GABA and glycine receptors are the only anionic channels in this family. The other family members in vertebrates are the nicotinic acetyl choline receptors (nAChR), the 5-HT_3 receptor and the zinc activated ion channel, which are all permeable to cations. Besides these receptors, the glutamate-activated chloride channel (GluCl), the MOD-1 serotonine receptor, the EXP-1 GABA receptor and the histamine-activated chloride channel that only occur in invertebrates are also members of the cys-loop superfamily. Of these invertebrate receptors, only the EXP-1 GABA receptor is permeant for cations (Lester et al. 2004). The presence of family members in prokaryotes was hinted by the presence of sequence homologues in prokaryotic genomes (Tasneem et al. 2005). Recently, one of these homologues was cloned and proved to be a functional proton gated channel, equally permeable to Na^+ and K^+ (Bocquet et al. 2007). These results imply a prokaryotic origin of the cys-loop receptor superfamily.
All cys-loop receptor superfamily members are pentameric in structure. The variation in subunit composition in heteropentameric receptors results in a variety of different receptors with specific properties. The hallmark of the cys-loop receptor superfamily, and the origin of their family name, is a conserved signature sequence of 13 amino acids, flanked by cysteine residues. These cysteine residues form a disulfide bond. This creates a loop in the receptor structure which is common for all members of the cys-loop receptor superfamily (Sine and Engel 2006).
Detailed structural knowledge of the cys-loop superfamily is based on structural homology studies with the Acetyl choline binding protein (AChBP) from the freshwater snail *Lymnaea stagnalis* and receptors from the *Torpedo* electric ray (Brejc et al. 2001; Miyazawa et al. 2003; Sine and Engel 2006; Unwin 2005). Cys-loop receptor subunits are believed to have an extracellular domain with mainly β sheets, a channel domain composed of α helices and an intracellular domain with an α helix (Sine and Engel 2006).

2.2.2 Structure of glycine receptors

2.2.2.1 Membrane topology of glycine receptors

In the case of the glycine receptor, 4 very homologous α subunits and one β subunit have been described. The β subunit (58 kDa) has about 47% homology with the α subunits (45 kDa) (Grenningloh et al. 1990) and although some cells express only β subunit mRNA (Malosio et al. 1991), it cannot form homomeric functional GlyR. Functional glycine receptors are made of a pentameric assembly

of α subunits or a heteromeric assembly with a stoichiometry of 2 α subunits and 3 β subunits (figure 2.5). This is only a recent revision of the long held idea of a 3α : 2β stoichiometry (Betz and Laube 2006; Grudzinska et al. 2005). However, this revision resolves the puzzling observation that kinetic modelling described two glycine binding sites in α/β heteromeric GlyR, while stoichiometry described three ligand binding α subunits (Legendre 1998). The exact ligand binding domains of GlyR are not entirely clear, but they are located in the N-terminal of the α subunits. A ligand-binding pocket is formed between distinct loops at the interface of the principal ligand binding surface of the N-terminal and an adjacent subunit. In contrast to $GABA_AR$ and nAChR, the disulfide loop motif of the N-terminal is not involved in agonist binding (Betz and Laube 2006; Legendre 2001; Lynch 2004).

Each subunit has 4 transmembrane domains, with TM2 contributing to the pore lining (figure 2.5). The N-terminal domain contains the ligand binding domain, which is in contact with TM1. TM1 is thought to be involved in gating, forming a linkage between the ligand binding site and the pore lining TM2. The TM2-TM3 domain has also been implicated in gating, forming a contact point between the ligand binding site and TM2. Binding of agonist induces a "wave" of conformational changes which are transmitted through these, and probably other, contact points and result in a conformational change in TM2, opening the pore (Lynch 2004). The exact location of the activation gate in GlyR is not known. There are two hypotheses, based on homology with other cys-loop members. The first is that the TM2 domains are kinked inwards, forming a centrally located gate. The second is that the gate is located at the same narrow pore region as the selectivity filter (Lynch 2004). The classical 4TM topology has been challenged by Leite *et al.* (2000) who state that TM1 and TM3 are too short to be membrane spanning domains, while TM2 and TM4 are true α helices. The issue of topology of the glycine receptor subunits thus is not yet settled.

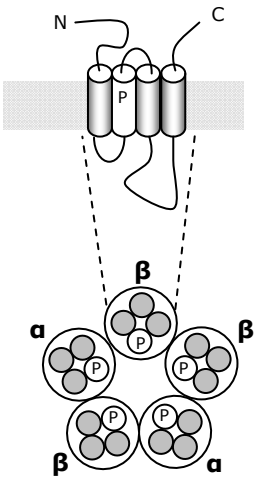

Figure 2.5 *Schematic presentation of glycine receptor structure.* Each glycine receptor subunit is composed of 4 transmembrane domains, with TM2 contributing to the pore. A functional channel is formed by 5 subunits. In heteromeric form, the stoichiometry is 2α:3β. Homomeric channels are also pentameres, but can only be formed by α subunits. Ligand binding occurs at the N-terminal of α subunits. N: amino terminal, C: carboxy terminal, P: pore

2.2.2.2 Glycine receptor subunits

The subunits α1-4 are coded by the genes *Glra1-4* and the β subunit is coded by the *Glrb* gene. Splice variants of each subunit have been described. α1 has two isoforms. The rat splice variant of α1 is called $\alpha1^{ins}$, which contains an extra phosphorylation site. It is expressed in brain stem and spinal cord and makes up 30% of the total α1 mRNA. For α2, two splice variants, α2A and α2B, have been reported. They have similar distributions, but α2A has higher expression during development, while in the adult α2A expression is undetectable. In the rat a third variant of α2 has been described, resulting from an amino acid substitution conferring strychnine insensitivity on this variant (α2*). However, this remains controversial since it was not confirmed to date. α3 then, has two alternative splice variants in human, α3K and α3L. α3K misses 15 amino acid residues in the large intracellular loop between TM3 and TM4. The variants have a similar distribution in the CNS but functionally differ in desensitization kinetics. In the rat, a hypersensitive α3 isoform, α3(P185L), was described to be the result from RNA editing (Meier et al. 2005). The α4 subunit has been described in mice (Harvey et al. 2000; Matzenbach et al. 1994), chick (Harvey et al. 2000) and zebrafish (Devignot et al. 2003), but the protein is not found in rat or human. In humans, the α4 sequence contains a stop codon, preventing functional protein expression. An α4 splice variant has been described in the zebrafish (Devignot et al. 2003). Recently a splice variant of the β subunit has been described in mouse by Oertel *et al.* (2007). Previously only a gene polymorphism of human *Glrb* was reported, which was not likely to result in a coding mutation (Milani et al. 1998). The novel splice variant, however, implies a profound change in subunit topology. With exon 7 lacking, the βΔ7 splice variant misses TM1 and TM2. Surprisingly, Oertel and colleagues did not find an influence on membrane integration and found βΔ7 to heteromerize with α1 and interact with gephyrin. βΔ7 expression did however not infer picrotoxin resistance, characteristic of α/β heteromeres, and the authors suggest that the α1/βΔ7 pore is formed by α subunits, consistent with the loss of the pore forming TM2 in βΔ7. They speculate about an until now unknown role of βΔ7 as an accessory subunit involved in membrane anchoring (Oertel et al. 2007).

2.2.2.3 The β subunit is involved in receptor clustering

β subunits are required for clustering of glycine receptors in the postsynaptic membrane. All GlyR subunits have a large intracellular loop between TM3 and TM4, and in β subunits this is even larger. It is most likely via this loop that β subunits interact with gephyrin to cluster the glycine receptor postsynaptically. Gephyrin is a 93 kDa protein which binds β on one hand and on the other hand anchors to the cytoskeleton. The binding of gephyrin to polymerized microtubules provides a mechanism of linking GlyR complexes to the

cytoskeleton and securing their place in the postsynaptic membrane (Fritschy et al. 2008; Legendre 2001; Lynch 2004).

2.2.3 Glycine receptor subunit expression during development

2.2.3.1 Developmental regulation of subunit expression

The different subunits are not evenly expressed at all developmental stages. Prenatally the dominant subunit is α2, which is then expressed throughout the CNS. Postnatally α2 expression sharply declines, but is nevertheless still present in the adult in some CNS regions like the retina, the auditory brain stem nuclei and some higher brain regions, such as in layer IV of the cerebral cortex and in the hippocampal region (Malosio et al. 1991). There is however indisputably a developmental switch from α2 to α1β (Malosio et al. 1991; Watanabe and Akagi 1995) (table 2.1). The study of Becker *et al.* (1988) was the first to describe a switch in glycine receptor type based on changing strychnine binding affinities during development, although they were not yet known as the α1 and α2 subunits at that time. Takahashi *et al.* (1992) also described this switch by comparing the functional properties of receptors in an expression system with those of native receptors in spinal cord neurons of different developmental stages. Besides α1 expression, α3 expression also augments postnatally, but α3 is expressed at lower levels. In the adult rat CNS, α1 and α3 are differentially located (Malosio et al. 1991). The β subunit is widely expressed in adult as well as in embryo, but the embryonic expression levels are lower than in adult.

Table 2.1 Glycine receptor subunit mRNA expression in developing rat spinal cord

Subunit	Developmental stage					
	E14	E19	P0	P5	P15	P20
α1	+	(+)	++	++	+++	+++
α2	+++	+++	+++	+	(+)	(+)
α3	-	(+)	(+)	(+)	(+)	(+)
β	+	+	+++	+++	+++	+++

Results from *in situ* hybridization. Relative expression levels:
- not detected, (+) very low, + low, ++ moderate, +++ high
E: embryonic day, P: postnatal day
From Malosio et al. (1991)

2.2.3.2 Physiological role of α2 glycine receptors

The presence of homomeric α2 GlyR in embryo raises the question whether these receptors could be involved in early synaptic transmission. It is not likely that they are, since their kinetics are too slow to be activated in synaptic transmission (Mangin et al. 2003) and they do not posess a β subunit, required for postsynaptic clustering (see below). The immature glycinergic synapses are thus probably mediated by α2/β heteromeric receptors. What the physiological

role of the homomeric α2 is, remains unclair. They could possibly mediate non-synaptic signalling involved in developmental processes such as synaptogenesis or differentiation (Legendre 2001; Lynch 2004; Mangin et al. 2003). This will be further discussed in section 2.3.

2.2.3.3 Glycine induces depolarization during development

An interesting functional feature of glycine receptors is that not only there is a developmental change in expressed subunits, but there is also a change in effect of GlyR activation. Glycine, as well as GABA, acts depolarising in the embryonic and immature brain. This is due to a depolarized Cl^- equilibrium potential (E_{Cl}). In neurons, the depolarized E_{Cl} shifts to a more hyperpolarized value because of expression of the K^+/Cl^- cotransporter KCC2 postnatally (Ben-Ari 2002). A glycine induced depolarization can cause Ca^{2+} entry through VGCC and evoke functional changes through Ca^{2+} signalling. The physiological role of these changes, induced by glycine, is poorly understood. Together with the fact that the embryonically expressed α2 homomeric channels are not likely to play a role in synaptic transmission, a role of extrasynaptic glycine signalling in development is a possibility certainly worth further investigation. The function of inhibitory neurotransmitters in neuronal and glial development will be discussed later.

2.2.4 Pharmacology and modulation of glycine receptors

2.2.4.1 Endogenous agonists of the glycine receptor

Besides glycine, GlyR can be activated by taurine and β-alanine (glycine > β-alanine > taurine). Both compounds are released in the CNS, but have not been shown to be involved in synaptic signalling. The subunits differ in their sensitivity to taurine and β-alanine. α1 is potently activated by β-alanine and taurine, while they are weak partial agonists for α2 and α3 (Betz and Laube 2006; Legendre 2001; Lynch 2004).

2.2.4.2 Physiological modulation of the glycine receptor

The functions of GlyR can be modulated by several mechanisms. Several phosphorylation sites have been identified on GlyR subunits and they are all located on the large TM3-TM4 intracellular loop. This loop is highly variable between subunits, which results in a subunit specific distribution of phosphorylation sites, and thus a subunit specific regulation. Consensus phosphorylation sites on GlyR have been reported for protein kinase A (PKA), protein kinase C (PKC), Ca^{2+}/calmodulin-dependent kinase (Cam-K) and protein tyrosine kinase (PTK) (Legendre 2001; Lynch 2004). PKA phosphorylation sites are not found on most α subunits, accept the $\alpha1^{ins}$ splice variant, and β subunits

have a cytoplasmatic PKA-site. The direct phosphorylation of these subunits and the functional consequences thereof still require investigation. PKC phosphorylation sites have been found on TM4 of α1 and on the TM3-TM4 loop of β, but its functional purpose is not clear since contradictory results on GlyR properties have been reported. CaMKII phosphorylation increases glycine evoked responses, but there is no known consensus site on α or β subunits, which is suggestive for indirect CaMKII actions. PTK phosphorylation seems to enhance GlyR currents in hippocampal and spinal neurons, possibly mediated through phosphorylation of the large intracellular loop of β subunits, since PTK phosphorylation effects were only seen in α1β heteromeric channels and not in α1 homomeres (Lynch 2004).

Besides phosphorylation, there are several modulators of GlyR with possible physiological relevance. The most important one seems to be zinc (Zn^{2+}), which is released in the synaptic cleft together with neurotransmitters. Glycinergic synapses have however not been shown to release Zn^{2+}, so to have an effect on GlyR, it would have to diffuse from other nearby terminals. This could form a compensatory feedback between the activation of glutamatergic synapses (excitatory, Zn^{2+} release) and the potentiation of glycinergic currents (inhibitory). The effects of Zn^{2+} on glycinergic signalling are concentration-dependent. Low [Zn^{2+}] potentiate GlyR currents, while high [Zn^{2+}] (> 10 μM) depresses GlyR currents. However even high Zn^{2+} concentrations evoke a transient (~ 1 s) potentiation of GlyR currents. Inhibition develops slowly thereafter. The actions of Zn^{2+} are allosteric, and two putative Zn^{2+} binding sites are found on α subunits (Legendre 2001; Lynch 2004; Lynch et al. 1998). Other physiological signals influencing GlyR include Ca^{2+} (rapid & transient potentiation), extracellular pH (alkalic pH causes rise of EC_{50} in α1 containing channels), neurosteroids (differential effects on subunits, which has a possible role in development) and G protein βγ subunits (decreases glycine EC_{50} for α1) (Lynch 2004).

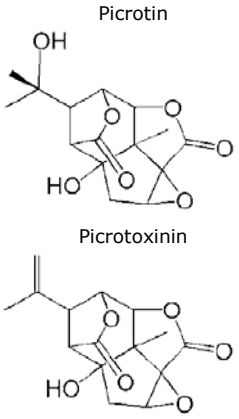

Figure 2.6 *Structure of picrotoxin components.* Picrotoxin is an equimolar mixture of picrotin and picrotoxinin, which only differ in hydration of the terminal isoprenyl group.

2.2.4.3 Antagonists of the glycine receptor

A potent and specific antagonist for GlyR is the plant alkaloid strychnine. Strychnine is a competitive antagonist but it does not completely share the binding site with glycine. The binding sites of strychnine and glycine seem to be distinct, but overlapping. Strychnine is a valuable research tool since strychnine antagonism is currently the most

convincing way to discriminate between glycinergic and GABAergic inhibition in synapses. Besides this, radiolabeled [^{3}H]strychnine has been used widely in studies investigating GlyR expression and its displacement is a tool in investigating potencies of other GlyR ligands. Strychnine is widely known for its use as a poison, especially against rodents.

Another blocker of GlyR is the plant alkaloid picrotoxin (PTX). PTX is a blocker of $GABA_AR$ as well as GlyR. The toxin actually is an equimolar mixture of picrotin (PTN) and picrotoxinin (PXN), which only differ in the terminal isoprenyl group that is hydrated in PTN (figure 2.6). PTX has characteristics of both competitive and non-competitive antagonists, making it difficult to define its exact blocking mechanism. Heteromeric channels containing the β subunit are markedly less sensitive to PTX block then homomeric channels. The work in this thesis aids to resolving the contributions of the PTX components PXN and PTN to α2 homomeric glycine receptor block, on which we will elaborate in chapter 3.

Besides strychnine and picrotoxin, other known compounds are cyanotriphenylborate (CTB) and ginkglosides, which are both pore blockers. These compounds are negatively charged, non-competitive, use- and voltage-dependent, which is in complete accordance with their pore-blocking mode of action. CTB is markedly more potent in α1 GlyR compared to α2 or α3 (Rundstrom et al. 1994). The use of CTB and its characterisation is currently limited because it is not commercially available. Ginkglosides are isolated from the ginkgo tree and share structural similarities with PXN. There are several ginkglosides, which differ in potency, but most are selective for glycine receptors over GABA receptors (Ivic et al. 2003). An overview of all pharmacological agents modulating the GlyR is provided by Lynch *et al.* (2004).

2.2.5 Functional properties of glycine receptors

2.2.5.1 Modelling of receptor kinetics

The activation of a receptor requires both binding of an agonist, and a conformational change inducing the opening of the channel, a process known as gating. In a simple form, activation of a receptor can be modelled as:

$$A + R \xleftrightarrow{K_A} AR \xleftrightarrow{E} AR^* \quad (1)$$

Where A is the agonist, R is the receptor, AR is the occupied but closed receptor and AR* is the activated receptor. The binding affinity (K_A) is given by:

$$K_A = [A][R]/[AR] = k_{off}/k_{on}$$

With k_{off} being the dissociation constant (s^{-1}) and k_{on} the association constant ($M^{-1}s^{-1}$). Efficacy (E), which is the equilibrium constant for gating, is given by:

$$E = [AR^*] / [AR] = \beta / \alpha$$

Where β is the opening rate constant and α is the closing rate constant (both have units s^{-1}). To correctly describe receptor kinetics, analysis using ultrafast flow applications (non-stationary) and analysis at the single channel level (stationary) are needed. For instance, the closing rate constant and closed time are function of the open time constant value of a receptor and the opening rate constant is function of the first latency, the time it takes a receptor to open after agonist binding. Using single channel analysis β and α can be calculated, from where E can be estimated, which can be used to calculate K_A given that:

$$EC_{50} = K_A / 1 + E$$

The concentration needed for half maximal activation of the receptor (EC_{50}) thus depends on both binding and on efficacy (gating). The theoretical concepts behind this relation have been reviewed by Colquhoun (1998).
In reality the model (1) is an oversimplification of glycine receptor activation. The receptor has five subunits, which make the possibilities more complex. Data suggest a coupled model for glycine receptor activation in which the subunits change conformation simultaneously, so that the receptor only exists in the closed or in the entirely activated state (Lynch 2004). Kinetic studies of α1, α1/β and α2 GlyR have all proposed a model for glycine receptor activation wherein two bound glycines are needed to activate the channel (Gentet and Clements 2002; Legendre 1998; Mangin et al. 2003). Surprisingly, Mangin *et al.* (2003) found that the open probability of the α2 homomeric channel after 1 ms application of ≤ 1 mM glycine was too low to account for synaptic functioning. This is probably due to a low glycine affinity of the α2 receptor. The authors speculate that, given the high efficacy of α2, it more likely has a role in paracrine signalling, responding to sustained low glycine concentrations (Mangin et al. 2003).

2.2.5.2 Unitary conductance of glycine receptors

Activated glycine receptors have several subconductance states. α1 receptors have five subconductance states between 20 and 85 pS (20, 30, 45, 65, 85 pS), with 85 pS being their main conductance state. α2 and α3 have the same main

conductance state but have an additional, frequently visited, 100-110 pS subconductance state. The α/β heteromeric channels generally have a lower principal conductance state, being about 45 pS. The 85 pS state is not seen in α1/β heteromeres. Subconductance states of α heteromeres are not known (Legendre 2001; Lynch 2004).
When GlyR open, they mainly conduct Cl^- ions. The GlyR are selective for anions over cations, but remain slightly permeable to K^+ and Na^+. The GlyR pore, which has a diameter of about 5.2 Å, contains at least two sites with positive charges for interactions with anions. Positive charges at the outer ring of the pore serve to concentrate anions in the outer vestibule, thereby increasing conductance. The main selectivity filter is thought to be located deeper within the pore, lined by positively charged arginines (Legendre 2001; Lynch 2004).

2.2.5.3 Desensitization kinetics: an illustration of the importance of perfusion speed

Desensitization of GlyR was long thought to be too slow (0.5-5 s) to influence synaptic events, but fast application studies have now described a very fast desensitization component (decay time constant about 5 ms). This component was induced by clustering of α1 receptors (Legendre et al. 2002) or coexpression of α1 with β (Mohammadi et al. 2003). The influence of this component on synaptic GlyR desensitization remains to be elucidated, but it illustrates the need for adequate speed of solution application in the study of ionotropic neurotransmitter receptors, if we are to describe receptor kinetics correctly.

2.3 Role of inhibitory neurotransmitter receptors during development of the central nervous system

In recent years evidence has been growing that fast synaptic signalling is not the only function of neurotransmitters. Their name proves rather misleading, since it has now been established that they also have a trophic function, mainly mediated through extrasynaptic signalling. Although inhibitory neurotransmitters will be discussed given the scope of this thesis, it is noteworthy that trophic effects are not limited to GABA and glycine. Studies concerning the effect of glutamate seem to contradict each other (Haydar et al. 2000; LoTurco et al. 1995; Luk and Sadikot 2004; Mochizuki et al. 2007). These apparent differential results mainly seem to depend on such factors as developmental stage (Luk and Sadikot 2004; Mochizuki et al. 2007), environment (Haydar et al. 2000) and receptor type involved (Luk and Sadikot 2004). Dopamine was also shown to affect proliferation, depending on the type of receptor (Ohtani et al. 2003). Activation of ATP receptors has recently been shown to positively influence neural stem cell proliferation (Mishra et al. 2006; Ryu et al. 2003). And finally,

acetylcholine stimulates neural stem cells proliferation and differentiation mainly through its metabotropic muscarinic receptors (Ma et al. 2000; Zhou et al. 2004)

2.3.1 Role of GABA in neuronal development

2.3.1.1 Influence of GABA on proliferation of neuronal precursors

GABA signalling occurs already before synapses have been formed in the developing CNS (Bordey 2007; Lujan et al. 2005; Nguyen et al. 2001; Owens and Kriegstein 2002; Represa and Ben-Ari 2005; Salazar et al. 2008). In mice, GABA immunoreactivity is seen as early as E10 (Haydar et al. 2000). In accordance with GABA signalling before synapse formation, a vesicle independent GABA release has been described in immature pyramidal neurons (Demarque et al. 2002). Extrasynaptic GABA signalling has several trophic actions, which seem to persist postnatally.

As mentioned earlier, GABA induces a depolarisation during development because of high intracellular Cl^- concentrations, a consequence of the actions of the NKCC1 transporter (Ben-Ari 2002; Ouardouz and Sastry 2005). Kinetics of $GABA_AR$ in immature cells are more adapted to the paracrine GABA signalling. They show a higher GABA affinity and are less sensitive to desensitization (Owens et al. 1999). Studies also report an outward current shift when bicuculline, a $GABA_AR$ blocker, was applied, suggesting a tonic activation of these receptors in neural precursors (LoTurco et al. 1995; Owens et al. 1999). The difference in receptor kinetics probably results from different subunit compositions of the $GABA_AR$ in proliferating precursor cells (Owens and Kriegstein 2002).

The depolarising effect correlates with an increase in intracellular Ca^{2+} and results in inhibition of proliferation (Liu et al. 2005; Nguyen et al. 2003). However, Haydar *et al.* (2000) report a region-specific effect with GABA indeed inhibiting proliferation in the subventricular zone, but they describe a proliferation-stimulatory effect in the ventricular zone. The proliferative effect of GABA on cerebellar granule cell precursors supports the idea of differential GABA effects according to CNS region and/or cell type (Fiszman et al. 1999). A receptor-dependent effect is also plausible since a recent study described a stimulation of proliferation by $GABA_B$ receptor activation in fetal neural progenitor cells from mice (Fukui et al. 2008).

There seems to be an interaction between the pathways of growth factor signalling and GABA signalling in precursor cells. Antonopoulos *et al.* (1997) propose a feedback between GABA and basic fibroblast growth factor (bFGF) signalling. They found that bFGF increases expression of the α1 $GABA_A$ subunit, which is widely expressed by proliferating neural precursors, and that GABA

inhibits bFGF induced proliferation. Epidermal growth factor was shown to decrease GABA production, adding to its direct proliferative effects (Nguyen et al. 2003).

2.3.1.2 Influence of GABA on the differentiation of neuronal precursors

Besides the effect on proliferation, GABA has been shown to influence morphological changes in developing neurons, associated with differentiation. Neuronal differentiation is stimulated by GABA and counteracted by GABA antagonists (Borodinsky et al. 2003; Cancedda et al. 2007; Maric et al. 2001; Marty et al. 1996; Tapia et al. 2001; Tozuka et al. 2005). The stimulatory effect on dendritic outgrowth is associated with increases in intracellular Ca^{2+} and activation of Ca^{2+} dependent kinases (Borodinsky et al. 2003; Maric et al. 2001). Cancedda *et al.* (2007) proved the depolarizing effect of GABA signalling to be essential for *in vivo* morphological maturation. Like with proliferation, there is again an interplay with growth factor signalling. GABA was shown to promote brain-derived growth factor (BDNF) release from neurons, promoting differentiation of interneurons early in development (Marty et al. 1996).

2.3.1.3 Influence of GABA on migration of neuronal precursors

After proliferation in the germinal zones, neuronal precursors migrate to their target tissues. There are two distinct modes of migration: tangential and radial (Heng et al. 2007; Nadarajah and Parnavelas 2002). Radial migration is the migration from the ventricular zone to the cortical plate and occurs through a combination of somal translocation and glial guidance. The relative prevalence of these two radial migration modes depends on developmental stage (Nadarajah and Parnavelas 2002). Tangential migration is mainly of importance for GABAergic interneurons, which originate in the ganglionic eminence (GE). These cells migrate from the GE to the ventricular zone and then switch to radial migration to reach their final destination (Nadarajah and Parnavelas 2002).
The influence of GABA on migration is dependent on concentration and receptor type involved. Behar *et al.* (1996; 2000; 1998) conducted a series of experiments providing some insight on this mechanism in the rat CNS. They described that, depending on concentration, GABA induced chemotaxis (directed cell migration) or chemokinesis (random cell movement) *in vitro* (Behar et al. 1996). These effects were respectively induced by femtomolar and micromolar GABA levels and were Ca^{2+} dependent. A later study from the same group established that picrotoxin-sensitive / bicuculline-resistant $GABA_C$-like receptors and saclofen-sensitive $GABA_B$ receptors mediate a migratory signal out of the ventricular zone and through the intermediate zone respectively (Behar et al. 2000). In light of the femtomolar concentrations directing migration *in vitro*

(Behar et al. 1996) the authors speculate about the possible existence of a so far unidentified G-protein coupled picrotoxin-sensitive GABA receptor. An earlier study already indicated an important role for G-protein coupled receptors in migratory influence of GABA (Behar et al. 1998). In that case, this novel receptor, and not the $GABA_C$ receptor, would mediate the migratory signal out of the ventricular zone. Once the cells reach the cortical plate, signalling through picrotoxin-sensitive / bicuculline-sensitive $GABA_A$ receptors then provides a stop signal (Behar et al. 2000). A reduction in migration speed, caused by $GABA_A$ receptor signalling was also described in an *ex vivo* study of the postnatal subventricular zone of mice (Bolteus and Bordey 2004), although Behar *et al.* (1999) state that during cortical developing in mice, glutamate might be the primary chemo-attractive neurotransmitter instead of GABA.

2.3.2 Role of glycine in neuronal development

In comparison to GABA, research into the role of glycine in neuronal development is rather limited. Nevertheless, there are several indications that glycine receptor signalling might be of importance in regulation of developmental processes. Glycine receptors are expressed throughout the developing nervous system (Flint et al. 1998; Malosio et al. 1991). The most abundant glycine receptor agonist in the developing neocortex is not glycine itself, but taurine (Agrawal et al. 1971; Rassin et al. 1977; Sturman et al. 1977). Taurine levels diminish postnatally, but still a role in cortical development is conceivable. Flint *et al.* (1998) show that glycine receptors are expressed on immature migrating and differentiating neurons in the embryonic and perinatal cortex and that these receptors are activated by a nonsynaptically released ligand, presumably taurine. Moreover, like GABA, GlyR activation results in a depolarisation and increased intracellular Ca^{2+}.

This opens up several interesting perspectives, if we take into account what is known about GABA. Taurine namely is not only a ligand for glycine receptors, but also for GABA receptors (Behar et al. 2001; Jia et al. 2008; Tang et al. 2008). Could it be that taurine acts as a general ligand influencing both GABA and glycine receptors? The study of Behar *et al.* (2000) used picrotoxin to prove involvement of $GABA_{A/C}$ receptors in migration. Could it then be that the observed effect is not purely mediated through GABA receptors, but also through glycine receptors? After all, picrotoxin is a GlyR antagonist. This issue was not discussed by the authors. Moreover, when GABA synthesis was knocked out, no apparent effects on histogenesis in the mouse CNS could be detected (Ji et al. 1999), which contrasts with the apparent CNS defects in taurine-deprived kittens (Palackal et al. 1986; Sturman 1986). Is GABA then of less importance to development compared to taurine? Of course there is a species difference in these studies, and although they provide no definite evidence, they do fit the

hypothesis that taurine is an important signal in the developing CNS, acting on both glycine and GABA receptors. Therefore it is remarkable that in comparison to GABA receptors, few studies are dedicated to elucidating the role of glycine receptors in developmental processes.

2.3.3 Signal transduction from neurotransmitter receptors to cell cycle modulation

When neurotransmitters bind to their receptors, they initiate a signal transduction cascade. In the case of ionotropic receptors, the ion flux either directly increases intracellular Ca^{2+} concentration or induces a change in membrane potential which can activate other processes and channels, such as voltage gated Ca^{2+} channels which indirectly increases intracellular Ca^{2+} levels.
Metabotropic neurotransmitter receptors are G-protein coupled. Activation of these receptors is coupled to adenylate cyclase (AC) activity and thus cAMP concentration. cAMP in turn activates protein kinase A. An alternative transduction pathway is the IP_3/Ca^{2+} cascade. This starts through G-protein stimulated cleavage of phosphatidylinositol bisphosphate (PIP2) by phospolipase C (PLC). The resultant inositol triphosphate (IP3) increases intracellular Ca^{2+}. Together with another product of PIP2 degradation, diacylglycerol (DAG), this Ca^{2+} promotes protein kinase C (PKC) activation. Ca^{2+} also directly activates Ca^{2+}/calmoduline dependent kinase (CaM-K). PKC and CaM-K both stimulate the mitogen activated protein kinase (MapK) pathway.
Eventually these phosphorylation cascades have an influence on the cyclin dependent kinases (CDK) through CDK activating kinases (CAK) or cyclin/CDK inhibitors (CKI). CDK activity phosphorylates transcription factors like retinoblastoma protein (Rb). This results in E2F release and S-phase progression, preventing cell cycle exit. The signal transduction pathways are summarized in figure 2.7 (Martins and Pearson 2008).

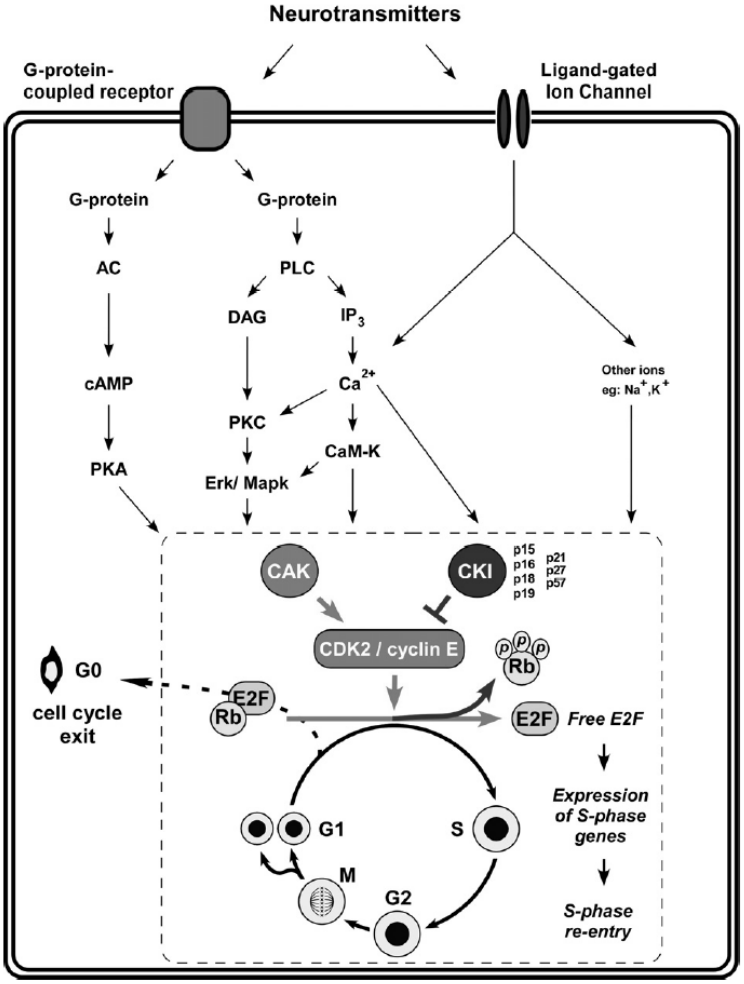

Figure 2.7 *From neurotransmitters to cell cycle.* Neurotransmitters activate ionotropic and/or metabotropic receptors which initiates signal transduction pathways with several phosphorylation cascades. This leads to changes in phosphorylation of transcription factors, resulting in cell cycle progression or cell cycle exit. AC: adenylate cyclase, cAMP: cyclic adenosine monophosphate, PKA: protein kinase A, PLC: phospholipase C, DAG: diacylglycerol, IP_3: inositol phosphate, PKC: protein kinase C, ERK: extracellular signal regulated kinase = MapK: mitogen activated protein kinase, CaM-K: Ca^{2+}/calmoduline dependent kinase, CAK: CDK activating kinase, CDK: cyclin dependent kinase, CKI: cycline/CDK inhibitors, Rb: retinoblastoma protein, E2F:family of transcription factors
From Martins and Pearson (2008), with permission.

Chapter 3 | Picrotoxinin and picrotin block of α2 homomeric glycine receptors

Results of this chapter are reported in Wang DS, Buckinx R, Lecorronc H, Mangin JM, Rigo JM and Legendre P. 2007. "Mechanisms for picrotoxinin and picrotin blocks of α2 homomeric glycine receptors." J Biol Chem 282(22):16016-35.

Abstract

Contrary to its effect on the γ-aminobutyric acid type A and C receptors, picrotoxin antagonism of the α1 homomeric glycine receptors (GlyRs) has been shown to be non-use-dependent and nonselective between the picrotoxin components picrotoxinin and picrotin. Picrotoxin antagonism of the embryonic α2 homomeric GlyR is known to be use-dependent and reflects a channel-blocking mechanism, but the selectivity of picrotoxin antagonism of the embryonic α2 homomeric GlyRs between picrotoxinin and picrotin is unknown.

Hence, we used the patch-clamp recording technique in the outside-out configuration to investigate the mechanism of picrotin- and picrotoxinin-induced inhibition of currents, which were evoked by the activation of α2 homomeric GlyRs stably transfected into Chinese hamster ovary cells.

Although both picrotoxinin and picrotin inhibited glycine-evoked outside-out currents, picrotin had a 30 times higher IC_{50} than picrotoxinin. Picrotin-evoked inhibition displayed voltage dependence, whereas picrotoxinin did not. Picrotoxinin and picrotin decreased the meanopen time of the channel in a concentration dependent manner, indicating that these picrotoxin components can bind to the receptor in its open state. When picrotin and glycine were co-applied, a large rebound current was observed at the end of the application. This rebound current was considerably smaller when picrotoxinin and glycine were coapplied. Both picrotin and picrotoxinin were unable to bind to the unbound conformation of the receptor, but both could be trapped at their binding site when the channel closed during glycine dissociation.

Our data indicate that picrotoxinin and picrotin are not equivalent in blocking α2 homomeric GlyR.

3.1 Introduction

Toxins have long been proven to be valuable tools in ion channel research. They allow researchers to discriminate easily between different responses in electrophysiological studies, and their blocking properties can provide insight into the functioning of ion channels. Picrotoxin (PTX) is such a substance that is amply used in cys-loop ion channel research. It is mainly known as a blocker of GABA receptors (Zhang et al. 1995) and glycine receptors (Legendre 2001; Lynch 2004; Wang et al. 2006), but also inhibits cation-selective nicotinic AChR (Erkkila et al. 2004) and 5-HT_3-R (Das et al. 2003). Since the β subunit confers a PTX resistance to the GlyR, this toxin can be used to discriminate between a functional expression of α-homomeric or α/β-heteromeric GlyR.

3.1.1 Characteristics of picrotoxin

3.1.1.1 Blocking mechanism of PTX

PTX has characteristics of both competitive and non-competitive antagonists, making it difficult to define its exact blocking mechanism. Moreover, it is likely that the inhibitory mechanism of PTX differs between different members of the cys-loop family (Lynch 2004). PTX is unlike a competitive antagonist in decreasing the mean open time and the relative frequency of the 85 pS main conductance state of homomeric GlyR. Moreover, it does not displace [H^3]strychnine, what should be expected from a competitive antagonist. What is competitive-like is that its potency decreased as agonist concentration increased and it is not use dependent (Legendre 2001; Lynch 2004).
The exact binding site of PTX is still unclear. There are several indications suggesting that PTX binds within the channel pore in α1 as well as in α2 GlyR (Hawthorne and Lynch 2005; Wang et al. 2006)., The 2'-6' pore lining amino acid residues of the TM2 domain are reported to be important for PTX binding (Shan et al. 2001; Zhorov and Bregestovski 2000). Heteromeric channels containing the β subunit are markedly less sensitive to PTX block then homomeric channels. This is related to a difference in TM2 between β and α subunits (Pribilla et al. 1992), again pointing out the pivotal role of the TM2 domain in PTX sensitivity. The binding of PTX in the channel pore is contradictory to the report of Lynch *et al.* (1995) who proposed that in α1, PTX acts as an allosteric competitive inhibitor altering the transduction process between agonist binding and channel gating.

3.1.1.2 PTX is a mixture of picrotin and picrotoxinin

PTX is in fact an equimolar mixture of picrotin (PTN) and picrotoxinin (PXN) (fig. 2.6). $GABA_AR$ and $GABA_CR$ are insensitive to PTN, but blocked by PXN, which

implies that the blocking effect of PTX on GABAR's stems from interaction with PXN (Qian et al. 2005). This is contradictory to the observations in α1 GlyR, which are equally inhibited by PXN and PTN (Lynch et al. 1995). $GABA_A$ and $GABA_C$ differ from α1 GlyR in the putative PTX binding domain by one residue in the 2' pore lining position. α1 GlyR's contain a glycine at that position, while GABAR's contain an alanine. This difference might explain the different sensitivities to PTN. Since α2 GlyR's also contain an alanine at the 2' pore lining position it is plausible that homomeric α2 GlyR's also display different sensitivities to the PTX components. The α2 GlyR is the most abundantly expressed embryonic GlyR subtype. It is believed to play a role in CNS development by influencing cell differentiation and synaptogenesis (see chapter 2) (Ben-Ari 2001; Flint et al. 1998; Nguyen et al. 2001).

3.1.1.3 Effect of PTX on homomeric α2 glycine receptors

The effect of PTX on homomeric α2 GlyR was investigated by Wang *et al.* (2006). The study found that PTX blocks α2 receptors in a concentration-dependent, voltage-independent manner and that PTX binds preferentially to the fully liganded conformation of the GlyR. PTX can thus not bind to the unliganded closed state but can however be trapped at its binding site when glycine dissociates from the receptor. The minimal Markov model proposed by Wang *et al.* (2006) explains some of the competitive-like behaviour of PTX. The observation that higher glycine concentrations result in faster PTX recovery is, according to this model, not the result of competitive inhibition. It is caused by the dependence of recovery from PTX block on glycine association. PTX can only dissociate from the channel in the fully liganded state. A PTX-dependent decrease in mean open time and a rebound current observed after termination of glycine-PTX co-application further support an open channel block by PTX, binding at the TM2 domain.

The entrapment of PTX in the pore has not been evidenced in the α1 GlyR (Hawthorne and Lynch 2005), but the larger single channel conductance (120 vs 80 pS) and the longer mean open time in α2 homomerics suggest a different open channel conformation between α1 and α2 (Wang et al. 2006).

3.1.2 Mechanisms of picrotin and picrotoxinin block

In this chapter, we describe work leading up to models for PXN and PTN inhibition of α2 homomeric receptors stably expressed in CHO cells. Hopefully this gives more insight into the functioning of the α2 homomeric receptor and provides researchers with extra tools to further elucidate the physiological role of α2 receptors in the developing CNS. All hereafter described experiments were performed using the outside-out patch-clamp configuration, with patches

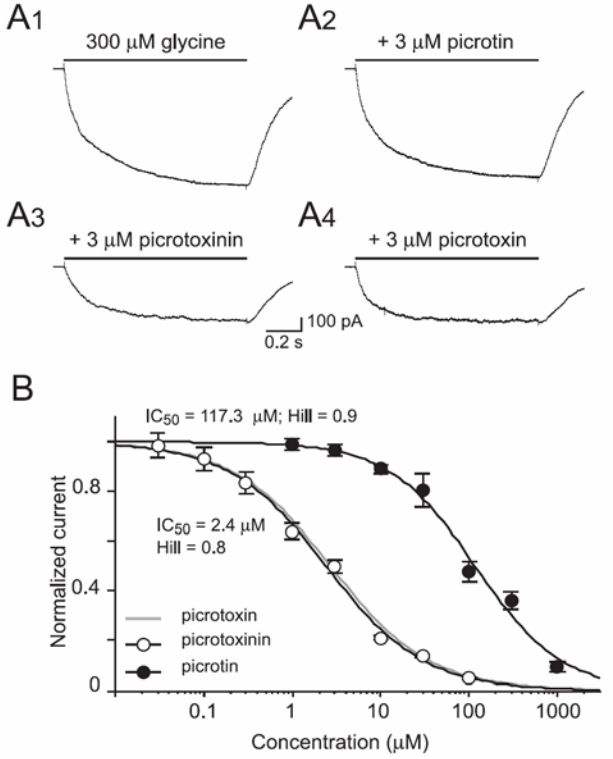

Figure 3.1 *Differential inhibition of α2 homomeric GlyR by picrotoxinin and picrotin.* **A1–A4**. Outside-out patch-clamp recordings showing current responses to glycine (300 μM) (**A1**) and to co-application of glycine with 3 μM of either picrotin (**A2**), picrotoxinin (**A3**), or PTX (**A4**) in one CHO cell transfected with the α2 GlyR subunit. No significant reduction in glycine-elicited responses was detected when picrotin was co-applied, whereas co-application of glycine with picrotoxinin produced a degree of inhibition comparable with that of PTX. Each trace represents the average of 10–15 responses. The *thick line* represents the application of drugs. **B.** Inhibition curves for picrotoxinin (○) and picrotin (●) on 300 μM glycine responses. The PTX inhibition curve (*gray line*), replotted from previous data (Wang et al. 2006), is included for comparison. Currents were normalized to the responses in the absence of picrotoxinin or picrotin. Each point is the average of values from 5 to 11 cells. In most instances multiple concentrations (three) of picrotoxinin or picrotin were applied to the same cell. Data were fitted with the Hill equation giving an IC_{50} of 2.4 ± 0.2 μM and a Hill coefficient of 0.8 ± 0.05 for picrotoxinin, and an IC_{50} of 117.3 ± 14.2 μM and a Hill coefficient of 0.9 ± 0.09 for picrotin.

derived from CHO cells stably transfected with α2 GlyR (see "Materials & Methods").

This work was done in cooperation with our collaborators at the "Université Pierre et Marie Curie" in Paris. We will now first discuss their findings, which will, together with our results, lead up to proposed Markov models for PXN and PTN inhibition of α2 homomeric glycine receptors. For detailed discussion of all results, we refer to our recently published paper (Wang et al. 2007).

3.1.2.1 Concentration and voltage dependence of picrotoxinin and picrotin block of α2 homomeric glycine receptors.

When 300 µM glycine was co-applied with different concentrations of PTX, PTN or PXN, it was clear that PXN and PTN did not block α2 GlyR to the same extent. EC_{50} and Hill coefficients were obtained by fitting concentration response curves with the Hill equation (fig. 3.1):

$$\frac{I}{I_{con}} = \frac{1}{1+\left(\frac{[P]}{IC_{50}}\right)^{n_H}}$$

Wherein I is the steady-state current in the presence of toxin, I_{con} is the control steady-state response, [P] is the concentration of PXN or PTN and n_H is the Hill coefficient.

The concentration of half-maximal inhibition (IC_{50}) of PTN was 117.3 ± 14.3 µM, which was more than 30-fold higher than the IC_{50} of PXN, being 2.4 ± 0.2 µM. So in contrast to the situation in α1 GlyR (Lynch et al. 1995), the PTN potency in α2 is not equal to the PXN potency. Hill coefficients of PXN and PTN were 0.79 ± 0.05 and 0.89 ± 0.09 respectively, which indicated that binding of one molecule of PXN or PTN is sufficient to mediate a blocking effect. The values obtained for PXN were similar to the values obtained for PTX (EC_{50} = 2.7 ± 0.2 µM and Hill = 0.80 ± 0.04), indicating that the effect of PTX on α2 GlyR is likely mediated by the PXN component (fig. 3.1). It is important to point out that PTN did still have a blocking effect, albeit less potent, which is different from the situation in $GABA_AR$'s where PTN does not have an inhibitory effect. When the glycine concentration was varied, it was seen that PXN and PTN block decreased with increased glycine concentration, so despite differences in efficiency, PXN and PTN also displays common features such as a competitive-like inhibitory mechanism.

PTX block of α2 GlyR was voltage independent (Wang et al. 2006), so the voltage dependence of the block by the PTX components was investigated. PXN block was voltage independent (fig 3.2A1-B1), demonstrating again the importance of PXN in the PTX blocking effect. PTN block however proved to be voltage dependent, increasing with more positive potentials (fig 3.2A2-B2). The voltage dependence of PTN was quite modest, but nevertheless significant. This voltage dependence indicates an open channel blocking mechanism for PTN, in which it is plausible PTN moves deeper into the pore, and thus the membrane electric field, than PXN. The open channel blocking mechanism of PTN was also hinted by the rebound currents seen at the end of PTN application (fig. 3.2A2). These rebound currents are caused by a sudden increase in single channel conductance after block relieve. They have been described earlier for the open

channel block effect of acetylcholine on nicotinic receptors (Legendre et al. 2000; Maconochie and Steinbach 1995).

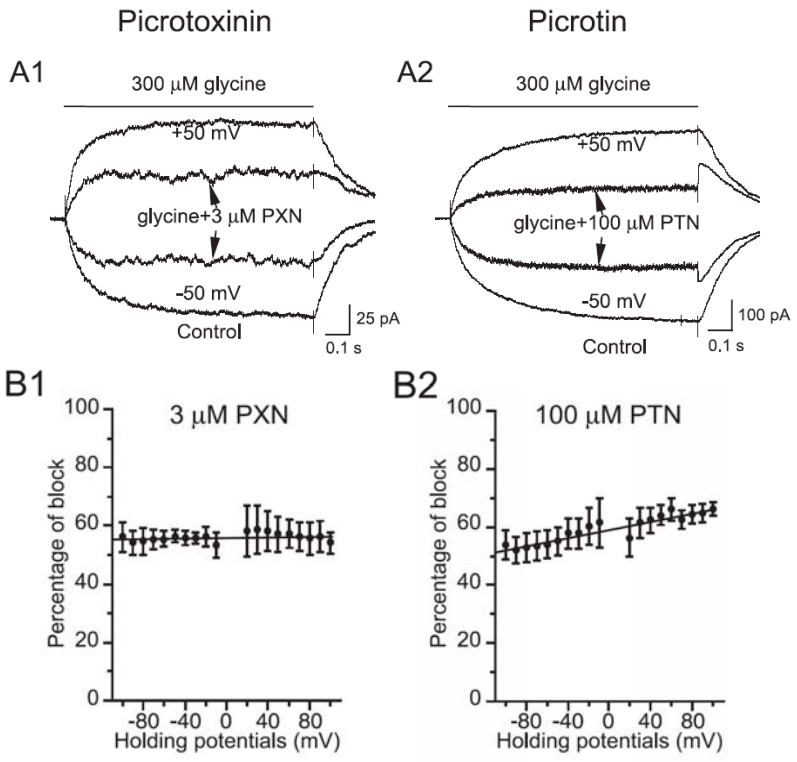

Figure 3.2 *Voltage-dependent inhibition of glycine response by picrotoxinin and picrotin.* **A1-A2**. Responses to 300 μM glycine (control) and to co-application of 300 μM glycine with either 3 μM picrotoxinin (**A1**) or 100 μM picrotin (**A2**) at a holding potential of +50 and -50 mV. Note that the rebound current at +50 mV is larger than that at -50 mV in the presence of 100 μM picrotin. Each trace represents the average of 10–12 trials. The *thick line* represents the application of 300 μM glycine. A1 and A2 were obtained from different patches. **B1-B2.** Plot of the percentage block by co-application of 3 μM PXN and 300 μM glycine (**B1**) or 100 μM PTN and 300 μM glycine (**B2**) at every 10 mV as a function of the holding potentials from -100 to +100 mV. Data were averaged from six patches and fitted by linear regression giving slope factors of 0.007 and 0.069 for 3 μM PXN and 100 μM PTN, respectively.

3.1.2.2 Acceleration of deactivation kinetics by picrotin and picrotoxinin

A short (1 ms) pulse of saturating (30 mM) glycine was applied with or without various concentrations of PXN or PTN to evaluate the influence of these compounds on deactivation kinetics. PXN decreased the deactivation time constant (τ_{decay}) in a concentration dependent manner that was not significantly different from PTX. Although PTN also decreased τ_{decay} in a concentration dependent manner, the necessary concentrations were higher than in the case of PXN. In both cases decay phases could be fitted with single exponentials.

If a short pulse of agonist is applied, deactivation reflects the number of channel reopenings before agonist dissociation. This stems from the relationship between the deactivation time constant and mean burst duration. Mean burst duration namely is dependent on mean open time, mean closed time, number of reopenings and number of closures of the channel. In the absence of blockers and in a kinetic model with one open state, which is the case for the α2 GlyR (Mangin et al. 2003), the relationship is described by:

$$\tau_b = \frac{N_o}{\alpha} + \frac{N_c}{(\beta + k_{off})}$$

With τ_b being mean burst duration, N_o number of reopenings ($1+\beta/k_{off}$), N_c number of closures (β/k_{off}), α closing rate constant (s^{-1}), β opening rate constant (s^{-1}) and k_{off} the dissociation rate constant of the agonist (s^{-1}) (Colquhoun and Hawkes 1995; Wang et al. 2006). The factors determining number of openings, β and k_{off}, thus also are of main influence on τ_b under these conditions. So, deactivation time, which is a measure for number of reopenings before agonist dissociation, becomes a measure for mean burst duration. In the presence of inhibitors, this relation is no longer so straightforward since the blockers add blocked states which can also influence mean burst duration.

The influence of channel blockers on mean burst duration and mean open time depends on the type of block. Fast blockers will decrease mean open time and increase mean burst duration. The lengthening of mean burst duration results from the need of channel reopening to resolve channel block. These influences result in a biphasic relaxation: a "normal" relaxation and a slower relaxation because of blocked channels that are reopening and relieve block. Slow blockers will also decrease mean open time, but instead of an increase in mean burst duration, it will be decreased. This is in fact a sort of analysis problem. Channels again have to reopen to recover from the channel block, but in the case of slow blockers, this reopening can be seconds away. This makes it impossible for the experimenter to distinguish whether it belongs to the earlier burst. In theory, there is again a biphasic relaxation but in practice the second component will be too slow for detection (Colquhoun and Hawkes 1995; Wang et al. 2006).

Deactivation in the presence of PXN or PTN could be described by a single exponential. As explained above, this indicates that both compounds are slow channel blockers. If that is the case, both compounds should decrease mean open time to the same extent. In order for PTN to cause a similar decrease in deactivation time constant as PXN, a ≈ 10 times higher concentration was needed. This could reflect a lower association rate for PTN or a higher number of reopenings in the presence of PTN compared to PXN, since mean burst duration determines the deactivation time constant. In order to resolve this issue, single channel properties were studied.

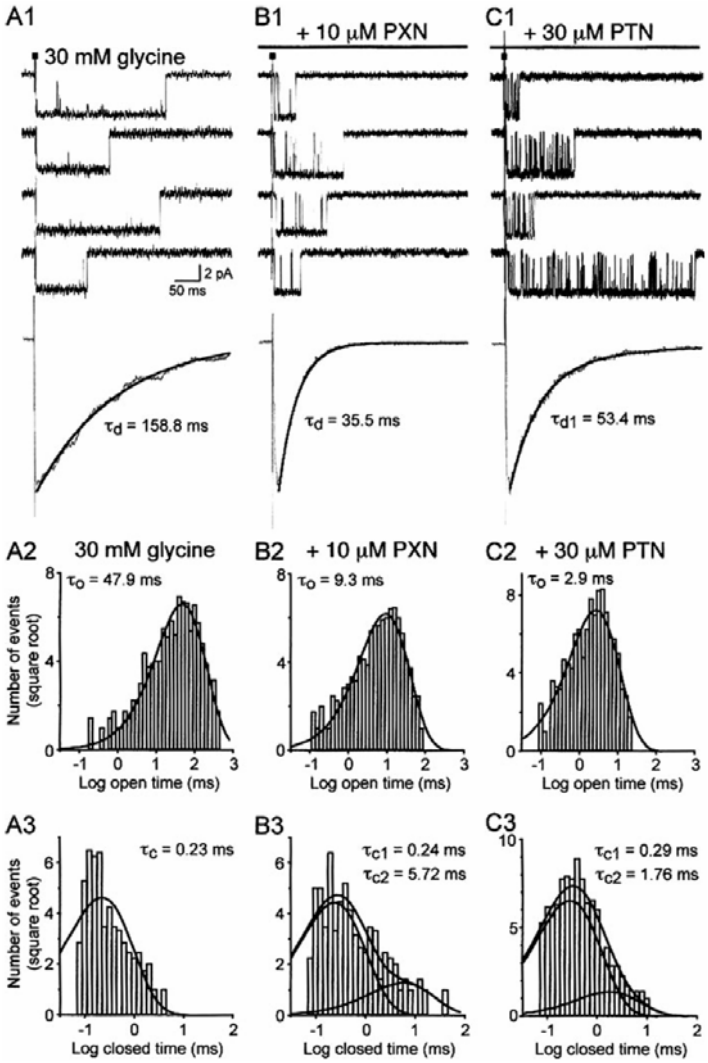

Figure 3.3 *Decrease in the mean open time in the continuous presence of both picrotoxinin and picrotin.* **A1, B1 and C1.** representative, nonconsecutive, single channel openings of a single α2 homomeric GlyR evoked by repetitive short pulses (1 ms) of 30 mM glycine in the absence (**A1**) and presence of continuous 10 μM picrotoxinin (**B1**) or 30 μM picrotin (**C1**) (cut-off filter frequency, 2 kHz). The *thick line* represents the application of 10 μM PXN or 30 μM PTN. The calibration bars in A1 also apply to B1 and C1. Lower traces show macroscopic like current obtained by averaging single openings. **A2, B2 and C2.** Open time duration histograms obtained in control (30 mM glycine, 1 ms pulse) (**A2**) and in the continuous presence of 10 μM PXN (**B2**) or 30 μM PTN (**C2**) are shown as a function of log intervals with the ordinate on a square root scale. Histograms were better fitted with a single exponential curve. **A3, B3 and C3.** Closed time histograms in control conditions (30mM glycine; **A3**) and in the continuous presence of 10 μM PXN (**B3**) or 30 μM PTN (**C3**) are shown as a function of log intervals, with the ordinate on a square root scale. Histograms were better fitted with a single exponential for control and with double exponentials for PXN and for PTN. Mean open and closed times were obtained by pooling single channel currents from 211 trials in nine different experiments for control, 164 trials in seven different experiments for 10 μM PXN, and 46 trials in three different experiments for 30 μM PTN.

Analysis of the mean open time distribution in the presence of PXN or PTN showed that both compounds decrease the mean open time (fig. 3.3). PXN decreased mean open time to a similar extent as PTX. PTN induced a stronger decrease in mean open time. At first sight this contradicts the slower deactivation in the presence of PTN, but this could be explained by a larger number of reopenings in the presence of PTN. In conclusion, the decrease of the deactivation time constants under the influence of PTN and PXN is mainly due to a decrease in mean open time. The shortening effect of PTN and PXN on mean open time proved to be concentration dependent and increased with higher toxin concentrations. This again supports an open channel block mechanism. From the relation between toxin concentration and mean open time, the association rate constant (k_{on}) and closing rate constant (α) can be estimated for each compound by fitting the data with the following function:

$$\frac{1}{\tau_o} = [P]k_{on} + \alpha$$

This fit gave similar k_{on} and α for PXN and PTN. In combination with the previously described findings, this implies that the longer decay time constant with PTN compared to PXN indeed can only be explained by a larger number of reopenings in the presence of PTN.

PXN and PTN both increased the closed time constants, with PXN behaving again very PTX-like. Where control closed time distribution could be fitted by one exponential, distribution in the presence of PXN or PTN is best described by two exponentials (fig. 3.3). The appearance of a longer closed time constant likely reflects an additional recovery pathway from PXN or PTN block.

3.1.2.3 Effects of picrotoxinin and picrotin on activation kinetics of glycine-induced currents

In control conditions, the rising phase responses to a saturating concentration of glycine could be described by two exponential curves, implying a fast (τ_{fast}) and a slow (τ_{slow}) component in this rising phase. It was already established that continuously applied PTX increased activation time constants. Again, it was tested how the two PTX components affect activation kinetics of α2 homomeric GlyR's. A concentration of 10 µM PXN or 300 µM PTN was applied in these experiments. When continuously present (before-during-after glycine application), both PXN and PTN slowed down the activation phase, but as with other results, PTN was less potent. This in addition to their effect on steady state currents (see chapter 3.1.2.1).

The effects of PXN and PTN depend on the way they are applied. Simultaneous application of PXN and glycine resulted in no significant change in rise time

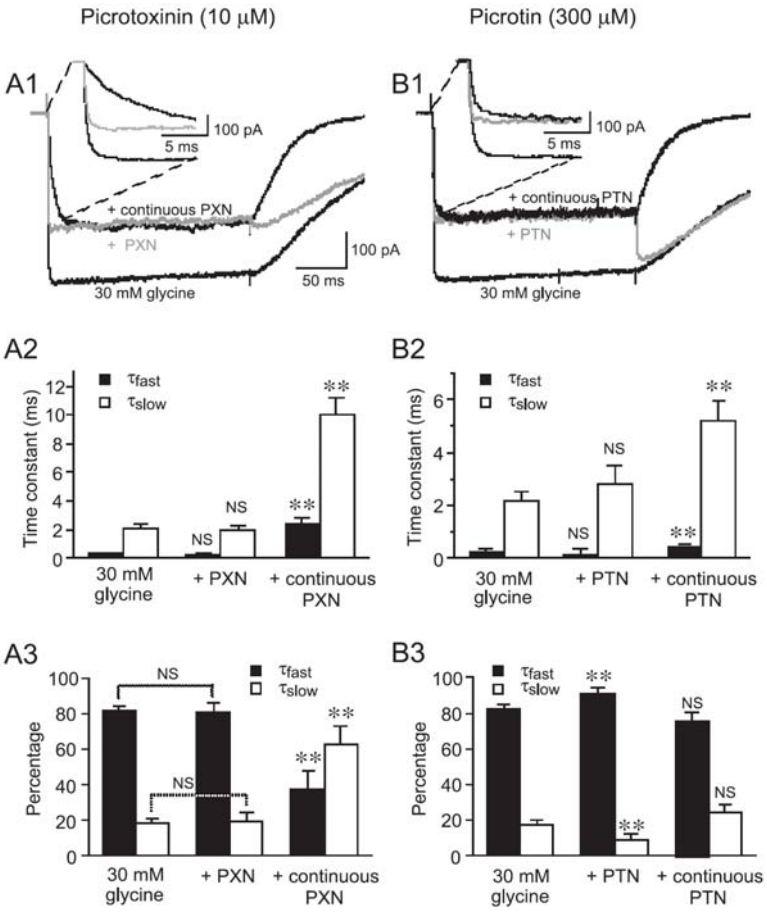

Figure 3.4 *Differential effects of picrotoxinin and picrotin on the onset of macroscopic currents activated by a saturating concentration of glycine.* **A1.** Averaged traces of currents (n=10–12) obtained from the same patch showing the activation phase of the responses activated by a saturating concentration of 30 mM glycine, by the co-application of 30 mM glycine and 10 μM PXN, and by glycine in the continuous presence of 10 μM PXN. Note that the activation phase of the glycine response was slowed down in the continuous presence of PXN. **A2-A3.** Summary of the time constant (τ_{fast} and τ_{slow}) values (**A2**) and their relative area (**A3**) obtained from the experiments shown in A1 (n=8). Note that the percentage of τ_{fast} significantly decreased (**A3**), whereas the relative amplitudes of both τ_{fast} and τ_{slow} significantly increased (**A2**) in the continuous presence of PXN (t-test; $p<0.01$) when compared with 30 mM glycine and co-application of 30 mM glycine and 10 μM PXN. **B1.** Averaged traces of currents (n=10–11) obtained from the same patch showing the activation phase of the responses activated by a saturating concentration of 30 mM glycine, by the co-application of 30 mM glycine and 300 μM PTN, and by glycine in the continuous presence of 300 μM PTN. Note that the activation phase of the glycine response in the continuous presence of picrotin was less slowed down than that in the continuous presence of picrotoxinin. The calibration bars in A1 also apply to B1. A1 and B1 were obtained from the same patch. **B2-B3.** Summary of the time constant (τ_{fast} and τ_{slow}) values (**B2**) and their relative area (**B3**) obtained from the experiments shown in B1 (n=8). Note that τ_{fast} and τ_{slow} were increased in the continuous presence of PTN only in B2 (t-test; $p<0.01$), although the relative area remained unchanged (B3). **: statistical significance $p<0.01$. NS: not significant.

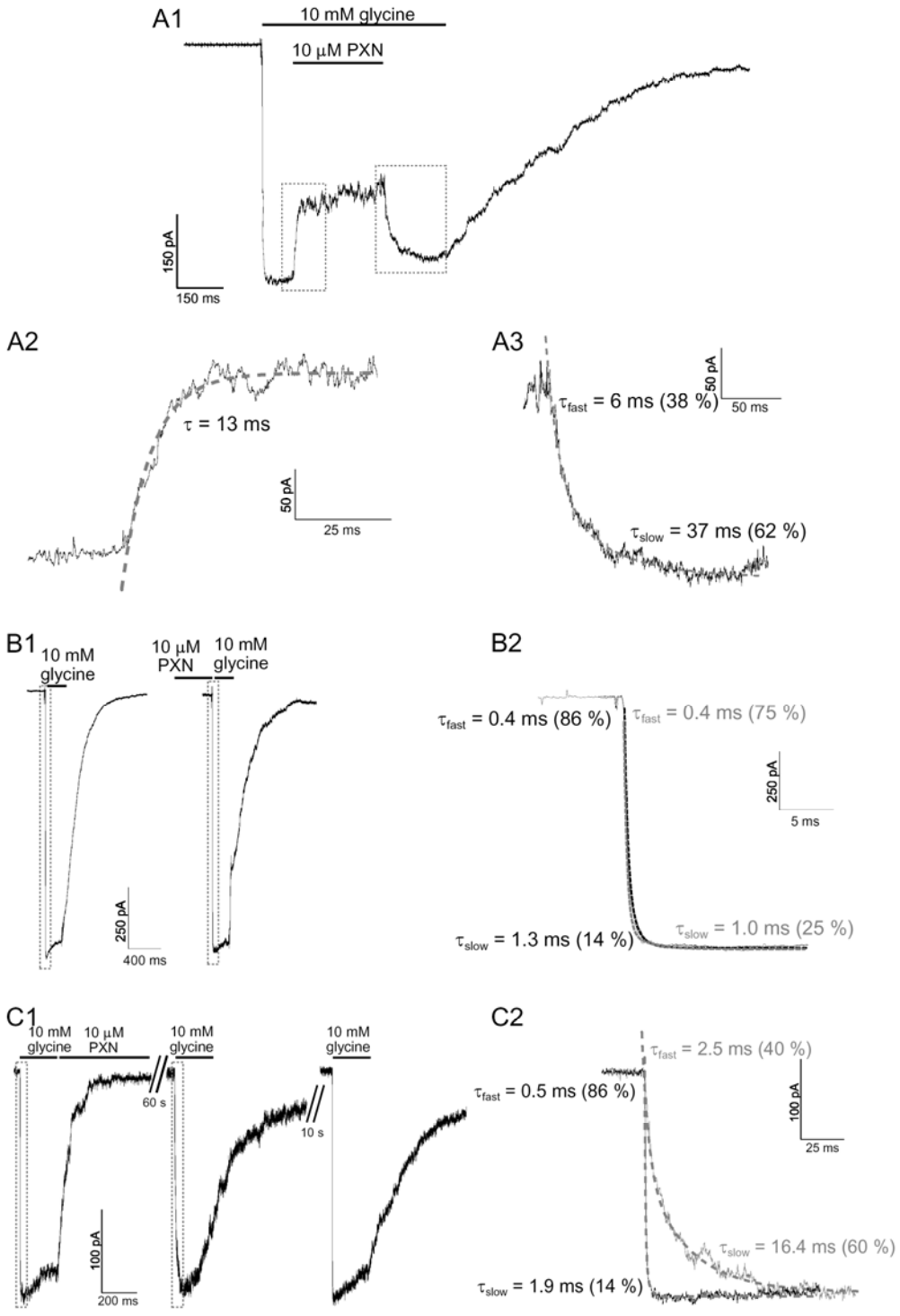
A1
10 mM glycine
10 µM PXN
150 pA
150 ms
A2
τ = 13 ms
50 pA
25 ms
A3
τfast = 6 ms (38 %)
50 pA
50 ms
τslow = 37 ms (62 %)
B1
10 mM glycine
10 µM PXN
10 mM glycine
250 pA
400 ms
B2
τfast = 0.4 ms (86 %)
τfast = 0.4 ms (75 %)
250 pA
5 ms
τslow = 1.3 ms (14 %)
τslow = 1.0 ms (25 %)
C1
10 mM glycine
10 µM PXN
10 mM glycine
10 mM glycine
60 s
10 s
100 pA
200 ms
C2
τfast = 2.5 ms (40 %)
τfast = 0.5 ms (86 %)
100 pA
25 ms
τslow = 16.4 ms (60 %)
τslow = 1.9 ms (14 %)

Figure 3.5 *Differential recovery from picrotoxinin block.* **A1.** Average of 5 traces of current obtained in response to a 600 ms step application of 10 mM glycine, transiently inhibited by a 300 ms step application of 10 µM picrotoxinin (PXN) with 10 mM glycine. The dashed boxes in A1 indicate parts of the trace enlarged in A2 (left box) and A3 (right box). **A2.** The onset of the picrotoxinin inhibition was well fitted by a mono-exponential curve (gray dashed line) giving a time constant of 13 ms. **A3.** The recovery from the inhibition by PXN was best fitted by a bi-exponential curve (gray dashed line) giving time constants τ_{fast} = 6 ms (38%) and τ_{slow} = 37 ms (62%). **B1.** Average of 4 traces showing currents evoked by a 300 ms step application of 10 mM glycine following a 300 ms step application of control solution (left black trace) or 10 µM PXN (right gray trace). Dashed boxes in B1 indicate the part of the traces enlarged in B2. **B2.** The onset of both responses was best fitted by a bi-exponential function. The fast and slow time constant values and their relative areas are respectively indicated in black (control pre-incubation) and in gray (PXN preincubation). Note the absence of effect of the PXN pre-incubation. **C1**. Example of three consecutive responses to 200 ms step application of 10 mM glycine where the first application was directly followed by a 500 ms step application of 10 µM PXN. The interval between each application is indicated between each trace. Note the quickening in the decay of the first glycine response during the PXN application and the slowing down in the onset of the second response. The third response exhibits an onset similar to the first response indicating a complete recovery from the PXN effect. Dashed boxes indicate the part of the two first traces enlarged in C2. **C2.** The onset of the first and second responses was best fitted by a bi-exponential function. The fast and slow time constant values and their relative areas are respectively indicated in black (first application) and gray (second application).

constants or the relative contributions of these constants. PTN applied together with glycine also did not change rise time constants, but did increase the relative contribution of τ_{fast} (fig. 3.4).

Continuous application of PXN or PTN had more extensive effects. PXN increased both τ_{fast} and τ_{slow} and increased the relative contribution of τ_{slow}, again very similar to PTX. PTN also increased τ_{fast} and τ_{slow} but had no effects on their relative contributions (fig. 3.4). There are, however, several aspects of these effects that demand further attention. The parameters τ_{fast} and τ_{slow} were significantly faster with PTN application than with PXN application. Could this be the result of a faster block recovery from PTN? Also, when continuously applying PXN or PTN, no effect on τ_{fast} or τ_{slow} could be seen with the first glycine application. It was consistently observed that the above described effects did not arise until the second glycine application. This raises the question whether PTN and PXN can bind to the unliganded receptor. The lengthening of the rise time probably results from an increase in first latency, as was observed for PTX (Wang et al. 2006). The question at hand is whether this lengthening reflects a recovery from channel block or is it the result of binding of PTN or PXN to the unliganded receptor? A series of experiments mainly conducted in our lab helped to resolve these issues and will be discussed in the next section.

3.2 Results

3.2.1 Onset and recovery of picrotoxinin and picrotin block of α2 homomeric glycine receptors

To evaluate recovery from open channel block, the recovery time constant of PXN and PTN block was measured. This was done by transiently applying 10 µM

PXN or 100 µM PTN during application of 10 mM glycine on the outside-out patch, which was clamped at -50 mV (fig. 3.5A1 and 3.6A1). A concentration of 10 µM PXN reduced glycine-evoked current with a time constant of $\tau_{(b)}$ = 17.6 ± 2.1 ms (n=11) (fig. 3.5A2). Steady state current in the presence of PXN was 41 ± 2% of control. Currents recovered from PXN block with a bi-exponential time course with time constants $\tau_{fast(r)}$ = 5.9 ± 1.0 ms and $\tau_{slow(r)}$ 38 ± 5 ms (n=11) (fig. 3.5A3). The relative contribution of these components was 40.0 ± 4.5% and 60.0 ± 4.5% respectively.
Application of 100 µM PTN blocked currents to 51 ± 3% of control. The onset of inhibition had a bi-exponential time course with $\tau_{fast(b)}$ = 0.5 ± 0.1 ms (69 ± 4%) and $\tau_{slow(b)}$ = 6.9 ± 1.7 ms (31 ± 4%) (n=11) (fig. 3.6A2). Recovery from PTN block was faster with time constants $\tau_{fast(r)}$ = 0.64 ± 0.08 ms and $\tau_{slow(r)}$ = 14 ± 3 ms (fig. 3.6A3). The relative contribution of these recovery time constants was 64 ± 5% and 36 ± 5% respectively.

3.2.2 Picrotoxinin and picrotin do not bind to unliganded closed α2 homomeric glycine receptors

To investigate whether the PTX components can bind to the unliganded receptor, we applied 10 µM PXN or 100 µM PTN immediately (< 0.1 s time interval) before applying 10 mM glycine. If PXN or PTN would bind to the unliganded state, this should influence the glycine evoked current. Without any toxin pre-treatment, the activation time constants of the glycine evoked currents were τ_{fast} = 0.50 ± 0.06 ms (90% ± 5%) and τ_{slow} = 1.97 ± 0.52 ms (10% ± 5%) (n=7). If 10 µM PXN was applied for 300 ms prior to 10 mM glycine the time constants of the rising phase were τ_{fast} = 0.50 ± 0.05 ms (81 ± 9%) and τ_{slow} = 1.71 ± 0.19 ms (n=5) (fig. 3.5B). With 100 µM PTN pre-incubation, these time constants were τ_{fast} = 0.55 ± 0.14 ms (87 ± 9%) and τ_{slow} 2.83 ± 1.25 ms (n=5)(fig. 3.6B). Hence pre-incubation with PXN or PTN did not have an effect on the amplitude of the glycine-evoked current.

3.2.3 Picrotoxinin and picrotin can be trapped after binding to the open channel

The above results indicate that PXN and PTN can bind to the open channel, but not to the unliganded closed state. We next asked whether PXN or PTN can become trapped when the channel closes after binding PXN/PTN binding. This was described to be the case for PTX (Wang et al. 2006), so would it be the case for both PTX components? To answer this question, we used a protocol in which toxin was applied during the deactivation phase of a first 10 mM glycine application. Toxins were applied for 500 ms, corresponding to the duration of the deactivation phase. We subsequently analysed the effect on the activation phases of successive outside-out currents evoked by 10 mM glycine. The second

glycine pulse was applied 60 s after toxin post-treatment. A last glycine pulse was applied 10 s after the second one (fig. 3.5C and 3.6C). Currents evoked 60 s after PXN (10 µM) post-treatment had a similar amplitude to control currents (< 4% decrease, n=5) but these currents showed a significant increase in rising time constants (t-test, $p<0.01$, n=5). The current activation after PXN post-treatment could be fitted by two exponential curves with time constants τ_{fast} = 4.15 ± 1.24 ms (54 ± 8%) and τ_{slow} = 18.5 ± 9.3 ms. Applying 10 mM glycine 10 s after this response restored the activation kinetics to values similar to control, being τ_{fast} = 0.42 ± 0.05 ms (66 ± 5%) and τ_{slow} = 2.31 ± 0.39 ms. The proportion of the fast component was significantly decreased in the glycine-evoked currents after PXN post treatment (fig. 3.6C).
PTN (100 µM) post-treatment also resulted in slower activation, with τ_{fast} = 0.53 ± 0.05 (53 ± 5%) and τ_{slow} = 4.95 ± 0.80 ms, albeit less pronounced than in the case of PXN. The effect is mainly due to a significant increase in τ_{slow} (t-test, $p<0.01$, n=8) and in the proportion of τ_{slow} (t-test, $p<0.05$, n=8). Kinetics of currents evoked by 10 mM glycine recovered to values similar to control, being τ_{fast} = 0.42 ± 0.05 ms (67 ± 5%) and τ_{slow} = 2.31 ± 0.91 ms (fig. 3.6C). As in the case of PXN, PTN post-treatment did not affect the amplitude of the glycine-evoked currents.

3.3 Discussion

A previous study has described the inhibitory mechanisms of PTX on α2 homomeric glycine receptors (Wang et al. 2006). PTX is however an equimolar mixture of PTN and PXN. The potencies of these compounds differ between ligand gated ion channels. Both have been described to have equal blocking potencies in α1 homomeric GlyR (Lynch et al. 1995) but PTN was not active in GABA-R block (Qian et al. 2005). The alanine on the 2' pore lining position that is thought to be responsible for PTN resistance in GABA-R's is also present in α2 GlyR's. This inspired us to evaluate the blocking mechanisms of PTN and PXN on α2 GlyR's. Results indicate that PXN is the main mediator of PTX block in α2 GlyR's, but α2 GlyR's are not completely insensitive to PTN. Our findings show PXN and PTN that bind to the open channel conformation, where they can become trapped if the channel closes.

3.3.1 Blocking mechanisms of picrotoxinin and picrotin on α2 homomeric glycine receptors

Contrary to the situation in α1 GlyR's, IC_{50} values indicated that PXN is more than 30 times more potent in blocking α2 homomeric GlyR's than PTN. The concentration dependence of block by PXN was similar to that of PTX, already hinting that PXN is the main mediator for PTX-block in α2 homomeric channels. An observation that was consistently confirmed in all other conducted

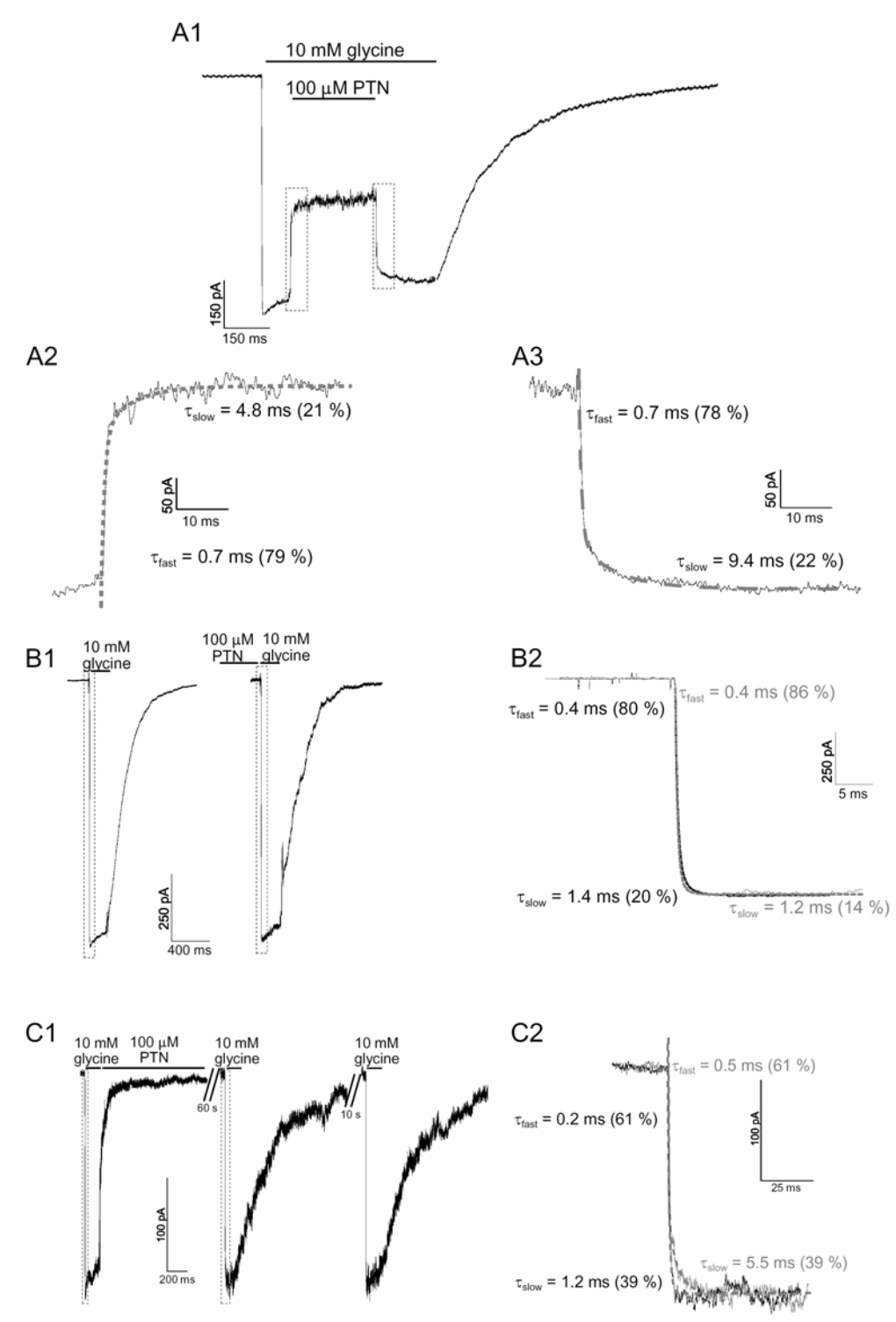
A1
10 mM glycine
100 µM PTN
150 pA
150 ms
A2
τslow = 4.8 ms (21 %)
50 pA
10 ms
τfast = 0.7 ms (79 %)
A3
τfast = 0.7 ms (78 %)
50 pA
10 ms
τslow = 9.4 ms (22 %)
B1
10 mM glycine
100 µM PTN
10 mM glycine
250 pA
400 ms
B2
τfast = 0.4 ms (86 %)
τfast = 0.4 ms (80 %)
250 pA
5 ms
τslow = 1.4 ms (20 %)
τslow = 1.2 ms (14 %)
C1
10 mM glycine
100 µM PTN
10 mM glycine
10 mM glycine
60 s
10 s
100 pA
200 ms
C2
τfast = 0.5 ms (61 %)
τfast = 0.2 ms (61 %)
100 pA
25 ms
τslow = 5.5 ms (39 %)
τslow = 1.2 ms (39 %)

Figure 3.6 *Differential recovery from picrotin block.* **A1.** Average of 5 traces of current obtained in response to a 600 ms step application of 10 mM glycine and transiently inhibited by a 300 ms step application of 100 µM picrotin (PTN) with 10 mM glycine. The dashed boxes in A1 indicate parts of the trace enlarged in A2 (left box) and A3 (right box). **A2.** The onset of the PTN inhibition was well fitted by a bi-exponential curve (gray dashed line) giving time constants τ_{fast} = 0.7 ms (79%) and τ_{slow} = 4.8 ms. **A3.** The recovery from the inhibition by PTN was also best fitted by a bi-exponential curve (gray dashed line) giving time constants τ_{fast} = 0.7 ms (78%) and τ_{slow} = 9.4 ms. **B1.** Average of 5 traces showing currents evoked by a 300 ms step application of 10 mM glycine following a 300 ms step application of control solution (left black trace) or 100 µM PTN (right gray trace). Dashed boxes in B1 indicate the part of the traces enlarged in B2. **B2.** The onset of both responses was best fitted by a bi-exponential function. The fast and slow time constant values and their relative areas are respectively indicated in black (control pre-incubation) and in gray (PTN pre-incubation). Note the absence of effect of the PTN pre-incubation. **C1.** Example of three consecutive responses to 200 ms step application of 10 mM glycine where the first application was directly followed by a 500 ms step application of 100 µM PTN. The interval between each application is indicated between each trace. Note the quickening in the decay of the first glycine response during the PTN application. Dashed boxes indicate the part of the two first traces enlarged in C2. **C2.** The onset of the first and second responses was best fitted by a bi-exponential function. The fast and slow time constant values and their relative areas are respectively indicated in black (first application) and gray (second application). Note that there is no significant difference for τ_{fast} and a slight lengthening of τ_{slow} between the first and the second application.

experiments. Although PXN and PTN have different potencies in blocking homomeric α2 GlyR, they also share similar properties, possibly hinting at least partly similar blocking mechanisms.

Both compounds slowed down the activation phase and speeded up the relaxation phase of glycine evoked currents. The slower activation phase probably results from longer first latencies, as was the case for PTX (Wang et al. 2006), but this was not explicitly measured. Important to point out is that the effect on the activation phase was not seen when PXN or PTN was applied simultaneously with glycine, and was only seen from the second glycine application onward when PXN or PTN was continuously applied. Pre-treatment experiments with PXN and PTN proved this to be the result from the inability of PXN or PTN to bind the unliganded closed state. The speeding up of the relaxation phase is the result from a reduction in mean open time and mean burst duration. Although PXN was again the most potent component in speeding up deactivation, PTN treatment also showed shorter mean open times. This can be explained by a larger number of reopenings in the presence of PTN, which is also consistent with the slower deactivation with PTN compared to PXN (longer mean burst duration). PTN block indeed induced more flickering in single channel traces. Moreover, mean open time analysis showed similar binding and opening rate constants for PTN and PXN, again pointing to a larger number of reopenings as the mechanism for slowing down relaxation in PTN block compared to PXN block. The decrease in mean open time was larger with increasing toxin concentration, indicating binding to the open conformation, as confirmed by the transient blocking effect when toxin was temporarily applied within the application of glycine. The faster deactivation in the presence of PXN or PTN compared to control further indicates that both compounds are slow channel blockers. This type of block does not allow the experimenter to

distinguish a late, block-relieving, reopening of the channel as part of the earlier burst. The result is an apparent speeding of the relaxation phase which can be described with one exponential, as was seen in our data.
This open channel block seems to be contradicted by the competitive-like behaviour of PXN and PTN. Block by both toxins was dependent on glycine concentration, which is typical for competitive antagonism. The mechanism of block by PXN and PTN can however explain this behaviour. Post-treatment experiments clearly showed that PXN and PTN can become trapped at their binding sites once the channel closes. This was exemplified by the persistence of effect on the activation phase of glycine-evoked currents, even 60 seconds after PXN/PTN post-treatment. With higher concentrations of glycine, the likelihood of the receptor being in the open state increases, thus also increasing the chance of reopening so that block can be relieved.
Nevertheless, the similarities between PXN and PTN go hand in hand with important differences. Besides an obvious difference in potency, PTN-inhibition was more voltage sensitive than PXN-inhibition. This indicated that PTN goes deeper in the electrical field of the membrane, hinting a different binding site for PTN than for PXN. In the case of α1 homomeric GlyR, PXN binding is thought to occur close to the 6' pore lining residue (Zhorov and Bregestovski 2000). It is possible that the PTN binding site is more near the 2' pore lining residue, which is deeper within the pore and could account for the different voltage sensitivities. This concept is supported by the apparent influence of the nature of the 2' pore lining residue on PTN-mediated inhibition. α1 GlyR's show equal sensitivity to PTN and PXN, in contrast to α2 GlyR's and $GABA_{A-C}$R's. Although α2 GlyR show high homology with α1 in the pore region, the 2' pore lining residue is an alanine in α2 (and GABAR's), while it is a glycine in α1 GlyR's. Another explanation for the different sensitivities between α1 and α2 might be a difference in conformational changes of the channel pore. Given that PXN and PTN are open channel blockers, channel conformation might influence access to binding sites, and hence blocking effects. The different kinetic properties of α1 and α2, with longer open times and higher main conductance states for α2 (Mangin et al. 2003), indeed indicate different open state conformations.

3.3.2 Minimal Markov model for picrotoxinin and picrotin block of α2 homomeric glycine receptors

Based on the experimental observations, our collaborators constructed Markov models describing PXN and PTN block. The earlier described minimal Markov model for α2 homomeric GlyR (Mangin et al. 2003) was used to fit control data of long glycine applications to obtain the different rate constants. This model describes 3 glycine binding sites, with each glycine-liganded state linked to a desensitized closed state. The α2 homomeric GlyR was modelled to have one

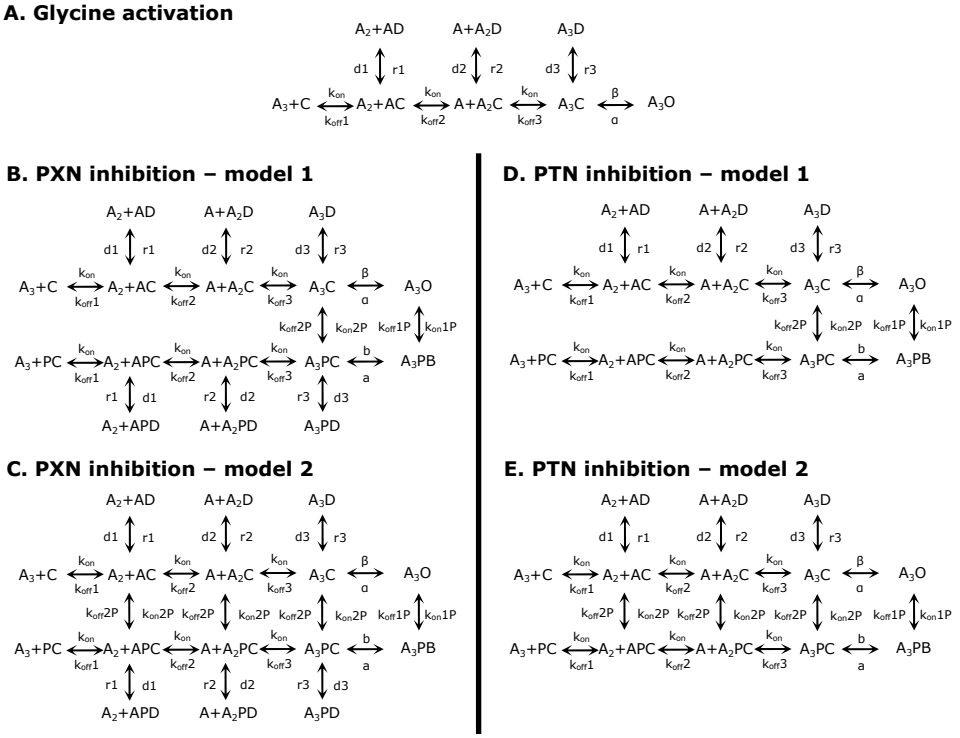

Figure 3.7 *Kinetic schemes used for fitting glycine responses in the absence and presence of picrotoxinin or picrotin.* **A.** This kinetic scheme was used for homomeric α2 GlyR in control conditions (without picrotoxinin or picrotin), from Mangin *et al.* (2003) **B.** Picrotoxinin can bind and unbind from the fully glycine-liganded closed state or from the open state of GlyR. In this scheme, picrotoxinin is trapped when glycine dissociates from the fully liganded closed state. **C.** In this alternative model, picrotoxinin can bind and unbind from all glycine-bound states, but picrotoxinin is only trapped when the receptor returns to the glycine-unbound closed state **D.** As in A, picrotin can bind and unbind from the fully glycine-liganded closed state or from the open state of GlyR only. In this scheme, picrotin is trapped when glycine dissociates from the fully liganded closed state. GlyRs cannot further desensitize from the glycine-bound closed states when picrotin is trapped on its binding site. **E.** This model is identical to C expect that, as in D, GlyRs cannot further desensitize from the glycine-bound closed states when picrotin is trapped on its binding site. A: agonist, P: picrotoxinin or picrotin, C: resting (closed) states of the receptor, D: desensitization states, O: open states.

fully liganded open state (fig. 3.7A). The thus predicted EC_{50} and Hill coefficient were in accordance with the earlier obtained values for α2 receptors (Mangin et al. 2003). Next, PXN and PTN effects on the same traces were analyzed with different models based on experimental data.

Given the similar inhibitory properties of PXN and PTX, the Markov model for PXN was based on the earlier described model for PTX (Wang et al. 2006). From experimental data it was known that (1) the Hill coefficient was near to 1, indicating one binding molecule (2) PXN binding occured in the open conformation (3) PXN could not bind to the unliganded closed state and (4) PXN could become trapped if the channel closes. These properties were modelled by incorporating three sequential PXN-bound, glycine-bound closed states linked to a glycine-unbound state (A_3PC, A + A_2PC, A_2 + APC and A_3 + PC) (fig. 3.7B).

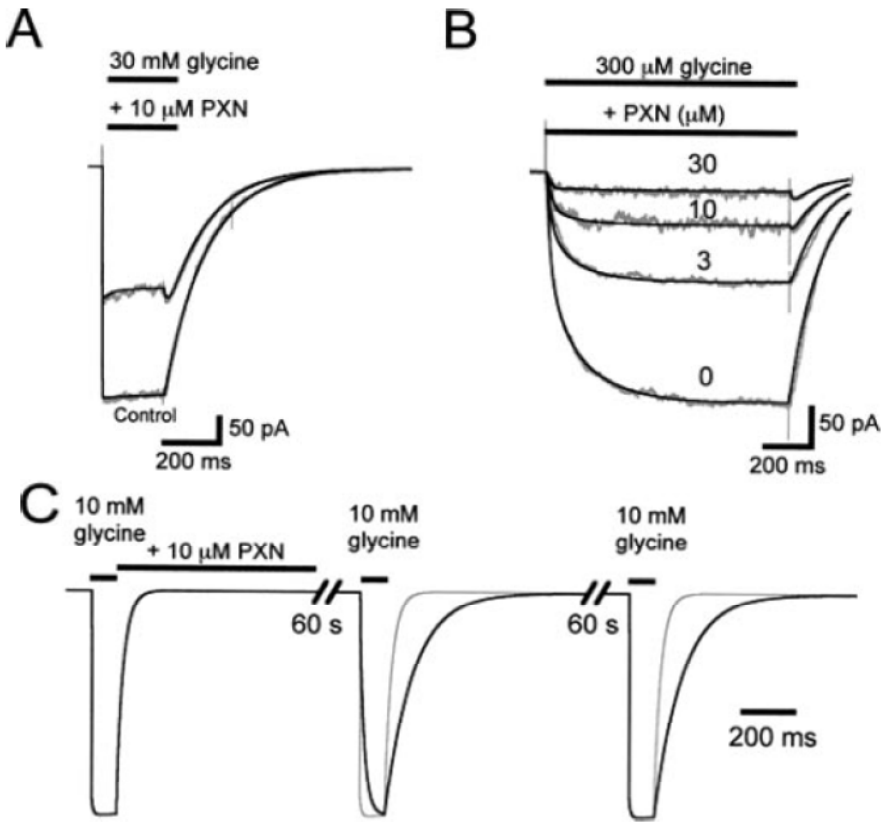

Figure 3.8 *Prediction of experimental results for picrotoxinin by kinetic models.* **A.** Outside-out currents (*gray traces*) elicited by glycine (30 mM) in the absence and presence of 10 μM PXN were superimposed on simulated currents using PXN model 1, as depicted in fig. 3.7B (*black line*) (V_H = -50 mV). **B.** Outside-out currents evoked by co-application of glycine (0.3 mM) and 0, 3, 10, and 30 μM PXN (*gray lines*) were superimposed on simulated currents using PXN model 1. PXN model 1 well predicts the concentration-dependent inhibitory effect of PXN and the time course of glycine-evoked currents when PXN and glycine are co-applied. **C.** Simulated traces generated using PXN model 1 showing that this kinetic scheme also predicts that when picrotoxinin was applied during the deactivation phase of the glycine-evoked current, and the lengthening of the rise time evoked by PXN can persist up to 60 s after washout of glycine and PXN. For time course comparisons, the control response on the left was superimposed (in gray) on the other simulated glycine-evoked currents.

Adding the states with both glycine and PXN bound accounts for the decrease in deactivation time when PXN was applied during relaxation (fig. 3.5C1). To properly fit the experimental data, each glycine- and PXN-bound state had to be linked to a desensitized state (A_3PD, A + A_2PD, A_2 + APD) (fig. 3.7B & fig. 3.8). Two variants of the model, which differed in the possibility of PXN to dissociate from the partly liganded state (fig. 3.7B-C), equally described experimental data. These models are similar to the PTX inhibition model (Wang et al. 2006). The obtained average rate constant values are also very similar to PTX values, confirming that PTX inhibition of α2 homomeric GlyR's is mainly mediated by

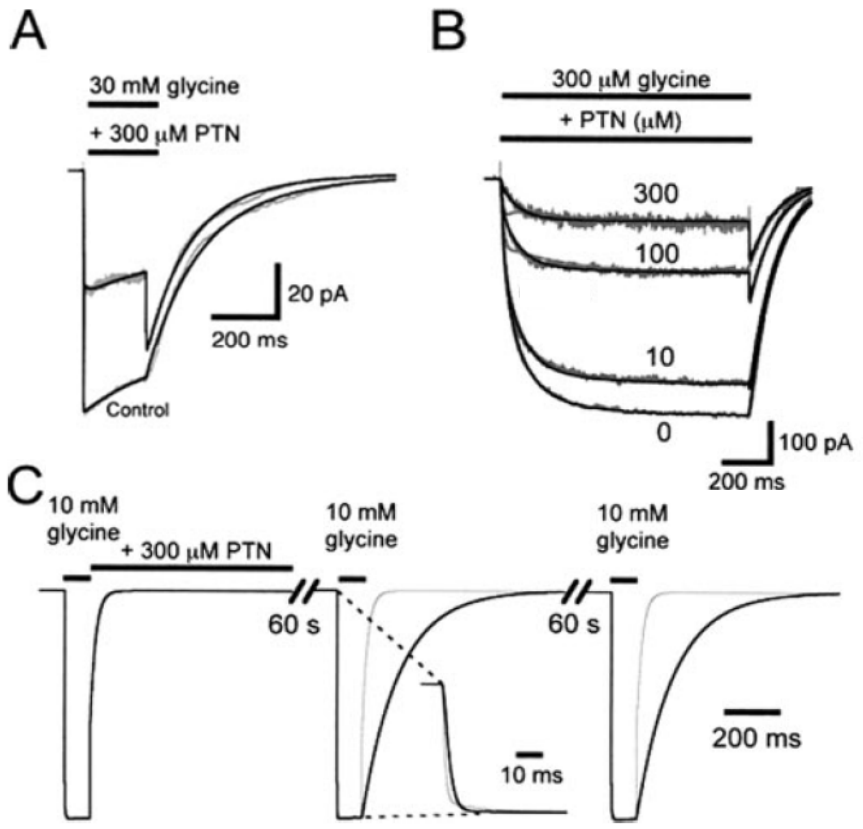

Figure 3.9 *Prediction of experimental results for picrotin by kinetic models.* **A.** Outside-out currents (*gray traces*) elicited by glycine (30 mM) in the absence and presence of 300 μM PTN were superimposed on simulated currents using PTN model 1, as depicted in fig. 3.7D (*black line*) (V_H = -50 mV). Note that this model well predicts the large rebound current observed at the end of the co-application of glycine and PTN. **B.** Outside-out currents evoked by co-application of glycine (300 μM) and 0, 10, 100, and 300 μM PTN (*gray lines*) were superimposed on simulated currents using PTN model 1 (*black line*) and a model were PTN is unable to bind to the fully liganded closed conformation. Note that both models well predict the concentration-dependent inhibitory effect of PTN but that PTN binding to the fully liganded closed state is necessary to predict the concentration-dependent effect of PTN on the activation time course of glycine-evoked currents. **C.** Simulated traces generated using PTN model 1 showing that this kinetic scheme also predicts that the lengthening of the rise time evoked by PTN can persist up to 60 s after washout of glycine and PTN (see inset). For time course comparisons, the control response on the left was superimposed (in *gray*) on the other simulated glycine-evoked currents.

PXN. The model showed the affinity of PXN for the open state to be lower than for the glycine-liganded closed state. This could mean that the binding site is not equally accessible in these states because of conformational changes associated with inglycine bding and gating.

Given the earlier mentioned common properties observed in PXN and PTN block, the model for PXN block was also tested for PTN block. The hypothesis was that

PTN is a weak agonist for the PXN-binding site. The PXN-model however failed to describe experimental data of PTN block. PTX is known to induce desensitized-like closed states in α1 GlyR's (Lynch et al. 1995). A model was constructed based upon the hypothesis that PTN can induce such desensitized-like states in α2 GlyR's. Given the likely deeper penetration of PTN in the channel pore, based upon voltage sensitivity of PTN block, a first model tested assumed PTN could only bind and dissociate from the open state of the receptor. It hypothesised that in the (partly) liganded closed states, the PTN binding site was inaccessible. However, this model did not describe experimental data (fig. 3.9). Therefore, as with PXN, two other models with PTN able to dissociate from and bind to at least the fully liganded and the open states were tested next (fig. 3.7D-E). Both these models successfully fitted experimental data (fig. 3.9). Again, the transitions between the monoliganded glycine-bound closed states (A_2 + AC to A_2 + APC) and the doubly liganded glycine-bound states (A + A_2C to A + A_2PC) proved not necessary to describe experimental data. As with PXN, the affinity for the glycine-bound closed states was higher than for the open state.
PXN data could not be fitted with the proposed models for PTN block, which makes it improbable that both PTX compounds share the same inhibitory mechanism. The rate constant values obtained from fitting procedures point out some of these differences. The PTN dissociation rate is somewhat 100 times faster than for PXN, so the affinity of PTN for the open state is rather low compared to PXN. This fast dissociation explains the fast recovery of PTN block (fig. 3.6A) compared to PXN block (fig. 3.5A). The on-rate constant and off-rate constant of the transition between PTN-bound open state (A_3PB) and PTN-bound fully liganded closed state (A_3PC) are slower than for PXN. In fact, in the case of PXN, the transition between A_3PB and A_3PC has a very large on-rate constant, suggesting that these states collapse when PXN binds to the receptor, as was also proposed for PTX (Wang et al. 2006). Although on- and off-rate constants for PTN were close to the range of the on- and off-rate constants of the GlyR itself, data could not be fitted correctly when they were set equal. This implies that the channel does not simply close upon PTN binding, but a specific conformational change leading up to channel closure is evoked by PTN binding. The larger value of the off-rate constant compared to the on-rate constant of the transition between A_3PB and A_3PC for both PXN and PTN indicates that when glycine and toxin are present, the receptor mainly resides in the blocked state, rather than the closed state.

3.3.3 Concluding remarks

The data we collected, together with our collaborators at the "Université Pierre et Marie Curie", shows that PXN and PTN are channel blockers that bind to the fully liganded closed state or open state of the α2 glycine receptors. Of these

compounds PXN is much more potent than PTN and is the main mediator of PTX inhibition. The binding of PXN and PTN to the fully liganded closed state might seem contradictory to the hypothesis that the toxins bind within the channel, but this was also observed for PXN block of GABAR's (Qian et al. 2005).

The lower association rate constant of PXN for the open channel state suggests an open conformation of α2 GlyR's with limited access of PXN to its binding site. PTN did not show such difference between association rates of the liganded closed states and the open state. This suggests that, contrary to PXN, access of PTN to its binding site is not strongly influenced by the conformational change accompanying channel gating. The slower association rate for PTN compared to PXN is an argument in favor of a binding site deeper within the channel pore, as was suspected from voltage dependence experiments. Although the association rates of PTN for the liganded closed states and the open state are similar, PTN dissociation rates are higher in the open state. This accounts for the increased affinity for the liganded closed state, since affinity is the ratio of dissociation rate ($\mu M^{-1}s^{-1}$) and association rate (s^{-1}). This fast dissociation in the open state however contrasts with the previous hypothesis that the slow association rate is caused by steric hindrance of the toxin on its way to the binding site, unless one supposes that the binding site of PTN makes the molecule more sensitive to thermal agitation caused by ion flux in the open conformation (Ramakrishnan and Hess 2005). The exact obtained values of the rate constants are reported and discussed in our publication, but not elaborated here because the modelling work was not performed in our lab.

In conclusion, PTN and PXN are not equally potent in their blocking properties of α2 homomeric glycine receptors. It is likely that both compounds work through different allosteric mechanisms, given that their kinetic properties depend on conformational status of the GlyR. A question that remains open is the exact location of the binding site for PTN and PXN on α2 homomeric GlyR's, although this work indicates that there are separate binding sites, with the PTN-site located deeper within the channel pore.

Chapter 4 | Effects of the β subunit on kinetics of the α2-containing glycine receptor

Results of this chapter will be reported in Buckinx R, Legendre P, Rigo JM. "Kinetics of the α2β heteromeric glycine receptor." (working title) – *in preparation*

Abstract

Glycine receptors (GlyR) are widely distributed in the central nervous system, even before establishment of synaptic contacts. The α_2 GlyR-subunit is mainly expressed in immature nerve cells, although homomeric α_2 GlyR are most likely not involved in synaptic neurotransmission due to the lack of a β subunit, needed for postsynaptic clustering, and due to slow activation kinetics. However, the β subunit is expressed at low levels during development and increases perinatally, creating a window of co-expression of high levels of α2 and β. We have investigated whether the incorporation of the β subunit changes the kinetic properties in favor of synaptic signalling.

Our results indicate a faster activation and faster inactivation in α2β-containing patches compared to patches containing only α2. The faster inactivation is consistent with shorter mean open times observed in single channel measurements. Using an ultrafast application system to mimic synaptic signalling, an increase in open probability was observed for the α2β-containing patches. However, patches always contained a mixture of α2 and α2β, making it possible that the observed effects are an underestimation of the real influence of β subunits. Altogether our findings make it plausible that the β subunit is needed for synaptic functioning of the α2-containing glycine receptor, although the exact extent of the influence of the β subunit remains to be elucidated.

4.1 Introduction

Glycine plays a highly important role as an inhibitory neurotransmitter in the central nervous system, especially in the spinal cord. However, the role of GlyR is probably not limited to pure synaptic neurotransmission, since glycine receptors already appear before establishment of synapses (Ben-Ari 2001). During development the α2 subunit is most abundantly expressed. Postnatally α2 expression declines sharply. In the spinal cord, α2 expression disappears, but some α2 expression remains in higher brain regions such as cortex, hippocampus and hypothalamus (Malosio et al. 1991). Several studies have directly or indirectly indicated an extrasynaptic role for α2 glycine receptors. Firstly, the lack of β subunits prevents GlyR anchorage to gephyrin molecules, which is necessary for postsynaptic clustering (Kirsch et al. 1995; Meyer et al. 1995). Nevertheless, perinatally β subunit expression rises significantly while α2 expression is still high, so a transient synaptic clustering is more likely during that period (Malosio et al. 1991). Secondly, α2 is expressed in regions that lack synaptic transmission (Flint et al. 1998; Mangin et al. 2002). Thirdly, glycine receptors can be extrasynaptically activated by endogenous agonists like taurine (Flint et al. 1998). And last but not least, direct functional evidence hinting a non-synaptic role for α2 comes from a study of α2 homomeric receptor kinetics by Mangin *et al.* (2003). It was shown that the kinetics of the homomeric α2 GlyR were too slow for synaptic activation. The open probability (P_o) after a 1 ms application of 1 mM glycine was about 0.1, which is very low compared to mature heteromeric GlyR (α1/β) which had a P_o of about 0.9 in similar experiments (Legendre 1998; Mangin et al. 2003).

This inevitably leads to the question of the role of α2 GlyR. Why is a neurotransmitter receptor expressed, if it cannot function in neurotransmission? At this moment, completely answering this question is not yet possible. The exact physiological significance of a possible extrasynaptic function for (α2) GlyR's remains unclear. The affinity of the α2 receptor for glycine is rather low, but the homomeric channels show a high efficacy (Mangin et al. 2003). This suggests a role in paracrine signalling, responding to sustained low glycine concentrations. Several functions in the regulation of processes such as migration, differentiation or synaptogenesis are possible, but not yet clearly proven. Homomeric α2 GlyR's thus are probably not involved in immature glycinergic synaptic signalling. However, the β subunit is also expressed during development, albeit at lower levels than in adult (Malosio et al. 1991).

This raises the question whether the β subunit can influence α2 subunit kinetics. Earlier studies have shown an influence of the β subunit on α1/β receptor desensitization (Mohammadi et al. 2003). It has also been proven that the combined stable expression of α2 and β in CHO cells evoked different

distribution of single channel conductance levels compared to CHO cells stably expressing only α2 (Mangin et al. 2002; Mangin et al. 2005). In both α2 and α2/β CHO cells conductance level distribution showed four conductance peaks, but α2-expressing cells mainly showed conductance levels around 100 pS, where for α2/β-expressing cells small conductance levels (≤ 50 pS) were dominant. This shift was believed to result from influence of the β subunit, possibly allowing a discrimination of homo- and heteromeric channels on the single channel level. However, a small proportion of single channel currents in cells expressing only α2 still showed conductances of 45 pS (Mangin et al. 2005). It is thus currently not possible to distinguish with a 100% certainty between α2 and α2/β GlyR, even on the single channel level.

In this chapter results are described of a first study on α2β channel kinetics using the CHO cell line permanently transfected with α2 and β subunits. The results indicate an influence of the β subunit resulting in a higher open probability of the α2β channels during synaptic transmission, but it has to be noted that these conclusions are still preliminary. The mixed expression of homomeric α2 and heteromeric α2β GlyR's makes it difficult to draw definite conclusions at this time. Nevertheless, experiments described hereafter, performed on CHO cells expressing both α2 and β subunits are indicative for a more likely synaptic role for the α2β GlyR.

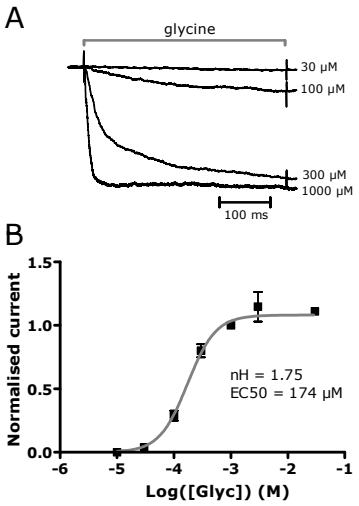

Figure 4.1 *Dose-response relationship of macroscopic currents from α2/β containing patches* **A.** Examples of normalised macroscopic currents elicited by 400 ms application of 30, 100, 300 and 1000 µM glycine. **B.** The dose-response relationship of α2/β containing patches (n=3 to 5 for each concentration) indicates an EC_{50} of 174 µM and a Hill coefficient of 1.75.

4.2 Results

4.2.1 Dose – response relationship of α2/β - containing patches

To investigate the response of α2/β containing patches to different concentrations of glycine we applied concentrations ranging from 10 µM to 30 mM to outside-out patches for 400 ms. To obtain a dose-response curve we analysed data from macroscopic currents of 3-5 patches for each concentration. Data from different patches were normalised to the response to 1 mM glycine. Dose-response analysis showed an EC_{50} of 174 µM and a Hill coefficient of 1.75 (fig. 4.1).

4.2.2 Open time constant of α2β single channel currents

As described by Mangin et al. (2005) the distribution of single channel conductance levels changes in cells expressing the β subunit in addition to the α2 subunit. However, different subconductance levels still exist. We compared mean open times of each of the most represented conductance levels most present in the transfected cells, being ≈ 100 pS in α2 expressing cells and ≈ 50 pS in α2/β expressing cells. Mean open times of the conductance level of ≈ 100 pS were very similar in α2 and α2/β expressing cells being 87 ms and 94 ms respectively (Fig. 4.2A-B). The ≈ 50 pS level, which is more prominently present in α2/β expressing cells, shows a marked decrease in mean open time in cells expressing α2/β compared to α2 expressing cells, being respectively 15.5 ms and 49 ms (Fig. 4.2C-D) .

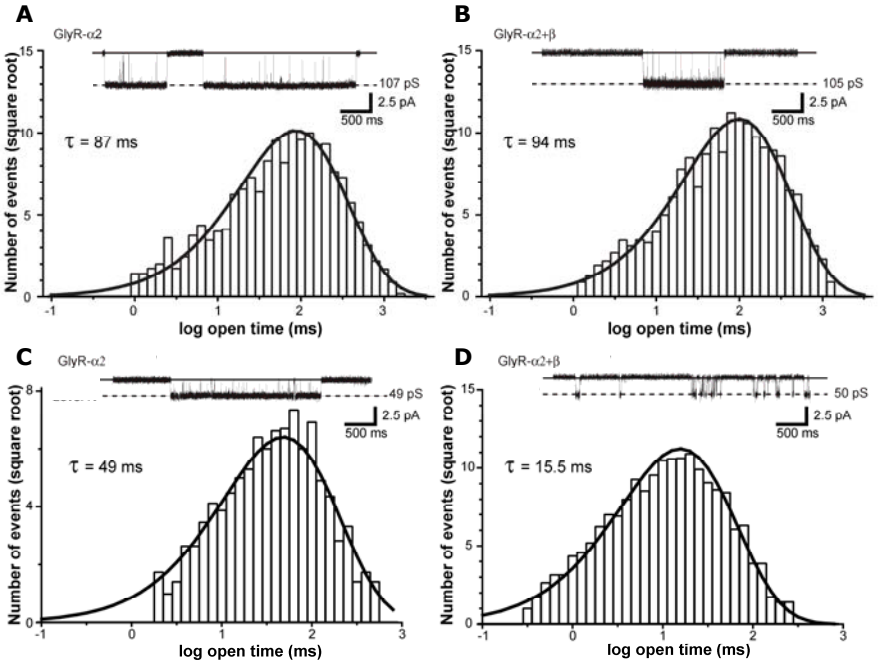

Figure 4.2 *Mean open time of α2 versus α2β glycine receptors* **A-B.** Mean open time analysis indicates the open rate constants of channels with around 100 pS, the homomeric form, does not differ between α2-expressing (A) and α2β expressing CHO cells (B). **C-D**. The open time constant of channels of the 50 pS conductance level, which corresponds with α2β heteromerics, decreases from 49 ms in CHO cells expressing only α2 (C) to 15.5 ms in CHO cells expressing both α2 and β (D).

4.2.3 Activation and inactivation kinetics of currents in α2/β - containing patches

To investigate the activation kinetics of macroscopic currents in α2/β transfected cells, we applied 1 mM of glycine, which is a physiological synaptic

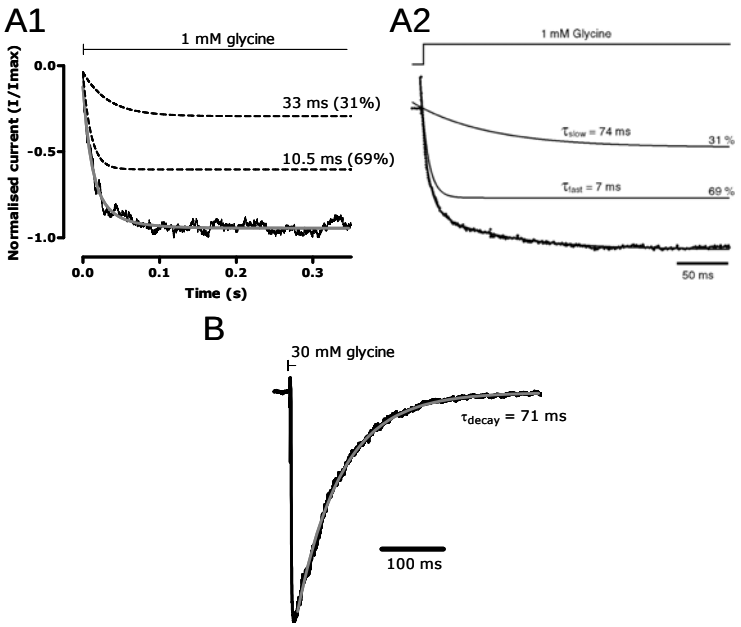

Figure 4.3 *Activation and inactivation of α2/β containing macroscopic currents* **A1.** Averaged response of an outside-out patch to 400 ms of 1 mM glycine. In this example the activation phase was best fitted by 2 exponentials with time constants 33 ms (31%, τ_{slow}) and 10.5 ms (69%, τ_{fast}). **A2.** Comparable experiment on a patch containing only α2 GlyR from Mangin *et al.* (2003), with permission. **B**. Fit of the decay phase of an averaged response of an outside-out patch to 1 ms application of 30 mM glycine. In this example the decay phase was best fitted by 1 exponential with decay time constant 71 ms (τ_{decay}).

concentration, to outside-out patches during 400 ms. The 20 to 80% rise time of the elicited currents was 12 ± 2 ms. Out of 9 tested patches, 5 showed an activation phase best fitted by two exponentials with time constants 6.5 ± 1.2 ms (τ_{fast}) and 49.3 ± 11.0 ms (τ_{slow}). The proportions of these exponentials were 77 ± 6% and 23 ± 6% respectively (fig. 4.3A1). Four patches were best fitted with a one phase exponential with 9.4 ± 1.7 ms activation time constant.
Deactivation of the current evoked by glycine in α2/β expressing cells was measured by shortly (1 ms) applying a saturating glycine concentration (30 mM) on an outside-out patch. The deactivation of elicited macroscopic currents could be fitted with a one phase exponential with a deactivation time constant of 64.2 ± 3.9 ms (n=6) (fig. 4.3B).

4.2.4 Open probability of α2β glycine receptors with synapse-like glycine application

To have an idea about how α2β receptors could function at synapses, we mimicked synaptic glycine signalling by applying 1 mM glycine for 1 ms on the

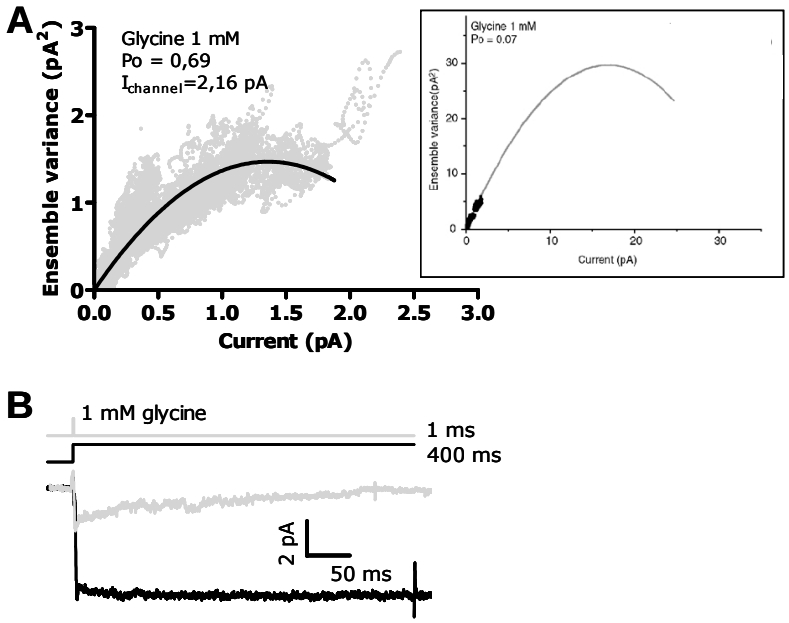

Figure 4.4 *Open probability in response to synaptic-like glycine application on an α2- and β-containing patch.* **A.** Averaged response of an outside-out patch to 400 ms of 1 mM glycine. In this example the activation phase was best fitted by 2 exponentials with time constants 33 ms (31%, τ_{slow}) and 10.5 ms (69%, τ_{fast}). *Inset* shows a comparable experiment conducted on a patch containing only α2 by Mangin *et al.* (2003), with permission. **B**. Fit of the decay phase of an averaged response of an outside-out patch to 1 ms application of 30 mM glycine. In this example the decay phase was best fitted by 1 exponential with decay time constant 71 ms (τ_{decay}).

outside-out patch using an ultrafast perfusion system. We next performed a variance analysis to estimate the open probability (P_o) of the receptors present in the patches. The 2 patches analysed had a P_o of 0.46 and 0.69 (see fig. 4.4 for example). The fits indicated a unitary current of about 2.5 pA, which corresponds to a unitary conductance of 50 pS at -50 mV holding potential, according to Ohm's law, suggesting the activation of heteromeric receptors.

4.2.5 Transient transfection as an alternative model

During experiments and analysis we noticed that patches from permanently transfected CHO cells still contained α2 homomeric glycine receptors in variable amounts, which made analysis and interpretation difficult. To reduce this problem in future experiments we checked whether transiently transfecting β subunits in CHO cells permanently transfected with α2 subunits was possible. To analyse successful transient transfection we compared the effect of picrotoxinin (PXN) on CHO cells with and without a transient β subunit transfection. As was

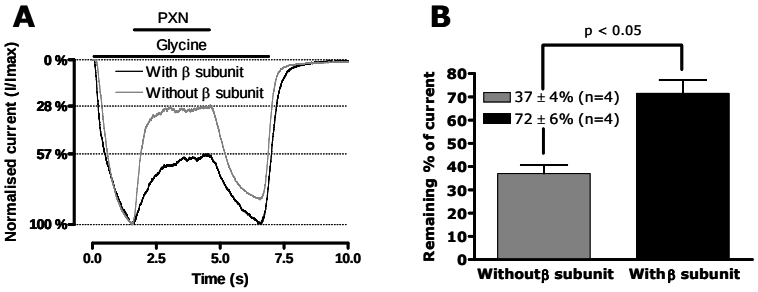

Figure 4.5 *Transient transfection of β subunit in α2 expressing CHO cells* **A.** Transient application of 2 μM PXN during glycine application indicates induction of resistance to PXN when β subunit is transiently transfected. The graph shows representative normalised responses of 2 patches. **B**. Transient transfection of the β subunit in α2 expressing CHO cells significantly reduces PXN effect by about 35% (t-test, $p<0.05$).

shown in the previous chapter, it is PXN that mainly mediates picrotoxin (PTX) effect. Since incorporation of the β subunit induces PTX resistance, succesfull functional transient transfection of β subunits should also induce PXN resistance. To investigate this, we transiently applied 2 μM PXN during glycine application, using the same protocol as shown in figure 3.5A1. In cells expressing only α2 subunits PXN application reduced glycine evoked current to 37 ± 4% (n=4) of control, while in α2 expressing CHO cells transiently transfected with β subunits PXN reduced currents only to 72 ± 6% (n=4) of control (fig. 4.5). PXN thus had a significantly smaller effect in cells transiently transfected with β subunit (t test, $p < 0.05$).

4.3 Discussion

In this chapter we performed preliminary analyses to estimate the kinetic characteristics of the α2β glycine receptor in light of its possible functions in- and outside of the synapse.

In contrast to earlier data (Mangin et al. 2005), we did not find an apparent difference in EC_{50} of currents from α2β containing patches or "pure" α2 containing patches. The EC_{50} value of 174 μM is very like the earlier described EC_{50} for α2 (Mangin et al. 2005). The Hill coefficient 1.75 is consistent with what is known for glycine receptors, and implies that binding of two glycine molecules is needed for channel opening (Gentet and Clements 2002; Mangin et al. 2003). The activation phase could be described by one phase or two phase exponentials, as was the case for α2 GlyR. Concerning the two phase activation, Mangin *et al.* (2003) reported a similar fast time constant but a slow time constant which appeared slower than the 49.3 ± 11.0 ms we report here for currents from α2β containing patches. This results in a 20-80% rise time of 12 ± 2 ms, which seems faster than activation in the case of α2 GlyR. In α2 GlyR, a 20-80% rise time of about 25 ms was observed (Mangin et al. 2003).

Inactivation of the currents elicited in α2β containing patches is clearly faster than inactivation in patches containing only α2 GlyR. The decay time constant of 64.2 ± 3.9 ms from our data is about one third of the time constant for "pure" α2 currents (Mangin et al. 2003). This faster inactivation corresponds with the shorter mean open time and indicates shorter mean burst duration after activation of α2β glycine receptors. In conclusion, our data so far support a faster activation and faster inactivation when the β subunit is present, although we could not find a shift in dose-response relationship. Once activated the α2β channels have a shorter mean open duration, which is consistent with the faster inactivation of the macroscopic currents.

The question at hand is now whether these apparent changes in kinetics are sufficient to enable the α2β glycine receptors to respond to synaptic glycine signalling, as opposed to the homomeric α2 receptors. Variance analysis indicates that there is indeed at least a 3-fold increase in open probability of α2β glycine receptors in experiments where synaptic transmission was mimicked. Where open probability was less than 10% with α2 homomeric channels (Mangin et al. 2003), our data indicate open probabilities in the range of 40 to 70%. This is however still less than the P_o described with α1β, the prevalent adult form of the glycine receptor, which was about 90% (Legendre 1998).

We did however notice from variance analysis and patches with few channels that the majority of the patches contained a mixture of α2 and α2β receptors in variable proportions. This makes analysis considerably more complicated. Since variance analysis gives a unitary current value, we can be sure the open probability described in our results is to be attributed to channels with a conductance of about 50 pS, which is the main conductance for α2β channels. Therefore we believe the increased P_o is likely to be a consequence of β subunit incorporation. In other cases the parabolic function could not be fitted because of a second variance component resulting from homomeric channels. The result of this complication is that the success rate of experiments, with analysis of pure α2β kinetics in mind, is rather low. Therefore we explored the possibility of using transient transfection of the β subunit in an α2 expressing CHO cell line. This transient transfection did result in functional α2β glycine receptors as shown by the induction of resistance to PXN. However, again there was a mixture of α2 and α2β present. Preliminary variance analysis experiments in this expression system again could not successfully be analysed due to the presence of α2. One of the possible future strategies is to increase the concentration of cDNA used, in order to increase the proportion of α2β receptors. Hopefully this will lead to a higher success rate. Nevertheless the results indicate that incorporation of the β subunit indeed speeds up kinetics of the α2-containing glycine receptor to such an extent that it is plausible to be involved in synaptic transmission. This would mean that the α2β glycine receptor could be responsible for glycinergic synaptic

transmission during development, possibly among other functions, but that its homomeric counterpart is likely involved in other processes. Our current data support this view, but do not allow to draw definite conclusions on the extent of the influence of the α2β subunit on kinetics because certainly the macroscopic currents are always a mixture of α2 and α2β in variable proportions. It is therefore possible that current data underestimate the effect of incorporation of the β subunit. Nevertheless, the β subunit seems to be important for the α2 glycine receptor to function in synaptic transmission. On the other hand, this mixture of α2 and α2/β might also be present *in vivo*.

Before synapse formation the influence of the β subunit on paracrine signalling might also be of importance for extrasynaptic physiological effects. It can be hypothesized that the steady increase of β expression, and the concomitant changes in response, could be of influence in slowly adapting the developing cell to other signalling modes. It is known that for instance GABA receptors also contain different subunits during development, which are accompanied by kinetic changes (Owens et al. 1999). It is still not clear what the purpose of this developmental regulation exactly is. Of course it should not be viewed as a phenomenon on its own. It could well be part of a carefully regulated process in a developing cell with concomitant changes in phosphorylation state and/or clustering, which are known to have an influence on receptor kinetics (Legendre et al. 2002; Lynch 2004). The developmental regulation of receptor kinetics could thus be of importance in understanding the influence neurotransmitters have during development of the nervous system. Our results indicate that the developmental regulation of β subunit expression has an influence on kinetics of the prenatally abundant α2 glycine receptor, which might be of importance for proper CNS development.

Chapter 5 | An introduction into oligodendroglial development

Oligodendrocytes are the glial cells responsible for myelination in the central nervous system (CNS). One oligodendrocyte can myelinate multiple axons, in contrast to the peripheral myelinating Schwann cell. The formation of the myelin sheath is crucial to allow saltatory conduction along axons and thus the fast signal transmission needed for efficient functioning of the CNS. The important role of the myelinating oligodendrocytes is demonstrated in demyelinating diseases, like Multiple Sclerosis. In MS, oligodendrocytes are damaged, which results in inefficient nerve conduction and the typical symptoms such as paralysis and problems of strength, vision, sensation and coordination.

5.1 The origin of the oligodendroglial cells

5.1.1 Exclusive ventral origin or not?

The developmental origin of oligodendrocytes (OLG) has been subject of intense debate. One view was that OLG in the developing CNS generate equally from all parts of the dorsal and ventral ventricular zones. The opposing theory was that the generation of OLG exclusively occurs in the ventral ventricular zone (Cameron-Curry and Le Douarin 1995; Pringle et al. 1998; Richardson et al. 2006). Recent findings, including *in vivo* experiments, have shown that both views were partly correct (Cai et al. 2005; Fogarty et al. 2005; Vallstedt et al. 2005). OLG arise indeed from both dorsal and ventral regions in spinal cord and brain stem, but the OLG from ventral origin normally suppress their dorsal counterparts (Calver et al. 1998; van Heyningen et al. 2001). In the cortex, the situation seems different for mammals than for birds. Experiments in chick-quail chimaeras show an exclusive ventral origin of OLG (Olivier et al. 2001), while in mice both ventrally and dorsally derived OLG have been described, depending on developmental stage (Kessaris et al. 2006).

The main source for OLG in the developing spinal cord thus remains the ventral ventricular zone and more in particular the motor neuron progenitor (pMN) domain, also the place of origin for motor neurons (figure 5.1) (Miller 2002; Richardson 2001; Richardson et al. 2006; Rowitch 2004). About 85% of oligodendrocyte precursors in the mouse spinal cord are derived from this region, with development starting at around embryonic day (E) 12.5. Oligodendrocyte precursor cells (OLP) migrate from this domain dorsally and laterally to populate the entire spinal cord.

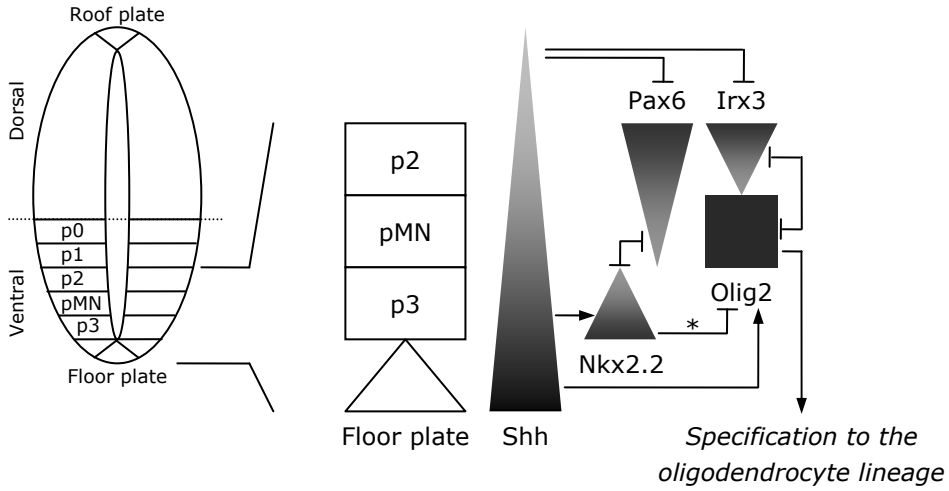

Figure 5.1 *Key players in the specification and localisation of oligodendrocyte precursors in the developing spinal cord.* Oligodendrocyte generation in the developing spinal cord occurs primarily in the ventral pMN domain of the spinal cord under the influence of Shh, which is at the basis of a complex interaction between transcription factors regulating time and localisation of the specification of neuroepithelial cells to the oligodendrocyte lineage. p0-3: place of origin of different types of interneurons, pMN: motor neuron progenitor domain, Shh: Sonic hedgehog, Pax6: paired box gene 6, Nkx2.2: NK2-transciption factor related, locus 2, Olig2: oligodendrocyte lineage gene 2, Irx3: Iroquois 3. Arrowhead lines indicate positive effects, T-shaped lines indicate inhibitory effects.

5.1.2 Oligodendrocyte generation in the ventral spinal cord

At the start of a hierarchy of protein-gene interactions that regulate the appearance of OLP is the Sonic hedgehog protein (Shh) (Ericson et al. 1997; Orentas et al. 1999; Pringle et al. 1996). Shh is first expressed by the notochord, thereby inducing floor plate formation, followed by a Shh-expression of the floor plate itself (Richardson 2001). The result is a Shh gradient in the ventral spinal cord which is the basis of formation of the five ventral progenitor domains, including the pMN domain. Important members of the transcription factor cascade influenced by Shh are oligodendrocyte lineage gene 2 (Olig2), NK2 transcription factor related (Nkx2.2), paired box gene 6 (Pax6) and Iroquois 3 (Irx3) (Briscoe et al. 2000; Fu et al. 2002; Novitch et al. 2001; Zhou et al. 2001). Olig2, probably indirectly induced by Shh through Nkx6 (Liu et al. 2003; Novitch et al. 2001), is crucial for the specification of OLP (and motor neurons) in the pMN domain. The fact that Olig2 expression is limited to the pMN domain is in turn the result from interactions with and between Nkx2.2, Pax6 and Irx3, which are involved in determining the boundaries of the pMN domain (Nicolay et al. 2007; Rowitch 2004). So where Olig2 is of vital importance for OLP specification, the area where embryonic cells are specified to OLPs is defined by these other transcription factors. This was proven by knockout experiments for

Nkx2.2 which resulted in a ventral expansion of the pMN domain (Lee et al. 2003; Qi et al. 2001). It is probable that a knockout of Irx3 would result in a dorsal expansion, given its inhibitory effect on Olig2 (Novitch et al. 2001), but this has not yet been experimentally confirmed. Pax6 turned out to have an inhibiting effect on Nkx2.2 and vice versa (figure 5.1) (Briscoe et al. 2000). As mentioned earlier, the pMN domain is the same domain that also generates motor neurons (Richardson 2001). These, however, arise earlier in development (around E9.5) than the OLP (Rowitch 2004). The neuron-glial switch is believed to be dependent on a down regulation of neurogenins (Ngn) 1 and 2, which in fact occurs shortly after motor neuron production and has experimentally been shown to be critical to allow OLP production. The suppression of Ngn1/2 expression is thought to be the result of Notch signalling (Rowitch 2004; Zhou et al. 2001).

5.1.3 Oligodendrocyte generation in the dorsal spinal cord

Although a lot of research has been done on transcription factors regulating OLP specification in the ventral spinal cord, not much is known about OLP generation in the dorsal spinal cord. In fact, the debated question whether OLP even arise dorsally has only recently been answered (Cai et al. 2005; Fogarty et al. 2005; Vallstedt et al. 2005). The answer is undoubtly yes, but the contribution of dorsally derived OLG (about 15%) in the spinal cord is markedly less than that of ventrally derived OLG (about 85%). The origin of the debate probably lies in the timing of the lineage specification. Ventral precursors appear at E12.5, which is earlier than their dorsal counterparts (E15), probably giving the ventral precursors the time to compete more efficiently for proliferation and survival signals (Richardson et al. 2006). The result is that dorsal OLP normally are suppressed by the ventral OLP. The signals controlling the OLG specification in the dorsal spinal cord are largely unknown. Dorsally derived OLP also express Olig2, but it is unlikely that this is under the influence of Shh at a distance that far from the floor plate and that close to the roof plate which expresses bone morphogenic protein (BMP), a Shh suppressant (Cai et al. 2005; Vallstedt et al. 2005). A candidate to take over the role of Shh in the dorsal spinal cord is fibroblast growth factor (FGF), but this issue is still under investigation (Abematsu et al. 2006; Chandran et al. 2003; Kessaris et al. 2004; Naruse et al. 2006).

5.1.4 Oligodendrocyte generation in the developing brain

The situation in the brain shows strong similarities with that in the spinal cord, but differs at some important points. Shh again plays a pivotal role, as do the Olig genes, although in some brain regions Olig1 is needed for OLG specification, which is not the case in the pMN region of spinal cord, where Olig2 is sufficient

to do the job (Lu et al. 2002; Zhou and Anderson 2002). As in the spinal cord, the first OLP arise ventrally, more exactly in the medial ganglionic eminence (MGE) from where they migrate into other parts of the developing forebrain. This results in the entire cortex being populated by ventrally derived oligodendroglial cells by E18 (Kessaris et al. 2006; Tekki-Kessaris et al. 2001). A second wave of OLP originates in the lateral and caudal ganglionic eminence (LGE/CGE). A striking difference with the spinal cord is however the disappearance of the ventral OLG in favor of precursors that have originated from the cortex itself after birth (Kessaris et al. 2006). This is at least the case in mammals, in birds the switch from the ventral to dorsal OLG does not occur and birds seem to cope with only OLG from ventral origin (Olivier et al. 2001). This species difference could be the result of the evolutionary requirement of a secondary OLP source accompanying the growth of the cortex in mammals. The possibilities that the dorsal source in spinal cord is merely a by-product of this evolutionary change or the source of OLG with an unknown specialised role do currently not exceed the status of hypotheses. It could be that the dorsal source is no evolutionary addition at all. Maybe both sources have always been there and the evolution possibly lies in the expansion of the ventral source under the demand for more OLG earlier in development. Another question one could ask is "Why are the MGE-derived oligodendrocytes eliminated in postnatal brain?". Indeed, why go through the trouble of populating the cortex with a cell lineage destined to disappear? Again a question to which the answers are purely speculative. It could be the result of a competition for growth factors or survival signals in favor of the local precursors. Another intriguing possibility is that the early OLG have an up till now unknown specific function. And last but not least, the disappearance of the ventrally derived OLG could be the result of some sort of turnover since the postnatal subventricular zone, birthplace of new OLG during life, is a descendant from LGE and cortex cells of the embryonic brain. The question whether OLG generated during life are to replace or to supplement the existing oligodendroglial cells still remains to be answered.

5.2 Maturation of the oligodendroglial cell lineage

5.2.1 Transcriptional control of maturation

The maturation of the oligodendrocyte lineage is also under control of different transcription factors. Olig1 and Olig2 are again key regulators in promoting OLG maturation, but the exact mechanisms involved are largely unknown (Fu et al. 2002; Lu et al. 2002; Xin et al. 2005). SRY-box containing gene 10 (Sox10) also plays a central role in the promotion of OLG maturation, since interfering with normal Sox10 expression results in severe decrease in myelin protein expression and failure of myelination (Dugas et al. 2006; Stolt et al. 2002).

Sox10 may work directly on myelin gene expression, but also induces Olig2 and Nkx2.2 expression (Liu et al. 2007). Nkx2.2, that has a limiting effect on early OLG specification, has a promoting effect on OLG maturation, likely by regulating expression of myelin protein genes (Qi et al. 2001). Next to these maturation-promoting factors, several inhibitory factors regulate the maturation process. Amongst them are BMP, having a negative influence on Olig expression through upregulating inhibitor of DNA binding (Id) (Samanta and Kessler 2004), and Sox5/6, interfering with Sox10 (Stolt et al. 2006). Some factors seem to have a dual role. Retinoic acid (RA) for instance has been shown to have an inhibiting effect on maturation, probably by enhancing polysialylated neural cell adhesion molecule (PSA-NCAM) (Laeng et al. 1994; Noll and Miller 1994), but has also been described to have a positive effect on OLG maturation (Barres et al. 1994; Pombo et al. 1999). Another example is the Notch signalling pathway, shown to inhibit OLG maturation through indirect inhibitory effects on Sox10 and myelin basic protein (MBP) expression (Wang et al. 1998), but also proven to promote OLG differentiation (Cui et al. 2004; Genoud et al. 2002; Hu et al. 2003). There are several explanations for these discrepancies in results, requiring further research to resolve them. The main issue seems to be differences in experimental protocols concerning different ages, CNS regions or transcription factor concentrations. Stage-specific different effects of transcription factors have been described in the past, e.g. Nkx2.2, and thus surely are worth further investigation. In the case of Notch signalling, there could be different effects depending on different Notch ligands, more specifically Jagged1/Delta1 versus F3/NB-3 (Nicolay et al. 2007). The mentioned transcription factors and their influences are summarised in figure 5.2. Let it be clear that a lot of research still needs to be done to untangle the complicated network of transcription factors regulating OLG specification and maturation. This introduction only scratches the surface of the known data in this field of research, going into more detail would be beyond the scope of this thesis. Current knowledge of

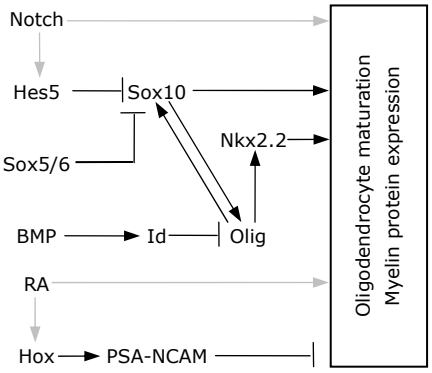

Figure 5.2 *Transcriptional control of OLG maturation.* Complex interactions between transcription factors influence the maturation of the oligendrocyte lineage. Key players are Sox10 and Olig. Hes: Mammalian homolog of *Hairy* and *Enhancer of split*, Sox: SRY-box containing gene, Nkx2: NK2 transcription factor related, locus 2, BMP: bone morphogenic protein, Id: inhibitor of DNA binding, Olig: oligodendrocyte lineage gene, RA: retinoic acid, Hox: homeobox, PSA-NCAM: polysialylated neural cell adhesion molecule. Arrowhead lines indicate positive effects, grey arrows indicate dual roles requiring further research, T-shaped lines indicate inhibitory effects.

transcriptional control of oligodendrogenesis has recently been reviewed by Nicolay *et al.* (2007).

5.2.2 Specification to the oligodendrocyte lineage

The result of these transcriptional controls is the differentiation of the oligodendrocyte lineage from their neuroepithelial origin to the myelinating oligodendrocyte. The maturation of oligodendrocytes is best characterized by studies of the developing spinal cord. The intermediate stages between neuroepithelial cells and precursors committed to the oligodendrocyte lineage are still subject of debate. In general, two hypotheses can be distinguished in this debate. The first is that oligodendrocytes derive from the same precursors as motoneurons, the second is the hypothesis that oligodendrocytes derive from glial restricted precursors (GRP) which also give rise to astrocytes (Liu and Rao 2003; Miller 2002). Moreover, the GRP-hypothesis and the motoneuron/oligodendrocyte-precursor-hypothesis are not mutually exclusive. It is possible that multiple ways lead to the same result. This is also seen in transcriptional control, with development of dorsally derived oligodendrocytes being Shh independent. The radial glia, a non-neuronal cell type in the developing CNS, important in supporting radial migration of neurons, have also been shown to have a precursor role. There seem to be distinct populations of radial glia having the potential to differentiate in either neurons or glia (Bonfanti and Peretto 2007; Hirano and Goldman 1988; Morest and Silver 2003; Pinto and Gotz 2007). Are these radial glia progeny from GRP or are they perhaps a separate lineage directly derived from neuroepithelium? That radial glia are not exactly the same cells as GRP is evidenced from the different marker expression in these cell types (Liu et al. 2002). An exact answer to the questions concerning the ancestors of oligodendrocytes thus cannot be given for the moment, but is an interesting issue with exciting prospects.

5.2.3 Maturation stages of the oligodendrocyte lineage

Once specified for the oligodendrocyte lineage, the image is clearer (Baumann and Pham-Dinh 2001; Richardson 2001). The migratory progenitor cell is a bipolar cell, which *in vitro* has the potential to give rise to oligodendrocytes, but also type-2 astrocytes, and for that reason is called the O2-A progenitor cell. The occurrence of type-2 astrocytes has not been proven in normal development, and it seems this cell type only develops *in vitro* or after transplantation of progenitor cells (Fulton et al. 1991). Main markers of the O2-A progenitor are the A2B5 antibody-staining (which stains several gangliosides), the proteoglycan NG2 and the platelet derived growth factor receptor α

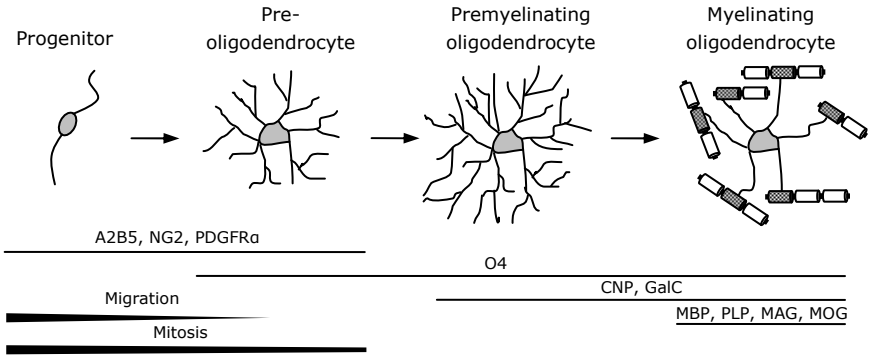

Figure 5.3 *Maturation of the oligodendrocyte lineage* Schematic overview of the main maturation stages of the developing oligodendrocyte. Progenitor cells are bipolar and gradually become more arborised. During myelination redundant processes retract. Each stage is characterised by expression of different markers. A2B5: ganglioside staining antibody, NG2: NG2 proteoglycan, PDGFRα: platelet derived growth factor receptor α, O4: sulfatide and glycolipid staining antibody, CNP: 2',3'-cyclic nucleotide-3'-phosphodiesterase, GalC: galactocerebroside, MBP: myelin basic protein, PLP: proteolipid protein, MAG: myelin associated glycoprotein, MOG: myelin oligodendrocyte glycoprotein

(PDGFRα). In the adult CNS, more branched NG2+/PDGFRα+ cells persist which are believed to have functions beyond being oligodendrocyte progenitor (Horner et al. 2002; Polito and Reynolds 2005). Some researchers see them as a fifth element of the CNS, next to neurons, astrocytes, oligodendrocytes and microglia, and distinguish them as a separate cell type called polydendrocytes, from which a subpopulation called synantocytes (from the Greek "synanto" meaning "contact") tightly interacts with synapses and nodes of Ranvier (Butt et al. 2005; Butt et al. 2002; Nishiyama 2007; Nishiyama et al. 2002). When O2-A progenitors develop further, they gradually become more arborised and can be marked with the O4 antibody (stains sulfatides and unidentified glycolipids). At this stage the cells still are mitotic but are less motile to even postmigratory. Where NG2+/O4- cells have a mitotic reaction to PDGF, O4+ cells no longer have this strong reaction and even tend to dedifferentiate in the presence of PDGF. Instead they show a mitogenic respons to FGF (Gard and Pfeiffer 1993). This O4+ stage is also called the pre-oligodendrocyte. When differentiating further into the lineage, cells lose their mitotic ability. These so-called pre-myelinating oligodendrocytes do no longer express the NG2 marker and start to express the first myelin protein 2',3'-cyclic nucleotide-3'-phosphodiesterase (CNP) and GalC, a galactocerebroside. The mature oligodendrocyte phenotype is characterised by sequential expression of other myelin proteins such as myelin basic protein (MBP), myelin associated glycoprotein (MAG), and proteolipid protein (PLP). The mRNA of DM-20, coding for an isoform of PLP, can be detected in the developing CNS at a very early stage of development. The actual myelin formation by the mature oligodendrocyte is correlated with the presence

of myelin oligodendrocyte glycoprotein (MOG). An overview of OLG maturation is provided in figure 5.3.

5.2.4 Control of oligodendrocyte number

During development, a lot more oligodendrocyte progenitors are generated than the demand for mature oligodendrocytes. Several processes serve to control oligodendrocyte cell number. The first one is mediated through paracrine survival factors such as FGF and PDGF. PDGF is a very strong survival signal for cells of the oligodendrocyte lineage, and competition for this factor, among others, is thought to be an important regulating basis for cell number (Barres et al. 1992; Calver et al. 1998). A second mechanism regulating differentiation concerns hormone signals such as thyroid hormone (triiodothyronine (T3)) which is required for timely cell cycle exit (Richardson 2001). A third mechanism involves contact with other cells or axons, and the surface molecules they express such as neuregulins (Colognato et al. 2002; Richardson 2001). Ultimately OLG that do not compete successfully for survival signals will be eliminated by apoptosis so there are not more myelinating oligodendrocytes than axons requiring this myelination.

5.3 Oligodendrocyte progenitors in the adult central nervous system

5.3.1 Progenitors in the subventricular zone

Oligodendrogenesis does not halt in the adult CNS. There are two main germinal zones within the adult brain, the subgranular zone and the subventricular zone (SVZ). The subgranular zone is located in the dentate gyrus (Ihrie and Alvarez-Buylla 2008; Melanson-Drapeau et al. 2003). Although glial progenitors are present in both germinal zones, most research has however focussed on the subventricular zone. The SVZ is the largest germinal zone in the adult CNS and lines the lateral ventricles. It stems from a layer of cells formed superficial to the embryonic ventricular zone. Glial progenitor cells can migrate out of the SVZ and give rise to astrocytes and oligodendrocytes (Levison et al. 1993; Levison and Goldman 1997; Luskin and McDermott 1994; Menn et al. 2006; Suzuki and Goldman 2003). The exact lineage progression of these cells is not yet completely understood. Gliogenesis from the SVZ seems to decrease with age, but progenitor cells of the SVZ keep their gliogenic potential (Aguirre and Gallo 2004; Cayre et al. 2006; Lachapelle et al. 2002). Moreover, demyelination stimulates migration and oligodendrogenesis by adult SVZ progenitors (Menn et al. 2006; Nait-Oumesmar et al. 1999; Picard-Riera et al. 2002).

5.3.2 NG2 expressing progenitors throughout the central nervous system

Apart from the SVZ progenitors, there is a pool of cycling cells in the adult CNS which have characteristics of oligodendrocyte progenitors. These cells are spread throughout the CNS. A hallmark of these cells is the expression of NG2, but expression of O4 and PDGF receptor α has also been described (Dawson et al. 2003; Wilson et al. 2006). Both O4 and PDGF receptor α are known to be expressed by immature oligodendrocytes. Despite the NG2 expression, they are not identical to the NG2+ cells that give rise to oligodendrocytes during embryonic development. The adult NG2+ have a more complex morphology than their embryonic counterparts, but can quickly revert to a simpler morphology after injury (Reynolds et al. 2002). The oligodendroglial precursor role of the adult NG2+ cell type has been well established now, but the question that remains is whether the function of the adult NG2+ cells is limited to oligodendrogenesis. The answer is likely to be "no". They have for example been described to also give rise to astrocytes and neurons (Belachew et al. 2003; Zhu et al. 2008), and to be involved in glutamatergic signalling (Paukert and Bergles 2006; Polito and Reynolds 2005). Several questions remain concerning this relatively new CNS cell type. Are the NG2+ cells a separate class of glial cells, the so-called "polydendrocytes", or "simply" multipotent precursors? Are the NG2+ cells throughout the CNS a homogenous cell population or not? What is their physiological role? Although some studies have started to address these questions, a lot of controversy remains and further research is needed (Butt et al. 2005; Chittajallu et al. 2004; Dawson et al. 2000; Greenwood and Butt 2003; Nishiyama 2007; Paukert and Bergles 2006; Polito and Reynolds 2005; Wigley et al. 2007).

5.3.3 Adult oligodendrocyte progenitors and remyelination

An important role of the adult oligodendrocyte progenitor pool is remyelination after CNS injury, for example in Multiple Sclerosis. Although complete remyelination does not occur in MS, "shadow plaques" with a certain degree of remyelination can be observed. Local oligodendrocyte progenitors, and not mature oligodendrocytes, are likely to be responsible for the repair response after demyelinaton (Blakemore and Keirstead 1999; Carroll et al. 1998; Gensert and Goldman 1997). The local oligodendrocyte progenitors that are activated in remyelination correspond to the NG2+ glial cells and their response can be stimulated by PDGF (Chang et al. 2000; Reynolds et al. 2002; Wilson et al. 2006; Woodruff et al. 2004). Once activated, the progenitors increase expression of the transcription factors Nkx2.2 and Olig2, which is important for the differentiation to myelinating oligodendrocytes (Fancy et al. 2004), analogous to the role of these transcription factors in embryonic development.

Despite the pool of NG2+ progenitor cells present and their capability to differentiate and provide a certain extent of remyelination, total repair is not seen in MS. The reason for the failure of remyelination is not clear. Clearly the presence of a progenitor pool is a prerequisite for repair. Moreover, the recruitment of progenitor cells seems to be a local response, with symmetrical cell division in which both daughter cells migrate and differentiate. These factors have led researchers to believe that repeated demyelination depletes the pool of NG2+ progenitors and ultimately leads to failure of remyelination (Blakemore and Keirstead 1999; Carroll et al. 1998; Franklin et al. 1997). However, other studies state that depletion of oligodendrocyte progenitors is not a limiting factor in the failure of remyelination (Chang et al. 2002; Chari and Blakemore 2002; Penderis et al. 2003). The hypothesis then is that failure of remyelination is not due to a shortage of progenitor cells, but a failure of these cells to fully develop into myelinating oligodendrocytes. Several reasons for this differentiation failure have been proposed. Firstly, cell-cell interaction with dysfunctional progenitors could inhibit differentiation (Chari et al. 2003). Secondly, axonal damage in MS could result in absence of necessary axonal signals or presence of inhibitory signals (Chang et al. 2002; Charles et al. 2002). Finally, signalling pathways inhibiting differentiation could be initiated (John et al. 2002). Acute inflammation on the other hand seems to promote remyelination (Foote and Blakemore 2005). The exact reasons for failure of remyelination seem to be complex and multifactorial. In short one can say that if progenitor depletion is not a limiting factor in remyelination, the environmental factors are of more importance and provide possible targets for therapeutic strategies to improve the remyelination process, warranting further research into the factors regulating oligodendrocyte progenitor differentiation.

5.4 Electrophysiological properties of oligodendrocytes

It was long thought that glial cells are quiet, non-excitable cells. Electrophysiological studies were mainly focussing on neurons and the mechanisms on neural signalling and neurotransmission. However, it has become clear that glial cells express a variety of voltage- and ligand-gated ion channels. Nevertheless, they remain non-excitable in the sense that they can not repeatedly fire action potentials. The physiological role of the ion channels in glia thus lies outside the classical fast neuronal signalling. There is strong emerging evidence that the electrophysiological properties of glial cells have an important influence on development of these cells. Therefore an overview will be provided of the electrophysiological properties of oligodendroglial cells and the changes herein during development.

5.4.1 Expression of voltage-gated ion channels during maturation

During maturation from the NG2+ oligodendrocyte precursor (OLP) to the mature oligodendrocyte, these glial cells display profound changes in expressed ion channels (table 5.1). OLP express voltage activated Na^+ currents *in vitro* (Barres et al. 1990; Sontheimer et al. 1989; Williamson et al. 1997) and *ex vivo* (Berger et al. 1992a; Chittajallu et al. 2004; Lin and Bergles 2002), but remain only capable of generating one action potential at the most (Barres et al. 1990; Lin and Bergles 2002; Williamson et al. 1997) due to the large K^+ permeability of their membranes. During maturation, oligodendrocytes lose the expression of Na^+ currents (Berger et al. 1995; Kettenmann et al. 1991; Sontheimer et al. 1989). The expression of voltage activated Ca^{2+} channels (VGCC) in OLP is somewhat controversial, with some studies being unable to measure Ca^{2+} current in cultured OLP (Barres et al. 1990; Sontheimer et al. 1989) while others report different types of Ca^{2+} currents in OLP in cell culture (Blankenfeld et al. 1992; Verkhratsky et al. 1990; Williamson et al. 1997) as well as in slices (Berger et al. 1992a). The developmental regulation of VGCC also is not clear. Blankenfeld *et al.* (1992) report a transient downregulation in the intermediate state between progenitor and mature oligodendrocyte in culture. Other studies confirm the presence of VGCC in mature oligodendrocytes using immunohistochemistry (Agrawal et al. 2000; Chen et al. 2000). Berger *et al.* (1992a) could however not detect any VGCC in mature oligodendrocytes *in situ*, as opposed to progenitors, a result which had also been reported in *in vitro* studies (Berger et al. 1995; Kettenmann et al. 1991; Sontheimer et al. 1989).
Membranes of cells of the oligodendrocyte lineage are however mainly permeable to potassium. Immature oligodendrocytes show delayed rectifying voltage activated K^+ currents, rapidly decaying A-type K^+ currents and Ca^{2+} sensitive potassium currents (Barres et al. 1990; Chittajallu et al. 2004; Knutson et al. 1997; Lin and Bergles 2002; Sontheimer et al. 1989; Williamson et al. 1997). Although inward rectifying K^+ currents have been described in the immature oligodendrocytes, they are small compared to the outward K^+ currents and difficult to isolate from these outward currents (Barres et al. 1990; Knutson et al. 1997; Lin and Bergles 2002; Williamson et al. 1997). This changes when the oligodendrocyte matures. The expression of delayed rectifier K^+ currents diminishes and cells display an inward rectifying potassium current profile (Attali et al. 1997; Barres et al. 1990; Gaillard and Bossu 1995; Hida et al. 1998; Sontheimer et al. 1989; Sontheimer and Waxman 1993). A correlation between potassium current profile on one hand and cell cycle progression and differentation on the other has also been described in other glial cell types such as astrocytes (MacFarlane and Sontheimer 2000a), Müller cells (Bringmann et al. 2000) and Schwann cells (Sobko et al. 1998).

Table 5.1 Voltage-gated ion channel expression during oligodendrocyte development

Channel type	*Developmental expression*	*Study models*	*References*
Na_v	Immat.	*in vitro*	(Barres et al. 1990; Sontheimer et al. 1989; Williamson et al. 1997)
		in situ	(Berger et al. 1992a; Chittajallu et al. 2004; Lin and Bergles 2002)
Ca_v	Immat.	*in vitro*	(Blankenfeld et al. 1992; Verkhratsky et al. 1990; Williamson et al. 1997)
		in situ	(Berger et al. 1992a; Lin and Bergles 2002)
	Mat.	*in vitro*	(Blankenfeld et al. 1992)
		in situ	(Agrawal et al. 2000; Chen et al. 2000)
	N.p.	*in vitro*	(Barres et al. 1990; Sontheimer et al. 1989)
K_v	Immat. > Mat.	*in vitro*	(Attali et al. 1997; Barres et al. 1990; Chittajallu et al. 2002; Gaillard and Bossu 1995; Knutson et al. 1997; Schmidt et al. 1999; Sontheimer et al. 1989; Tiwari-Woodruff et al. 2006; Williamson et al. 1997)
		in situ	(Chittajallu et al. 2004; Chittajallu et al. 2002; Lin and Bergles 2002; Tiwari-Woodruff et al. 2006)
Kir	Mat. > Immat.	*in vitro*	(Attali et al. 1997; Barres et al. 1990; Gaillard and Bossu 1995; Knutson et al. 1997; Sontheimer et al. 1989; Williamson et al. 1997)
		in situ	(Lin and Bergles 2002)

Na_v: voltage-gated Na^+ channel, Ca_v: voltage-gated Ca^{2+} channel, K_v: voltage-gated outward rectifying K^+ channel, Kir: inward rectifying K^+ channel, (Im)mat.: (im)mature, N.p.: not present

5.4.2 Potassium channels expressed in oligodendroglial cells

Especially in immature cells of the oligodendrocyte lineage an important part of the outward potassium current is mediated by the *shaker* family of potassium channels, of which several members (Kv1.1-1.6) have been described to be expressed on mRNA level as well as on protein level (Attali et al. 1997; Chittajallu et al. 2002; Schmidt et al. 1999). There is however not a consensus in these studies to the nature and functionality of the expressed *shaker* subunits. Atalli *et al.* (1997) report Kv1.2, 1.4, 1.5 and 1.6 transcripts and proteins *in vitro* but only find functional evidence for Kv1.5 and report downregulation of Kv1.5 and Kv1.6 during oligodendrocyte development. Schmidt *et al.* (1999) describe a heterogeneous expression *in vitro* in immature oligodendrocyte using single-cell RT-PCR with most of the cells expressing Kv1.2, 1.5 and 1.6 and rare expression of Kv1.1, 1.3 and 1.4. On the protein level, they report ubiquitous expression of Kv1.4, 1.5 and 1.6, rare expression of Kv1.1 and no expression of Kv1.2 and 1.3, which does not completely coincide with their mRNA data. From functional studies they conclude that K^+ currents in immature oligodendrocytes are mediated through Kv1.5 homomeric channels or heteromeric channels containing this subunit. Chittajallu *et al.* (2002) show presence of Kv1.3, 1.4, 1.5 and 1.6 subunits in cultured oligodendrocyte

progenitors, with a PDGF or bFGF induced upregulation of Kv1.3 and 1.5 and a downregulation of these subunits with maturation. Their electrophysiological experiments *in situ* show an important contribution of Kv1.3 in oligodendrocyte progenitor membrane currents. Kv1.5 could not be directly studied due to lack of specific antagonists.

Although this shows the shaker family to be important in cells of the oligodendrocyte lineage, its role is not exclusive, since also Kv3.1, member of the *shaw* family of voltage gated potassium channels, has been reported to have a significant role in oligodendrocytes. The same study also points out expression of Kv2.1, 3.2, 3.3 and 3.4 and confirms expression of Kv1.3 and Kv1.5 (Tiwari-Woodruff et al. 2006).

The other major potassium current component in oligodendrocytes, albeit mainly at more mature stages, is an inward rectifying current, which seems to be mainly mediated by Kir4.1, a Kir type that is almost exclusively expressed in glia in the CNS (Butt and Kalsi 2006; Kalsi et al. 2004; Neusch et al. 2001). Other Kir subtypes that have been established in oligodendroglial cells are Kir 2.1, 2.2 and 2.3 (Stonehouse et al. 1999), which possibly heteromerize with Kir4.1 (Butt and Kalsi 2006). Expression of the ATP-sensitive Kir6.2 has been reported (Zhou et al. 2002) although another study contradicts this observation (Dunn-Meynell et al. 1998). And to conclude there is indirect evidence that G-protein regulated Kir (probably Kir3.x) are present in mature oligodendrocytes (Karschin and Wischmeyer 1995; Karschin et al. 1994).

5.4.3 Expression of ligand-gated ion channels during maturation

Not only expression of voltage activated ion channels changes during oligodendrocyte development. There is increasing evidence for an important role of ligand-gated ion channels, more exactly neurotransmitter receptors, in the life and death of oligodendrocytes (Belachew and Gallo 2004; Belachew et al. 1998a; Butt 2006; Karadottir and Attwell 2007; Nguyen et al. 2001; Verkhratsky and Steinhauser 2000). There is a wide range of neurotransmitter receptors described in oligodendrocytes, such as receptors for glutamate (Glu), γ-amino-buteric acid (GABA), serotonine (5-HT), acetylcholine (ACh), glycine (Gly) and ATP (table 5.2). Not all are characterised to the same extent, and their function often remains obscure. Nevertheless, their functional presence on these "non-excitable" cells opens up a wealth of interesting possibilities outside of synaptic functioning, where neurotransmitters are widely known for. The oligodendroglial function of the neurotransmitter receptors will be overviewed below. The focus in this chapter lies on the expression of ionotropic neurotransmitter receptors and their developmental regulation.

The most investigated neurotransmitter receptors in glia are probably the glutamate receptors (GluR). It has long been thought that only the AMPA/KA GluR were expressed in oligodendrocytes. AMPA/KA receptors have been established in OLP in culture (Barres et al. 1990; Borges et al. 1994; Gallo et al. 1994a; Patneau et al. 1994) and in slices (Berger 1995; Berger et al. 1992b; Yuan et al. 1998). The regulation of these receptors during development is somewhat controversial. Most studies in culture describe a downregulation of AMPA/KA receptors in mature oligodendrocyte as compared to progenitors (Belachew et al. 1998a; Borges et al. 1994; Deng et al. 2003; Itoh et al. 2002; Rosenberg et al. 2003). Other studies in culture and in slices see no difference between maturation stages (Berger et al. 1992b) or an upregulation with maturation (Karadottir et al. 2005; Patneau et al. 1994). Although the presence of NMDA receptors in cells of the oligodendrocyte lineage was described by some studies late in the nineties (Wang et al. 1996; Ziak et al. 1998), the dogma that oligodendrocytes do not express NMDA receptors was upheld until recent work described NMDA receptors in oligodendrocytes at different maturation stages and linked them to ischemic damage processes (Karadottir et al. 2005; Micu et al. 2006; Salter and Fern 2005). The presence of NMDA receptors in oligodendrocytes of the white matter (Karadottir et al. 2005; Salter and Fern 2005) and optic nerve (Micu et al. 2006) suggests that, contrary to the previous dogma, NMDA receptors are in fact omnipresent in oligodendrocytes. Karadottir and Atwell (2007) speculate that there are several reasons NMDA receptors were overlooked until recently. Firstly, most studies were done in cultured cells, which might downregulate NMDA receptor expression because of culture conditions. The study of Berger *et al.* (1992b), which was in the more physiological setting of a brain slice, might have overlooked the receptor because they were studying corpus callosum slices. In corpus callosum, NMDA receptors desensitise more quickly and might already have been in a desensitised state during the experiments. Secondly NMDA receptors are mostly located in processes, making them easier to miss, compared to the AMPA/KA receptors which are located in the soma of the cells (Salter and Fern 2005). And finally, many studies used NMDA receptor blockers in their control solutions to avoid bias of NMDA receptor activation in the gray matter, given the dogma that NMDA receptors were not expressed in the oligodendroglial cells of interest.

Receptors for GABA are also present in the oligodendrocyte lineage. Several studies have described them in culture (Belachew et al. 1998a; Kettenmann et al. 1984; Von Blankenfeld et al. 1991; Williamson et al. 1998) as well as in slices (Berger et al. 1992b; Pastor et al. 1995; Steinhauser et al. 1994). There are two types of ionotropic GABA receptors, namely $GABA_A$ and $GABA_C$, but to date only $GABA_A$ is found in glial cells. During oligodendrocyte development, GABA receptors are downregulated, as has been establish *in vitro* as wel as *in*

Table 5.2 Ligand-gated ion channel expression during oligodendrocyte development

Channel type	*Developmental expression*	*Study models*	*References*
AMPA/KA GluR	Immat.	*in vitro*	(Barres et al. 1990; Gallo et al. 1994a)
		in situ	(Berger 1995; Chittajallu et al. 2004; Yuan et al. 1998)
	Immat. > Mat.	*in vitro*	(Attali et al. 1997; Belachew et al. 1998a; Borges et al. 1994; Deng et al. 2003; Itoh et al. 2002; Rosenberg et al. 2003; Ziak et al. 1998)
	Immat. = Mat.	*in situ*	(Berger et al. 1992b)
	Mat. > Immat.	*in vitro*	(Patneau et al. 1994)
		in situ	(Karadottir et al. 2005)
NMDA GluR	Immat.	*in situ*	(Salter and Fern 2005; Wang et al. 1996)
	Immat. > mat.	*in situ*	(Ziak et al. 1998)
	Immat. & mat.	*in situ*	(Karadottir et al. 2005)
	Mat.	*in situ*	(Micu et al. 2006)
	N.p.	*in vitro*	(Berger et al. 1992b; Gallo et al. 1994a)
$GABA_AR$	Immat. > Mat.	*in vitro*	(Belachew et al. 1998a; Von Blankenfeld et al. 1991; Williamson et al. 1998)
		in situ	(Berger et al. 1992b; Pastor et al. 1995; Steinhauser et al. 1994)
GlyR	Immat. > Mat.	*in vitro*	(Belachew et al. 1998a; Belachew et al. 1998b)
		in situ	(Kirchhoff et al. 1996; Pastor et al. 1995)
P2X	Mat.	*in situ*	(James and Butt 2001; James and Butt 2002; Kirischuk et al. 1995)
nAChR	Immat. > Mat.	*in vitro*	(Belachew et al. 1998a; Rogers et al. 2001)
	N.p.	*in situ*	(Karadottir et al. 2005)
5-HTR	Immat. = Mat.	*in vitro*	(Belachew et al. 1998a)
	N.p.	*in situ*	(Karadottir et al. 2005)

GluR: glutamate receptor, $GABA_AR$: GABA receptor, GlyR: glycine receptor, P2X: purinoreceptor, nAChR: nicotinic acetyl choline receptor, 5-HTR: serotonine receptor, (Im)mat.: (im)mature, N.p.: not present

situ (Belachew et al. 1998a; Berger et al. 1992b; Kettenmann et al. 1991; Pastor et al. 1995; Von Blankenfeld et al. 1991).

Glycine evokes a current in progenitors and mature oligodendrocytes. This is the case in oligosphere culture (Belachew et al. 1998a; Belachew et al. 1998b) as well as in spinal cord slices (Kirchhoff et al. 1996; Pastor et al. 1995). Belachew *et al*. (1998b) report that the GlyR activated in an oligosphere culture derived from cerebral cortex is pharmacologically distinct from the neuronal GlyR, based on an apparent second binding site for strychnine and sensitivity for picrotoxinin and cyanotriphenylborate despite expression of respectively β and α_2 subunits, which normally induce insensitivity for these antagonists. Like for GABA receptors, the GlyR evoked currents decline with oligodendrocyte maturation (Belachew et al. 1998a).

The expression of other neurotransmitter receptors has to date not been extensively described. A few studies describe ionotrophic purinoreceptors (P2X)

in oligodendrocytes, mainly $P2X_7$, but their metabotropic counterparts (P2Y) seem to play the dominant role in oligodendrocyte physiology (James and Butt 2001; James and Butt 2002; Kirischuk et al. 1995). Kastritsis and McCarthy (1993) suggest an upregulation of ATP signalling with differentiation, although they do not specify the underlying receptor type. Functional nicotinic acetylcholine receptors (nAChR) have been demonstrated in oligospheres, with a downregulation in oligodendrocytes with a mature phenotype (Belachew et al. 1998a) and in oligodendrocyte progenitors *in vitro*, although in that study immunoreactivity for nAChR was no longer detected when maturation was induced in the cells (Rogers et al. 2001). Káradóttir *et al.* (2005) could however not confirm the presence of functional nAChR in slices, regardless of maturation state. Their study also looked for 5-HT receptors, but again there was no elicited current in the slice preparations. Belachew *et al.* (1998a) did see a serotonine elicited current, which remained stable during differentiation. The contradictions between the studies of Belachew *et al.* and Káradóttir *et al.* probably arise from differences in the preparations studied. There is not only the obvious difference between oligospheres and *in situ* preparations, but the source tissue of the oligospheres, cerebral cortex, also differs from the studied slice preparations of Káradóttir *et al.*, being white matter from cerebellum and corpus callosum. Although both preparations were from rats, experimental procedures or differences in brain regions probably account for the different results. More studies regarding neurotransmitter receptor expression in oligodendrocytes, correlated with development, are necessary. This research area is at the beginning of exploration, which is also reflected in the outstanding questions regarding the role of this neurotransmitter expression. Nevertheless, several studies hint interesting roles in neuron-glia interaction and glial development, giving rise to several interesting hypotheses, which will be discussed later in this introduction.

5.5 Role of potassium channels in oligodendrocyte development

5.5.1 The shaker family of K^+ channels strongly influences developmental processes in the oligodendrocyte lineage

The fact that glial cells expressing the outward rectifier K^+ current are in an immature proliferating state is no coincidence, since the channels that mediate this current are involved in regulation of proliferation. This has been described in several glial cell types for the outward potassium current in general (Chiu and Wilson 1989; Fieber et al. 2003; MacFarlane and Sontheimer 1997; Pappas and Ritchie 1998; Sobko et al. 1998; Wilson and Chiu 1993) and for the delayed rectifier shaker potassium channels (Kv1.x) in particular (Allen et al. 1998;

Kotecha and Schlichter 1999; MacFarlane and Sontheimer 2000b; Pannasch et al. 2006).

The group of Gallo has extensively studied the role of potassium channels in oligodendrocyte proliferation (Gallo et al. 1996; Knutson et al. 1997) and shows a mitogenic role for Kv1.3 and 1.4, with Kv1.3 probably being the most important subtype for this effect. Another *shaker* subtype, Kv1.6, was found to have an anti-proliferative effect (Chittajallu et al. 2002; Vautier et al. 2004).

The influence of K^+ channels on oligodendrocyte proliferation is thought to be mediated by a regulation of cyclin dependent kinase (cdk) inhibitors, $p27^{Kip1}$ and $p21^{CIP1}$, which are upregulated when potassium channels are blocked (Ghiani et al. 1999b) and mainly affect the cyclinE-cdk2 complex (Belachew et al. 2002; Ghiani and Gallo 2001). CyclinE-cdk2 controls G_1/S phase transition (initiation of DNA duplication) in the cell cycle and works mainly through phosphorylation of pocket protein Rb (pRb), thereby relieving pRb inhibitory effects on E2F transcription factors (Moroy and Geisen 2004)(figure 2.7). Interesting to mention is that factors inhibiting proliferation do not automatically promote differentiation and differentiation inducing factors (PDGF withdrawal, T3 treatment) are not counteracted by an overexpression of cdk2-machinery. This indicates that in the oligodendrocyte lineage, proliferation and differentiation are uncoupled events to a certain extent (Belachew et al. 2002; Tikoo et al. 1998; Tokumoto et al. 2002; Vautier et al. 2004; Zezula et al. 2001). So far the signal transduction pathway between direct K^+ channel blockade and upregulation of cdk inhibitors remains to be elucidated but direct K^+ channel blockade can be induced by activation of glutamate receptors and concomitant elevation of intracellular Na^+, a mechanism which is Ca^{2+} independent (Gallo et al. 1996; Ghiani et al. 1999b; Yuan et al. 1998). Indirect modification of K^+ currents has also been implicated in regulation of OLP proliferation, for instance in long-term treatment with retinoic acid (Knutson et al. 1997), activation of metabotropic β-adrenergic receptors (Ghiani et al. 1999a) or downregulation of PDGF-receptor mediated tyrosine kinase activity (Chittajallu et al. 2005), which all coincide with downregulation of K^+ currents and attenuation of proliferation.

The above mentioned studies have special interest in *shaker* channels, but data linking K^+ channel expression and oligodendrocyte development are not limited to the *shaker* subtype. Tiwari-Woodruff *et al.* (2006) prove Kv3.1 of the *shaw* family to be involved in normal oligodendrocyte proliferation and migration with *Kv3.1-/-* knockout mice showing reduced myelination. The inward rectifying channels, mainly expressed in mature oligodendrocytes, also are implicated in oligodendrocyte development. Knockout of Kir4.1 in mice results in strongly reduced oligodendrocyte maturation accompanied by hypomyelination (Butt and Kalsi 2006; Neusch et al. 2001). This role of Kir in oligodendrocytes is in

addition to the longer known function in regulation of extracellular K^+ ($[K^+]_o$) and setting of the resting membrane potential (V_m) (Butt and Kalsi 2006).

5.5.2 Potassium channels are involved in remyelination

Recent studies have pointed out that the influence of K^+ channels on oligodendrocyte biology could be of importance for remyelination. Bacia *et al.* (2004) reported that administration of 4-aminopyridine (4-AP) in mice impaired remyelination. This study could however not exclude indirect effects, rather than direct K^+ channel blockade on oligodendroglial cells, due to the systemic administration of 4-AP (Bacia et al. 2004). More direct evidence comes from a study by Herrero-Herranz *et al.* (2007) which showed that Kv1.4 was re-expressed around EAE-lesions. This re-expression was linked to lesion repair processes. These findings make it interesting to investigate potassium channels as possible therapeutic targets to ameliorate remyelination in demyelinating diseases. However, the drawback is that immune cells also express potassium channels which are involved in their activation. For example Kv1.3 is a channel type that promotes proliferation in oligodendrocyte progenitors (Vautier et al. 2004), but also in immune cells (Beeton et al. 2001; Rus et al. 2005). Since MS is an auto-immune disease, this overlap is of concern. Kv1.4 is not expressed in immune cells, but to date no specific blockers or agonists of this channel type are known (Herrero-Herranz et al. 2007).

5.6 Role of neurotransmitters in oligodendrocyte development

The search for the functional role of neurotransmitters in the oligodendrocyte lineage is still only at the beginning. The emerging data indicate roles of neurotransmitters outside of their classical synaptic function and involve them especially in processes concerning proliferation and maturation of oligodendrocytes. The myelinating function of oligodendrocytes inherently demands a close interaction with neurons of the CNS. In fact, it is known that electrical activity from axons promotes oligodendrocyte proliferation, survival, differentiation and myelination (Barres et al. 1993; Barres and Raff 1993; Demerens et al. 1996; Stevens et al. 2002). The mediating substances for this interaction between neuronal axons and oligodendroglial cells are growth factors such as PDGF (Barres and Raff 1993), but there is increasing evidence neurotransmitters, especially glutamate, adenosine and ATP, are involved (Karadottir and Attwell 2007).

5.6.1 Glutamate signalling in oligodendrocyte development

The likely source for neurotransmitters acting on surrounding glial cells are neurons. In the case of glutamate, several mechanisms of glutamate release have been proposed. A first mechanism is the synaptic release of glutamate by the axons, given that it is now established that oligodendrocyte progenitors receive glutamatergic synaptic input (Bergles et al. 2000; Karadottir et al. 2005). A second mechanism is the reversal of sodium dependent glutamate transporters (EAAT). This is possible since in the developing nervous tissue, axonal firing can lead to a greater increase in $[K^+]_o$ than in mature nervous tissue, as has been shown for the optic nerve (Connors et al. 1982; Foster et al. 1982). A rise of intracellular Na^+ and the increase of extracellular K^+ make it possible for the EAAT to reverse (Fern and Moller 2000; Li et al. 1999). EAAT normally transports Na^+ inside and K^+ outside for every glutamate molecule that is transported to the intracellular environment (Tzingounis and Wadiche 2007). A reversal would thus result in glutamate release. Activation of the glutamate receptor subsequently can affect oligodendrocyte physiology. Ionotropic glutamate receptors are reported to stimulate oligodendrocyte progenitor migration in culture (Gudz et al. 2006; Wang et al. 1996), although Gudz *et al.* (2006) only found this effect for AMPA receptors, in contrast to Wang *et al.* (1996). On the other hand, AMPA/KA receptors block lineage progression and proliferation. This latter effect is mediated through a block of K^+ currents resulting from an increase in intracellular Na^+ concentration. The concomitant depolarisation leads to a Ca^{2+} independent proliferation block, which involves upregulation of cdk-inhibitors $p27^{Kip1}$ and $p21^{CIP1}$ (Gallo et al. 1996; Knutson et al. 1997; Yuan et al. 1998). The exact signal transduction cascade involved in this effect remains to be elucidated. Although the effect involving K^+ channel blockade is clearly Ca^{2+} independent, activation of glutamate receptors can induce a rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The AMPA receptors in oligodendrocytes express the GluR3 en GluR4 subunit proteins but lack the subunit GluR2 making them Ca^{2+} permeable (Holtzclaw et al. 1995; Li and Stys 2000; Puchalski et al. 1994). NMDA receptors are Ca^{2+} permeable, also making direct Ca^{2+} entry possible. Next to that there is of course the excitatory effect of GluR activation, which combined with the presence of VGCC, can trigger Ca^{2+} influx. The intracellular Ca^{2+} rise can in turn activate a number of proteins which regulate gene expression and oligodendrocyte physiology (Baron et al. 1998; Holtzclaw et al. 1995; Liu and Almazan 1995; Pende et al. 1994). The resultant effect is inhibiting for oligodendrocyte proliferation. It is therefore at first sight puzzling to see that mitogens as PDGF and FGF actually promote expression of AMPA/KA receptor subunits in oligodendrocyte precursors (Chew et al. 1997; Gallo et al. 1994b). However, taking into account that growth factors spread further than glutamate, it is conceivable that the growth factors stimulate

oligodendrocyte progenitors to proliferate and migrate but at the same time induce GluR expression so that proliferation is inhibited upon arrival at the target axons, where glutamate is present (Karadottir and Attwell 2007).

5.6.2 Adenosine and ATP signalling in oligodendrocyte development

The knowledge about glutamate is by far the most profound of all neurotransmitter signalling in oligodendrocyte development. A lot less is known about other transmitter molecules in this setting. Whereas glutamate is mostly involved in early oligodendrocyte development, adenosine and ATP mainly act at later stages of development and myelination. The main effect of ATP does not seem to arise from activation of ionotropic P2X receptors, but from activation of metabotropic P2Y receptors (James and Butt 2001; James and Butt 2002; Kirischuk et al. 1995). An indirect effect of ATP comes from stimulating leukaemia inhibitory factor (LIF) release by astrocytes, which promotes myelination (Ishibashi et al. 2006). Apart from this, ATP is of course also a source of adenosine when degraded by ATPases. Adenosine also induces a rise in $[Ca^{2+}]_i$ which inhibits oligodendrocyte proliferation, but stimulates migration, differentiation and myelination (Othman et al. 2003; Stevens et al. 2002).

5.6.3 "Inhibitory" neurotransmitters in oligodendrocyte development

The role of GABA in development is not clear, but it is known that activation of $GABA_A$ receptors causes a $[Ca^{2+}]_i$ increase in oligodendrocyte progenitors and mature oligodendrocytes (Bernstein et al. 1996; Kirchhoff and Kettenmann 1992; Schmidt et al. 2000). Like glutamate receptors, activation of GABA receptors has been reported to downregulate K^+ delayed rectifier currents in oligodendrocyte progenitors (Pastor et al. 1995), and thus it is tempting to imagine a similar effect on oligodendrocyte development. Indeed, GABA receptor agonists inhibit proliferation and lineage progression of oligodendrocytes (Yuan et al. 1998). GABA is known as an inhibitory neurotransmitter, but in oligodendrocytes it causes a depolarisation (Gilbert et al. 1984; Hoppe and Kettenmann 1989; Kettenmann et al. 1984; Kirchhoff and Kettenmann 1992). The outward Cl^- gradient responsible for this depolarisation is possible because of the actions caused by a $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and Cl^-/HCO_3^- exchanger in these cells (Verkhratsky and Steinhauser 2000; Wang et al. 2003). Given this outward Cl^- gradient in oligodendrocytes, the neurotransmitter glycine also causes depolarisation when activating Cl^- permeable GlyRs and Na^+-dependent glycine transporters. This depolarisation induces an influx of Ca^{2+} through VGCC (Belachew et al. 2000). The influence of this mechanism on oligodendrocyte development is yet to be determined.

5.6.4 Signal transduction pathways remain unclear

Other neurotransmitters shown to trigger depolarisation in oligodendrocytes are ACh and serotonine. nAChR trigger a depolarisation and are Ca^{2+} permeable, thus possibly influencing, again, $[Ca^{2+}]_i$ (Belachew et al. 1998a; Nguyen et al. 2001). Serotonine also has an excitatory effect and is implicated in stimulation of glial proliferation and myelination (Belachew et al. 1998a; Chubakov et al. 1986; Nguyen et al. 2001). The emerging data keep pointing at a pivotal role of intracellular Ca^{2+} signals. The downstream signalling involved is however largely unknown. Does the oligodendrocyte respond to all neurotransmitter-triggered Ca^{2+} signals in the same way or are there different cascades involved? If there are, how do the cells distinguish between the different neurotransmitters eliciting a Ca^{2+} signal? Is there a developmental regulation of downstream factors to aid in this purpose? A lot of questions remain unanswered, a lot of possibilities remain to be explored. Moreover, if one wants to study direct neurotransmitter effects *in situ*, he faces the challenge to avoid bias from indirect effects of these neurotransmitters on other cell types in these preparations.

5.6.5 Detrimental effects of neurotransmitters on oligodendrocytes

To make matters even more complicated, neurotransmitters are also named in detrimental effects, requiring careful regulation of neurotransmitter signalling to avoid damage. The presumed lack of NMDA receptors in oligodendrocytes gave these cells the reputation of being quite resistant to excitotoxic damage, but it is now known that not only the AMPA receptors in oligodendrocytes are Ca^{2+} permeable, but they also express the Ca^{2+} permeable NMDA receptors, greatly increasing their sensitivity to ischemic damage (Dewar et al. 2003; Karadottir et al. 2005; Micu et al. 2006; Salter and Fern 2005). The small intracellular volume in myelin sheets, in combination with the preferential expression of NMDA receptors in processes additionally makes these cells prone to damage occurring through osmotic phenomena after ion influx. Detrimental effects mediated through glutamate receptors are not only mentioned in ischemic insults, but also in diseases such as periventricular leukomalacia (Volpe 2001) and multiple sclerosis (Matute et al. 2001). And again glutamate is not alone in its possible damaging effects. GABA also has a potential damaging effect to oligodendrocyte processes (Sidhu et al. 1997).

The roles of neurotransmitters in the oligodendrocyte lineage appear very broad. Research in this field however is rather limited. If we are to understand the regulation of oligodendrocyte development and survival, it is imperative to expand our knowledge on the functions of neurotransmitters. This is a challenge for "glial" research, but nevertheless an interesting one.

5.7 Cell lines as a model to study oligodendroglial biology

5.7.1 Commonly used oligodendroglial cell lines

To study cell biology, researchers often turn to cell lines. These are continuously proliferating cells that can easily be kept in culture for long periods. Cell lines are often derived from cancer cells, but can also be the result of spontaneous transformation *in vitro*. In the study of oligodendroglial cells, there are a couple of commonly used cell lines we will briefly overview here.

The human oligodendroglioma cell line (HOG) was originally reported to express oligodendrocyte markers MBP and CNPase, but not astrocyte markers GFAP and glutamine synthetase (Post and Dawson 1992). This was later confirmed by Buntinx *et al.* (2003) who also reported possible differentiation induction with increased expression of MBP and MOG mRNA, process outgrowth and a decrease in proliferation.

The MO3.13 cell line is an immortalized human-human hybrid cell line, generated from the fusion of rhabdomyosarcoma cells with adult human oligodendrocytes (McLaurin et al. 1995). This cell line expresses oligodendrocyte markers MBP and PLP, but also GFAP. Induction of differentiation by serum deprivation reduced the GFAP expression and increased MBP and PLP expression (Buntinx et al. 2003; McLaurin et al. 1995). Buntinx *et al.* (2003) could not confirm GFAP expression by MO3.13, but reported MOG expression which was enhanced by differentiation induction. When cultured in differentiation inducing medium, MO3.13 showed profound process formation and decreased proliferation (Buntinx et al. 2003).

The OLN-93 cell line was first described by Richter-Landsberg & Heinrich (1996). This cell line is derived from spontaneously transformed cells in a primary rat brain glial culture. Immunocytochemical characterization showed the OLN-93 cells to be A2B5 negative, and positive for several myelin components such as GalC, PLP, MBP and MAG. When cultured in 0.5% FCS, no increase in MBP staining was found. Furthermore, the OLN-93 cell line is negative for the astrocyte markers vimentin and GFAP. When the cells were grown in DMEM with 0.5% FCS on PLL coated surfaces, they show reduced proliferation and a more arborized morphology, which resembles differentiation. We will present functional data, based upon electrophysiological measurements, which will aid answering the question of the maturation stage of OLN-93 oligodendroglial cells.

5.7.2 Pro's and contra's in the use of cell lines

Cell lines are widely used in research, but caution is to be taken. The hallmark and big advantage of cell lines, namely their immortalized state and continuous

proliferation, is at the same time their biggest disadvantage. The continuous proliferation provides the researcher with means to obtain large amounts of a homogenuous cell population. This is not always the case with primary cell cultures which can contain different cell types or different differentiation stages and cannot always be easily grown *in vitro*. On the other hand this proliferative behaviour of cell lines implies genomic changes that might influence aspects of cell biology which are the subject of the research. The key word here is "characterization". The better a cell line is characterized, the more useful it gets to compare data obtained from the cell line with data from primary cell culture or other models. In this way cell lines can provide an easy and low-cost tool to obtain proof of concept and to pave the way for other experiments in more complicated models. In this way, cell lines can be a valuable addition to the researcher's tool, alongside primary cultures, *ex vivo* and *in vivo* models.
Therefore this part of the thesis is devoted to characterizing the OLN-93 cell line, which has been introduced in our lab as a model to study oligodendroglial processes such as membrane dynamics and differentiation.

Chapter 6 | Glycine receptors and transporters in the OLN-93 oligodendroglial cell line

Abstract

Neurotransmitters can act as regulators of development for oligodendrocytes. This has already been demonstrated for glutamate. The glycine receptor mainly expressed during development, which is an α2 homopentamere, has kinetic properties that do not allow an efficient functioning in synaptic signalling. It is therefore interesting to speculate about an extrasynaptic role for glycine or other agonists of the glycine receptor. We investigated the expression of glycine receptors and transporters in an oligodendroglial model, the OLN-93 cell line. We furthermore compared functional neurotransmitter receptor expression by this cell line with that in primary immature oligodendrocytes.

Despite the presence of glycine receptor mRNA, we could not establish functional glycine receptor expression in the OLN-93 cell line. Glycine application did induce a sustained current, probably arising from activation of the glial glycine transporter 1. The absence of clear receptor-mediated responses of the OLN-93 cell line to glycine could be expanded to other neurotransmitters. Although the OLN-93 cell line shares its unresponsiveness to glycine with the primary cells, there were remarkable differences in the response of primary immature oligodendrocytes to other neurotransmitters. OLN-93 did not react to glycine, glutamate, GABA, ATP or 5-HT. The primary cells showed receptor activation by glutamate and GABA application in physiological conditions.

The observed differences between OLN-93 and primary cells question the usability of OLN-93 as an (immature) oligodendrocyte model and necessitate further characterization of this model if it is to be used in future studies.

6.1 Introduction

Recently neurotransmitters have come into sight as possible modulators of oligodendrocyte development. Indeed, cells of the oligodendrocyte lineage express several neurotransmitter receptors, which moreover seem to undergo developmental regulation. The expression of neurotransmitters in oligodendrocytes and their possible role in development are discussed in the introduction (chapter 5).

In the relatively young field of neurotransmitter signalling on glial cells, and oligodendrocytes in particular, glutamate signalling has been particularly studied. This interest in glutamate is probably sparked by its long established role in excitotoxic mechanisms in neurons, which could equally play an important role in glial pathologies. The knowledge concerning effects of other neurotransmitters is rather limited, although many receptors have been described in oligodendroglial cells (see chapter 5 for an overview).

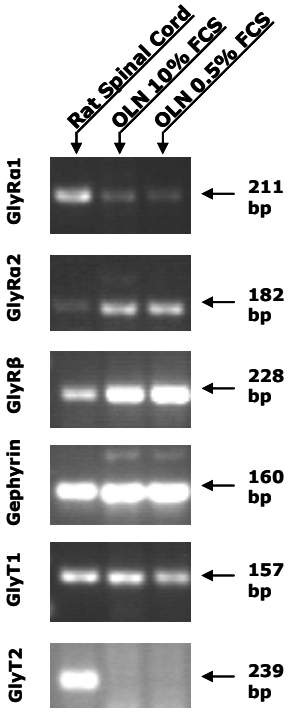

Figure 6.1 *mRNA of glycine-related proteins in OLN-93 cells.* OLN-93 cells express mRNA transcripts for glycine receptor subunits α1, α2 and β but not for α3 (data not shown). mRNA for the scaffolding protein gephyrin was detected. The message for GlyT1, but not GlyT2, was present. Positive controls are extracts from rat spinal cord. *Experiments conducted in cooperation with S. SahebAli*

Given previous results indicating a possible extrasynaptic role for glycine during development (Mangin et al. 2003 and chapter 4), we decided to study expression of glycine receptors and transporters in an oligodendrocyte model, the OLN-93 cell line. The ultimate goal is to investigate the influence of α2 glycine receptors and their agonists on developmental processes in oligodendrocytes. Glycine receptors have previously been shown to be developmentally regulated in cortical and spinal cord oligodendrocytes, decreasing with maturation (Belachew et al. 1998a; Kettenmann et al. 1991). Activation of glycine receptors has a depolarizing effect in oligodendrocytes, due to the activity of the NKCC cotransporter and the Cl^-/HCO_3^- exchanger in these cells. Moreover, glycine transporter activity is Na^+ dependent and can also induce depolarisation. This can result in an increase of intracellular Ca^{2+} (Belachew et al. 2000).

Given this possible signal transduction pathway triggered by glycine or other GlyR agonists, it is

then intriguing to hypothesize roles for these receptors in regulation of proliferation and development of oligodendrocytes. It has already been established that the other main ligand activated Cl^- channels, GABA receptors, can downregulate K^+ delayed rectifier currents (Pastor et al. 1995), possibly inhibiting proliferation and lineage progression in oligodendrocytes (Vautier et al. 2004).
We investigated expression and functionality of glycine receptors and glycine transporters in the OLN-93 cell line as a model for immature oligodendrocytes, and compared functional neurotransmitter receptor expression in OLN-93 with that of primary rat oligodendrocyte progenitors. In OLN-93 cells a functional GlyR expression could not be found, although GlyR mRNA was present. A comparison with neurotransmitter responses in primary immature oligodendrocytes evidenced a profound difference in response between these two models. We therefore felt it was necessary to further characterize the OLN-93 model in light of future studies, which will be discussed in chapter 7.

6.2 Results

6.2.1 Expression of glycine receptors and transporters in the OLN-93 cell line.

Expression of the glycine receptor subunits α1, α2, α3 and β was checked by RT-PCR on the OLN-93 cells. mRNA for α1, α2 and β was present, α3 expression could not be observed. Concerning glycine transporters, the message for GlyT1 was picked up, but was absent for GlyT2. Gephyrin, a protein linking GlyR to the cytoskeleton, was also expressed on the mRNA level. Positive controls used were extracts from rat spinal cord. RT-PCR results are summarized in figure 6.1. To investigate whether the mRNA message was translated to protein expression, indirect immunocytochemical fluorescence staining was conducted. OLN-93 cells stained positive for GlyR, gephyrin and GlyT1, but were negative for GlyT2. The expression pattern of these proteins did not change with different serum concentrations in OLN-93 culture (fig. 6.2), so electrophysiological experiments were only conducted in OLN-93 cultured in 10% FCS.

6.2.2 OLN-93 express functional glycine transporters, but not glycine receptors

After establishing protein expression of glycine receptors and transporters, functionality of these glycine activated proteins was investigated using whole-cell patch-clamp. Application of 10 mM glycine using a fast solution exchange system resulted in a sustained inward current of -0.6 ± 0.1 pA/pF (n = 10) at a holding potential of -60 mV (fig. 6.3A,B). This inward current had a small but

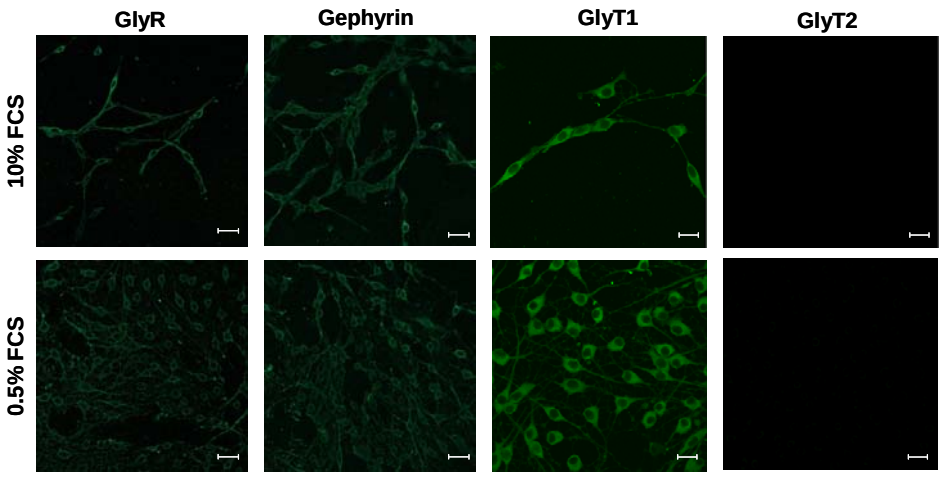

Figure 6.2 *Expression of glycine receptor and transporter proteins in OLN-93 cells.* OLN-93 cells are immunopositive for glycine receptor subunits, the gephyrin scaffolding protein and glycine transporter 1 but do not express glycine transporter 2 proteins. Bars represent 40 µm in GlyR and gephyrin pictures and 20 µm in GlyT pictures.

significant depolarising effect of 3.0 ± 0.5 mV (t-test, $p < 0.05$) (fig. 6.3C). When applying 10 µM strychnine, currents were not significantly inhibited (t-test, $p > 0.05$) and were 91 ± 7% of control (n = 3) (fig. 6.3A,D). Substituting extracellular NaCl by choline chloride significantly reduced the glycine evoked current to 19 ± 2 % (n = 4) of control (t-test, $p < 0.05$) (fig. 6.3B,D). The glycine evoked current could not be elicited at positive holding potentials and did not reverse (data not shown).

6.2.3 OLN-93 show limited response to neurotransmitters, compared to primary rat oligodendrocyte progenitors

10 mM glycine was also applied to primary rat oligodendrocyte progenitors, but did not evoke any current in five tested cells. Primary cells did however react to fast application of other neurotransmitters. 1 mM concentrations of acetylcholine, glutamate, GABA, serotonine and Na_2ATP were applied to the cells. None of the primary oligodendrocyte progenitors reacted to 1 mM acetylcholine. 1 mM glutamate induced a fast inactivating respons of -16 ± 2 pA/pF (n = 13) at a holding potential of -60 mV (fig. 6.4Aa). Glutamate elicited peak currents which could be enhanced by co-application of 1 µM glycine (data not shown). This glutamate induced current evoked a peak depolarizing shift in the membrane potential of 43 ± 7 mV (n = 4), followed by a sustained slightly depolarized potential (Δ V = 6 ± 3 mV (n = 4)) for the duration of the glutamate application (fig. 6.4Ab). Out of 9 tested primary oligodendrocyte progenitors, 6 reacted to 1 mM GABA at holding potential -60 mV with an inactivating inward current of -16 ± 7 pA/pF. This elicited a peak depolarisation of 24 ± 12 mV (n =

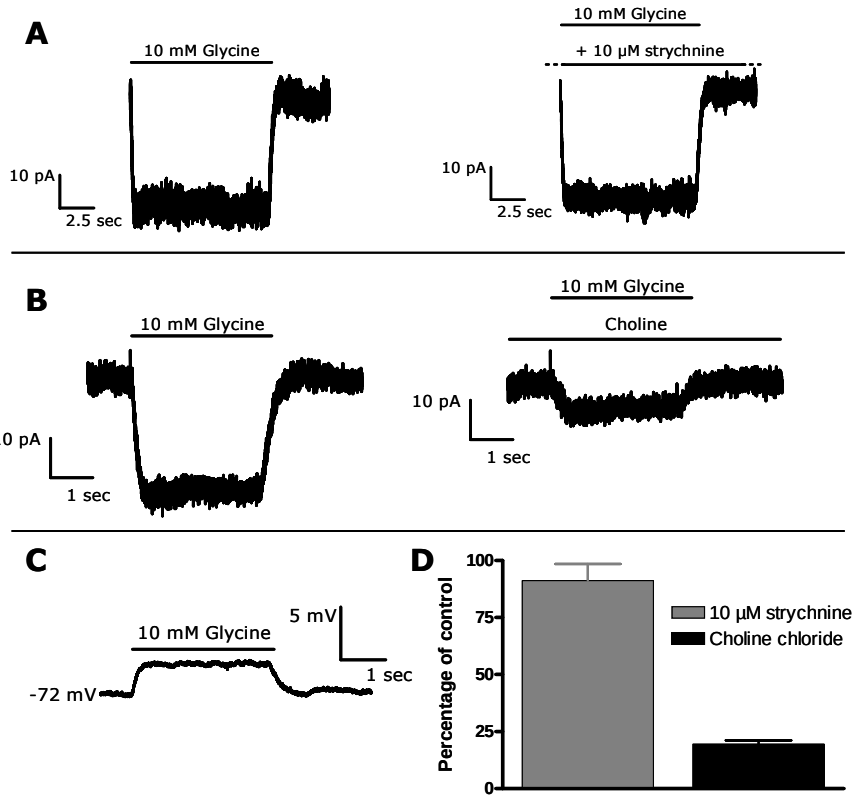

Figure 6.3 *Characterisation of glycine-evoked current in OLN-93.* **A.** Representative example of a sustained inward current in OLN-93 cells evoked by 10 mM glycine, which is insensitive to 10 µM strychnine. **B.** The glycine-evoked current is blocked by substituting extracellular NaCl by choline chloride. **C.** Application of 10 mM glycine evokes a small depolarisation. **D.** 10 µM strychnine does not significantly block control currents, but choline chloride significantly reduces current to 19 ± 2 % of control.

4) amplitude (fig. 6.4B). Cells that did not react to GABA at -60 mV also showed no response at other holding potentials. At a holding potential of -60 mV, 1 mM serotonine evoked a small non-inactivating current of -0.6 ± 0.1 pA/pF in 4 out of 10 tested cells. Of these non-responding cells, 5 were also clamped at lower holding potentials. A holding potential of -120 mV did elicit a current in 2 originally non responsive cells, but this current was outward. Two 5-HT responsive cells at -60 mV that were tested at -120 mV also showed outward currents at that holding potential, giving an average response of 2.0 ± 0.5 pA/pF in 4 out of 6 cells tested at -120 mV (fig. 6.4Ca). In current clamp, 5-HT caused a small depolarisation in 2 of 4 cells (fig. 6.4Cb). 1 mM Na_2ATP evoked a sustained inward current of -1.4 ± 0.6 pA/pF at holding potential -60 mV in 4 of 9 cells. Clamping the membrane at -120 mV evoked potentiated the Na_2ATP-evoked inward current to -5.0 ± 1.0 pA/pF in 6 of 8 tested cells (fig. 6.4Da), of

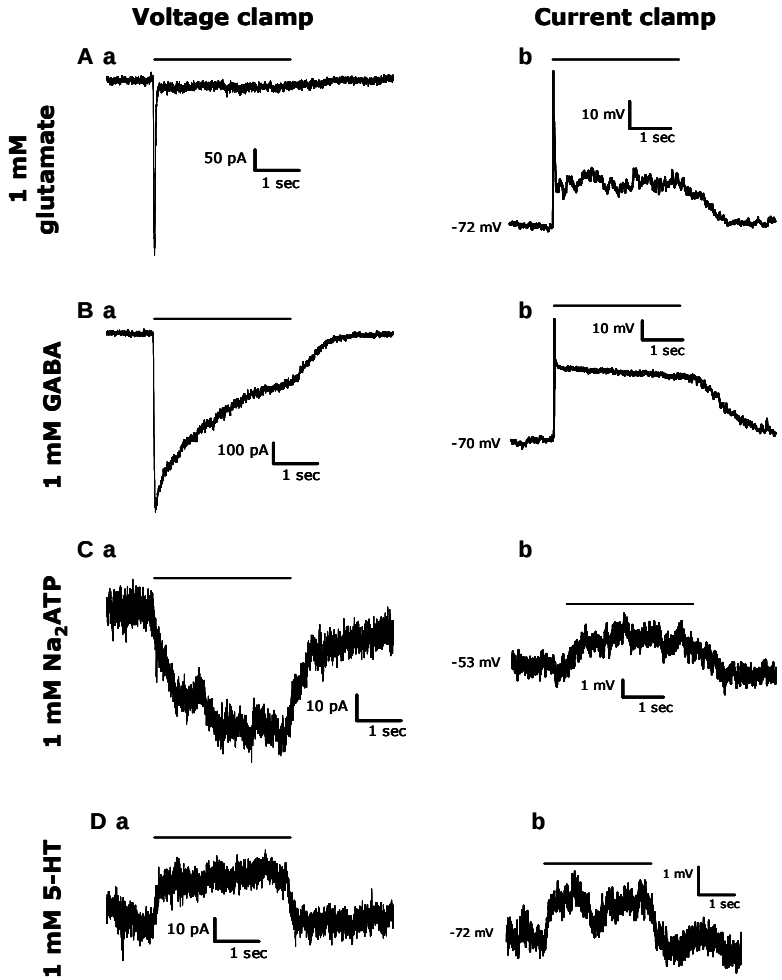

Figure 6.4 *Neurotransmitter responses in primary rat oligodendrocyte progenitors* Examples of responses to 1 mM glutamate (A), GABA (B), Na_2ATP (C) and serotonine (D) in voltage clamp (a) and current clamp (b). Holding potential in Aa and Ba was -60 mV, in Ca and Da it was -120 mV.

which 2 cells did not previously show a current at -60 mV. Applying 1 mM Na_2ATP while clamping the cell to 0 pA evokes a depolarizing shift in the membrane potential of -10 ± 6 mV (n = 3) (fig. 6.4Db). These responses to 1 mM glutamate, GABA, 5-HT and Na_2ATP in primary oligodendrocyte progenitors are in sharp contrast to the absence of responses to these neurotransmitters in OLN-93 cells (fig. 6.5). The only effect was seen in 4 out of 11 tested OLN-93 cells which showed a small outward current in response to 1 mM Na_2ATP of 0.4 ± 0.1 pA/pF at -60 mV holding potential.

6.3 Discussion

In light of investigating the influence of glycine on oligodendroglial development, we investigated whether the OLN-93 cell line expressed the necessary machinery to respond to glycine signals. OLN-93 was immunopositive for glycine receptors, but the used antibody cannot distinguish between different subunits. RT-PCR hinted the possible subunit composition of these proteins. mRNA of α1, α2 and β could be detected, indicating the possible presence of homomeric and heteromeric glycine receptors. The presence of the gephyrin anchorage molecule on the mRNA as well as on the protein level is additionally supportive for a possible functional presence of glycine receptors. It is then somewhat surprising that it proved to be impossible to activate glycine receptors in the whole-cell patch-clamp configuration using a fast solution exchange system. The speed of this application system allows detection of glycine receptor activity as is proven by experiments on CHO cells described in chapters 3 and 4 of this work. The conclusion that is on hand here is that although glycine receptor subunits are present in OLN-93 cells, they do not form functional receptors. The absence of functional glycine receptors in cultured glial cells, in contrast to cells in situ, was mentioned earlier by Kirchhoff *et al.* (1996). The reason for not forming functional GlyR, with the necessary components at hand, is not clear. For instance, it is possible that the trafficking of the GlyR to the cell membrane is defective in OLN-93. In light of this, it is noteworthy that immunocytochemical staining for GlyR seemed to be primarily intracellular. Defective trafficking could possibly result from an alteration of the gephyrin molecule, which is involved in GlyR trafficking (Kneussel 2005; Kneussel and Loebrich 2007; Maas et al. 2006). Another possibility is a defective multimerisation of glycine receptor subunits. However, this all

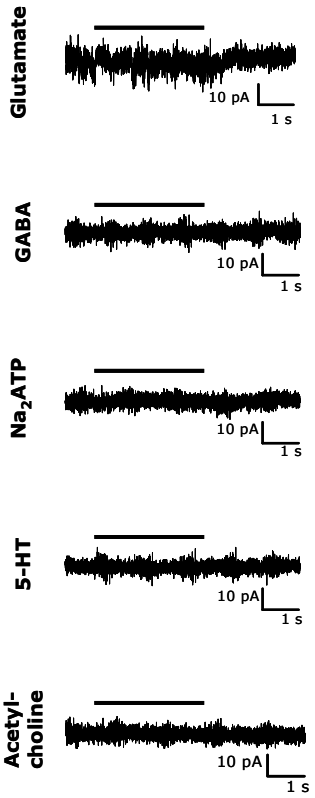

Figure 6.5 *Absence of response to neurotransmitters in OLN-93 cells.* Traces show that OLN-93 did not react to application of various neurotransmitters. Holding potential in these traces was -80 mV. Bars indicate application of 1 mM neurotransmitter.

remains very speculative, but it is clear that glycine receptors are not functional in OLN-93 cells.

Glycine did evoke a current in OLN-93, but this was a non-receptor like sustained current. Strychnine could not block the inward current, ruling out the possibility that it was mediated by glycine receptors. RT-PCR and immunocytochemistry indicated the presence of GlyT1, not GlyT2. This is what could be expected, since GlyT1 is known as the glial glycine transporter. It is thus tentative to speculate that the glycine-evoked current was mediated by the influx of Na^+ as a result of GlyT1 activity, which cotransports 2 Na^+ and 1 Cl^- with a glycine molecule (Roux and Supplisson 2000). The fact that glycine activated current evoked a small depolarization is supportive for an influx of Na^+. Moreover, substituting extracellular NaCl by choline chloride strongly inhibited the glycine evoked inward current, further supporting the hypothesis of Na^+ influx through glycine transporter activity. A final argument in favor of GlyT activity is the observation that current did not reverse, what normally is the case in currents mediated by ion channels.

The unresponsiveness of OLN-93 to glycine could be expanded to glutamate, acetylcholine, GABA, Na_2ATP and 5-HT. This is in sharp contrast with earlier findings on neurotransmitter receptor expression in oligodendrocytes (see chapter 5 for overview) and our own findings on primary rat oligodendrocyte progenitors. Primary rat oligodendrocyte progenitors showed responses to GABA, 5-HT, glutamate and Na_2ATP. Primary cells were unresponsive to acetylcholine and glycine. The sustained glycine evoked current observed in OLN-93 was also not present in primary cells. The inactivating responses to GABA and glutamate correspond to receptor activation by these neurotransmitters. Currents in response to 5-HT and Na_2ATP were sustained currents, unlike what is expected from receptor activation (see for example (Belachew et al. 1998a)). The exact nature of the molecules responding to the application of 5-HT and Na_2ATP remains unclear.

In conclusion, we can state that the OLN-93 cell line expresses functional GlyT1, but no functional glycine receptors. They also express no functional glutamate or GABA receptors, which is in contrast to the primary rat oligodendrocyte progenitors. Therefore the question arises whether the OLN-93 is a suitable model for (immature) oligodendrocytes in the study of the effect of neurotransmitters in oligodendrocyte physiology. To provide an answer to this question we conducted a biochemical and functional characterization of this cell line, which will be described in the next chapter.

Chapter 7 | Characterisation of maturation stage of OLN-93 cells by means of electrophysiological properties.

Part of the results presented in this chapter are reported in Buckinx R, Smolders I, SahebAli S, Janssen D, Smets I, Ameloot M and Rigo JM "Morphological changes do not reflect biochemical and functional differentiation in OLN-93 oligodendroglial cells." *Submitted*

Abstract

OLN-93 cells, a cell line established from spontaneously transformed rat brain glial cultures, are used as a model for oligodendrocytes. These cells are known to undergo morphological changes upon serum deprivation. The objective of the present study is to investigate a possible correlation between these morphological changes and (1) the loss or gain of oligodendrocyte markers and (2) the electrophysiological properties of these cells.
Using RT-PCR and immunocytochemistry, we demonstrate that the OLN-93 cell line expresses a broad range of markers (NG2, CNP, MAG, MOG) both when cultured in medium containing 10% or 0.5% fetal calf serum. Whole-cell patch-clamp recordings demonstrate that, regardless of the culture conditions, OLN-93 cells mainly express delayed-rectifying K^+ currents, a characteristic of immature oligodendrocytes. These findings indicate that the morphological changes of OLN-93 cells when cultured in low serum conditions, do not translate biochemically in a loss or gain of differentiation markers nor functionally in a change in their electrophysiological profile. Based on our data, we conclude that the OLN-93 cell line can be situated in the range of a late pre-oligodendrocyte to late immature oligodendrocyte, regardless of serum concentration.

7.1 Introduction

Our previous results concerning expression of glycine receptors and other neurotransmitters in OLN-93 showed discrepancies with the expression of these proteins in primary oligodendrocyte progenitors. OLN-93 cells is a cell line established from spontaneously transformed rat brain glial cultures (Richter-Landsberg and Heinrich 1996). It has been described as an immature cell line, capable of a certain degree of differentiation, based on biochemical and morphological criteria (Richter-Landsberg and Heinrich 1996; van Meeteren et al. 2005). However, to correctly interpret results from future experiments in this model, we felt it was necessary to conduct a more rigorous characterization of the maturation stage of OLN-93. For this purpose we investigated morphological, biochemical and functional oligodendrocyte differentiation criteria in OLN-93 when these cells were subjected to serum deprivation, which has been described to induce differentiation in oligodendrocyte cultures (McCarthy and de Vellis 1980).

When grown in DMEM supplemented with 10% FCS a high proliferation rate with a doubling time of 16-18 hours was determined. These cells are known to undergo morphological changes upon serum deprivation (Richter-Landsberg and Heinrich 1996). These morphological changes resemble the transition from bipolar progenitors to multi-processed immature oligodendrocytes also seen in primary cultures of oligodendrocytes (Baumann and Pham-Dinh 2001).

Immunocytochemical characterization showed the OLN-93 cells to be A2B5 negative, and positive for several myelin components such as GalC, PLP, MBP and MAG. When cultured in 0.5% FCS, no increase in MBP staining was found. Furthermore, the OLN-93 cell line is negative for the astrocyte markers vimentin and GFAP. Based on these data, Richter-Landsberg & Heinrich (1996) report this cell line to have passed the O-2A progenitor stage and have entered an oligodendrocyte-restricted lineage, without losing proliferation capacity. A study by van Meeteren *et al.* (2005) proposes that the OLN-93 cell line goes through early differentiation in serum-deprived culture and that IGF-I treatment can induce further maturation.

The data presented in this chapter intend to verify whether the morphological changes seen in OLN-93 really reflect biochemical and functional differentiation in this oligodendrocyte model. For this purpose, the loss or gain of oligodendrocyte differentiation markers and the electrophysiological properties of OLN-93 are investigated in high and low serum conditions.

In oligodendrocytes, the profile of potassium currents is correlated with differentiation stage (see chapter 5), a correlation we used to investigate functional maturation of OLN-93. The main K^+ channel family in oligodendrocyte progenitors, which is downregulated during oligodendrocyte maturation, is the

shaker subfamily of voltage-gated K^+ channels (Kv1). The subunits of Kv channels have six transmembrane domains. Besides Kv1 there are seven other subfamilies in the group of Kv channels. Functional channels are composed of four subunits. A lot of functional K^+ channels are in fact heteromultimeres of different subunits from the same subfamily. Add to this the occurrence of splice variants of the subunits and auxiliary subunits like Kvβ, which influence channel kinetics, and it is clear that there is a large functional diversity among potassium channels (Coetzee et al. 1999).
In this work, we investigated biochemical and functional expression of the Kv1 family in OLN-93 and correlated it with possible differentiation induction by serum deprivation. The results give a clear indication of the maturation state of OLN-93, facilitating the correct use of this cell line as an oligodendrocyte model.

7.2 Results

7.2.1 OLN-93 cells undergo morphological changes upon serum deprivation

Upon changing from medium containing 10% FCS to 0.5% FCS, OLN-93 cells alter their morphology to a more complex appearance, similar to some differentiation stages observed for oligodendrocytes in primary cultures. While cells left in 10% FCS medium mainly keep a simple bipolar morphology with a more ovoid cell body (fig. 7.1Aa), the cell body of cells cultured in 0.5% FCS flattens and the cells acquire multiple processes with fine branches (fig. 7.1Ab).

7.2.2 OLN-93 cells express oligodendrocyte lineage markers

By means of RT-PCR, the OLN-93 cells were checked for the expression of mRNAs of markers characteristic for the known developmental phases of oligodendrocytes. Results presented in fig. 7.1B indicate that mRNAs for NG2, CNP, MBP and PLP/DM20 are expressed by the OLN-93 cell line. Two amplification products are seen for MAG in rat spinal cord (positive control), corresponding to large MAG (L-MAG) and small MAG (S-MAG). Only S-MAG protein mRNA was seen in OLN-93 cells. The message for MOG could also be picked up and the cells did express the mRNA for glial fibrillary acid protein (GFAP), an astrocytic marker. No difference in the mRNA expression profile was observed between cells cultured in 0.5% FCS and cells maintained in 10% FCS.
The translation of the relevant mRNA was investigated by using immunocytochemistry. As illustrated in fig. 7.1C, NG2, CNP and MAG were expressed by OLN-93 cells irrespective of the serum content of the medium. MOG or GFAP immunostainings were never detected (fig. 7.1Cd to 7.1Ce') although primary cultures of rat astrocytes could be labeled with the same anti-

GFAP antibody and MOG-eGFP transfected OLN-93 cells with the anti-MOG one (data not shown).

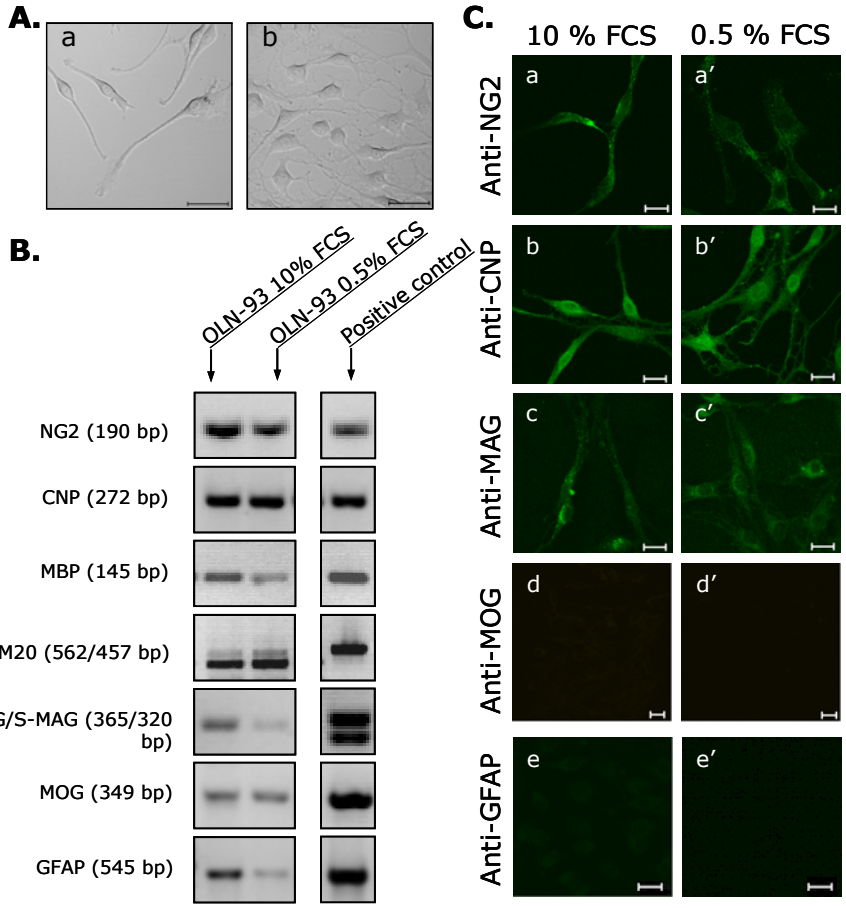

Figure 7.1 *Morphology and marker expression in OLN-93 cells.* **A.** Morphological appearance when grown in medium containing 10% FCS (a), and after switching the medium to 0.5% FCS (b). Bars represent 50 µm. **B.** Non quantitative RT-PCR analysis of total RNA extracted from OLN-93 cells grown under high serum conditions (left lane) and OLN-93 cells grown in low serum medium (middle lane). Positive controls are spinal cord/brain or a plasmid (in case of MBP, PLP/DM20) (right lane). **C.** Using indirect immunofluorescence staining, cells were labeled using antibodies directed against NG2 (a and a'), CNP (b and b') and MAG (c and c'). Anti-MOG and anti-GFAP staining are shown in d and d' and e and e', respectively. a-e are OLN-93 cells grown in 10% FCS conditions whereas a'-e' represent cells grown in 0.5% FCS. Bars represent 20 µm. *Experiments performed by Smolders I.*

7.2.3 Voltage-gated currents in OLN-93 cells are mainly potassium currents

Under our recording conditions, cultured OLN-93 cells had a mean membrane potential of -79 ± 1 mV (n=26) when cultured in 10% FCS and of -76 ± 1 mV (n=15) in 0.5% FCS. All patch-clamp experiments were performed in the whole-

cell configuration. The mean membrane capacitance (C_m) was 35 ± 1 pF (n = 36) in 10% FCS and of 43 ± 3 pF (n = 33) in 0.5% FCS (fig. 7.2A). These values are significantly different (t-test, $p < 0.05$). Input resistances were 67 ± 3 MΩ (n = 36) in 10% FCS and 80 ± 4 MΩ (n = 33) in 0.5% FCS, which is also significantly different (t-test, $p < 0.05$) (fig. 7.2B). When voltage-clamped at a holding potential of -60 mV, OLN-93 cells displayed outward rectifying currents in response to voltage steps ranging from -120 mV to +40 mV (by 20 mV intervals), both in 10% and 0.5% FCS (fig. 7.2C). Tail currents (fig. 7.2D), induced by clamping the cell at +20 mV for 20 ms before applying 20 mV voltage steps from -120 mV to +20 mV reversed at -59 ± 2 mV (n = 24) in 10% FCS and at -64 ± 1 mV (n = 19) in 0.5% FCS. These values are not significantly different (t-test, $p > 0.05$).

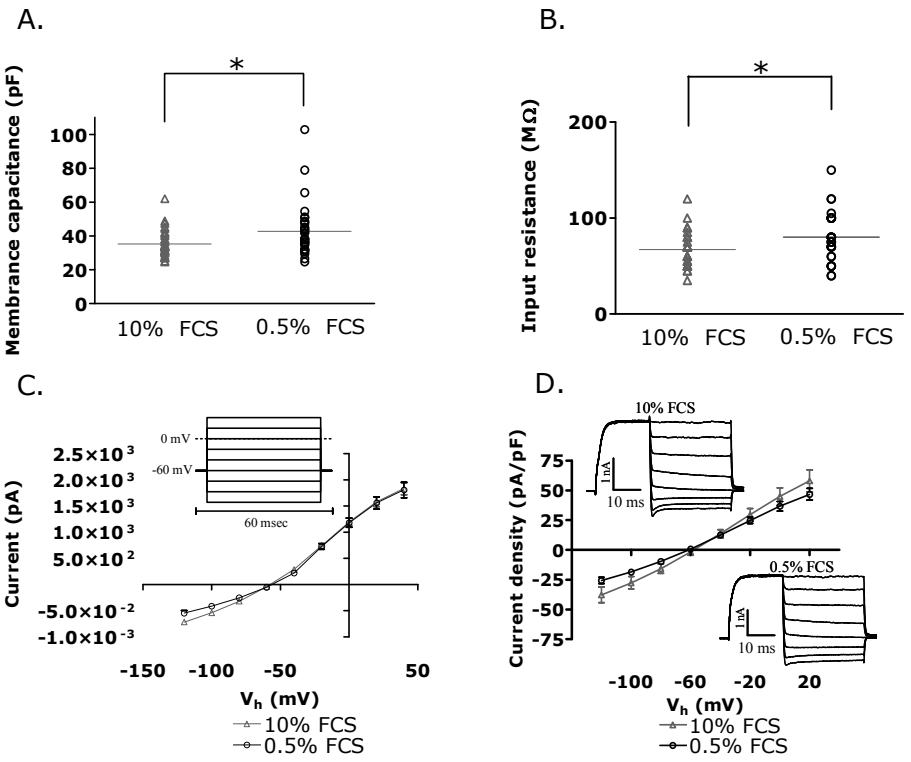

Figure 7.2 *Electrophysiological properties of OLN-93 cells.* **A.** Whole-cell membrane capacitance of OLN-93 in 10% (open triangles, n = 36) and 0.5% FCS (open circles, n = 33) conditions. **B.** Input resistance of OLN-93 cells in 10% (open triangles, n = 36) and 0.5% FCS (open circles, n = 33) conditions. **C.** Current-voltage relationship of absolute voltage-activated whole-cell currents in OLN-93 in 10% (open triangles, n = 25) or 0.5% (open circles, n = 20) FCS, recorded from a holding potential (V_h) of -60 mV. Inset shows the different voltage steps used in this protocol. **D.** Tail currents (induced by clamping the cell at +20 mV for 20 ms before applying 20 mV voltage steps from -120 mV to +20 mV) of OLN-93 cells in 10% FCS (open triangles, n = 24) and 0.5% FCS (open circles; n = 19). Insets are representative examples of tail currents expressed in OLN-93.

To further characterize the outward rectifying currents, 25 mM of TEA, a non-specific potassium channel blocker, was applied (fig. 7.3A and 7.3B). TEA-sensitive voltage-activated currents were calculated as the difference between currents in control conditions and currents recorded in the presence of 25 mM TEA. As shown in fig. 7.3C, these TEA-sensitive currents activate from -40 mV and display a clear outward rectifying profile, regardless of serum concentration. TEA inhibition of voltage-gated currents is smaller in OLN-93 cultured in 10% FCS than in 0.5% FCS, with TEA-sensitive current being respectively 45 ± 4 % (n = 13) and 63 ± 5 % (n = 10) of control current at +40 mV (I_{max}) (t-test, $p < 0.05$) (fig. 7.3C). The activation curve of TEA-sensitive currents (fig. 7.3D) shows no significant change in half-maximal activation (V_{50}) between 10% FCS and 0.5% FCS with values of -30 ± 8 mV (n = 13) and -30 ± 5 mV (n = 10), respectively (F-test, $p > 0.1$).

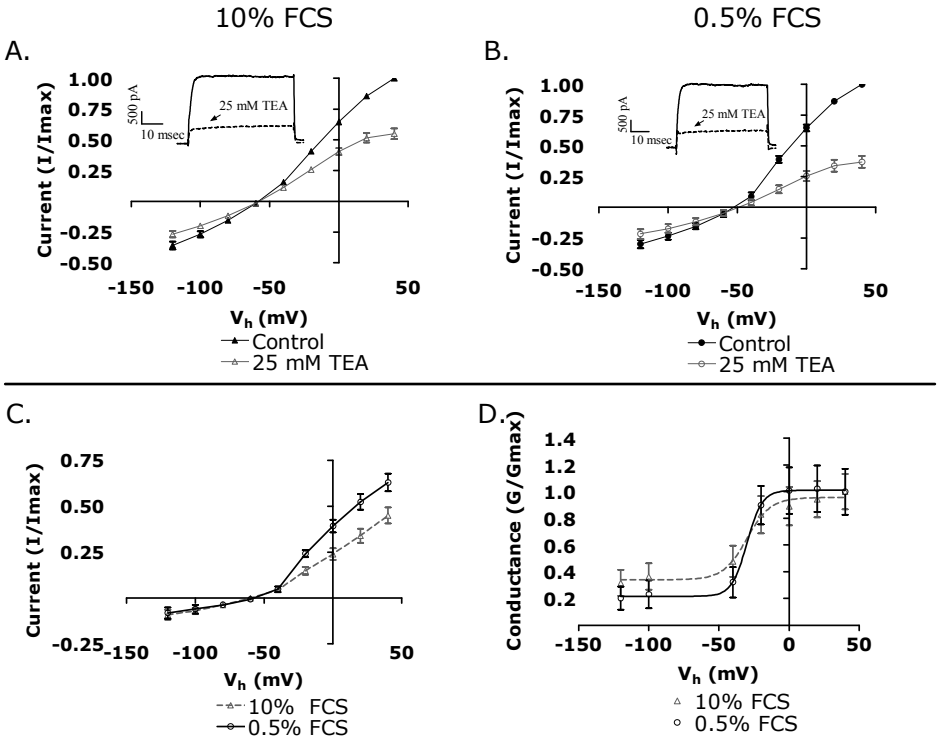

Figure 7.3 *TEA-sensitive currents.* **A, B.** Effect of 25 mM TEA on voltage-activated currents in 10% (A) and 0.5% (B) FCS. Cells were clamped at different membrane potentials (V_h) starting from a holding potential of -60 mV. Insets show examples of current activated at +40 mV and the effect of TEA. **C.** TEA-sensitive currents, relative to control currents at +40 mV (I_{max}), were expressed in function of the step potential as in A and B. . **D.** Normalised conductances of TEA-sensitive currents were calculated for cell using Ohm's law ($I = G.(V_h-V_{rev})$; V_{rev} being obtained form the tail current protocol shown in fig 7.2D). A Boltzmann fit yields V_{50} = -30 ± 8 mV (n = 13) in high serum conditions and V_{50} = -30 ± 5 mV (n = 10) in low serum conditions.

7.2.4 OLN-93 express potassium channels of the shaker family

Since the shaker family of potassium channels has been described to play an important role in proliferation and differentiation processes of oligodendroglial cells (Vautier et al. 2004 & chapter 5), we investigated their expression in OLN-93 cells. Using RT-PCR, mRNAs for Kv1.1 and Kv1.3 were found both in 10% and in 0.5% FCS (fig. 7.4A). No expression of mRNAs for Kv1.2, 1.4, 1.5 and 1.6 in OLN-93 could be observed (data not shown). Quality of our primers was confirmed by product amplification in the positive controls, which were from rat brain and spinal cord. The expression of Kv1.1 and Kv1.3 was confirmed at the protein level by means of fluorescent immunolabeling of OLN-93 cells using anti-Kv1.1 and anti-Kv1.3 antibodies (fig. 7.4B).

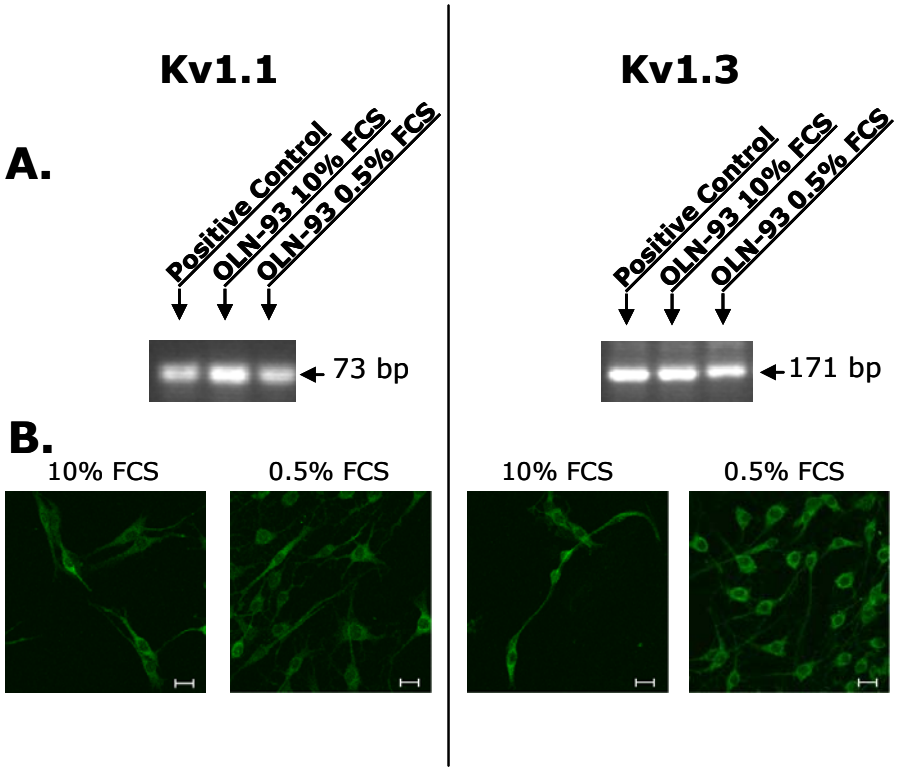

Figure 7.4 *Kv1 expression.* **A.** Reverse transcriptase PCR showed mRNAs for Kv1.1 and Kv1.3 (left lane: positive control as in Fig 6.1B, middle lane: OLN-93 cells cultured in 10% FCS, right lane: OLN-93 cells cultured in 0.5% FCS). **B.** Immunocytochemical staining with primary anti-Kv1.1 and anti-Kv1.3 and secondary anti-rabbit alexa647. Bars represent 20 µM.

Whole-cell patch-clamp experiments were then performed to evaluate whether the expressed Kv channels were functional. To date no specific blocker of Kv1.1 channels is known. Margatoxin (MgTx), a specific Kv1.3 blocker, was applied to OLN-93 cells at a concentration of 10 nM when recording voltage-gated currents in the whole-cell patch-clamp configuration. As shown in fig. 7.5A and B, MgTx

inhibited voltage-gated currents by 31 ± 5 % in 10% FCS and by 30 ± 3 % in 0.5% FCS at +40 mV. MgTx-sensitive currents activate from -40 mV and were not statistically different in 0.5% FCS compared to 10% FCS (2 way ANOVA, $p>0.1$; fig. 7.5C). V_{50} values obtained from conductance-voltage relationships (fig. 7.5D) were -33 ± 10 mV (n = 10) in 10% FCS and -28 ± 11 mV (n = 12) in 0.5% FCS, which is not significantly different (F-test, $p > 0.1$).

7.2.5 Primary oligodendrocyte progenitor cells show more pronounced outward currents

To correctly position the OLN-93 cell line in oligodendrocyte proliferation, we compared voltage elicited currents of OLN-93 in 10% FCS with a primary oligodendrocyte culture from rat, cultured in an undifferentiated state (see materials and methods). C_m of primary cells was 12 ± 1 pF (n = 30), significantly lower than 35 ± 1 pF (n = 36) in OLN-93 cultured in 10%

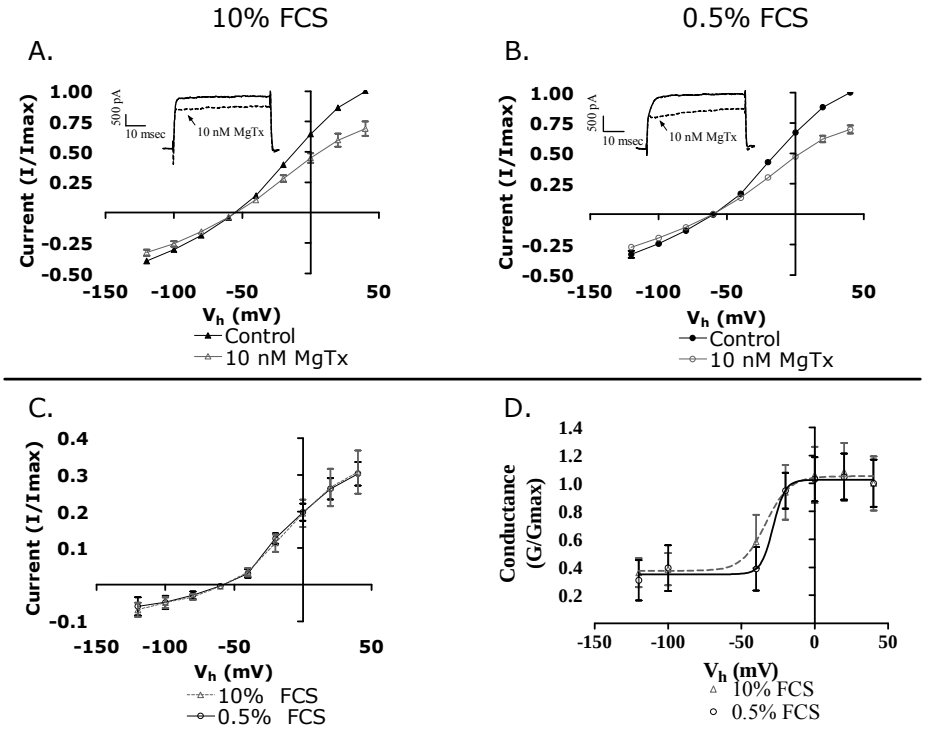

Figure 7.5 *MgTx-sensitive currents.* **A, B.** Effect of 10 nM MgTx on voltage-activated currents in 10% (A) and 0.5% (B) FCS. Cells were clamped at different membrane potentials (V_h) starting from a holding potential of -60 mV. Insets show examples of current activated at +40 mV and the effect of MgTx. **C.** MgTx-sensitive *shaker* currents, relative to control currents at +40 mV (Imax), were expressed in function of the step potential as in A and B. **D.** Normalised conductances of MgTx-sensitive currents were calculated as in fig 7.3D. A Boltzmann fit yields V_{50} = - 33 ± 8 mV (n = 12) in 10% FCS and V_{50} = 28 ± 11 mV (n = 10) in 0.5% FCS.

FCS (t-test, $p < 0.0001$). Input resistance was 391 ± 36 MΩ, which is significantly higher than that of OLN-93 cells (t-test, $p < 0.0001$). Resting membrane potential (RMP) was -64 ± 3 mV (n = 15), significantly higher than in OLN-93 (t-test, $p < 0.0001$), but primary oligodendrocyte progenitors show a higher heterogeneity in RMP (fig. 7.6B).

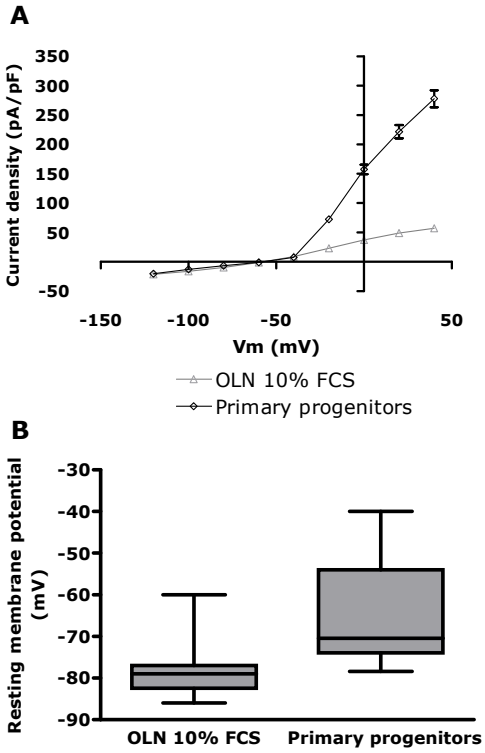

Figure 7.6 *Electrophysiological properties of OLN-93 compared with primary oligodendrocyte progenitors.* **A.** Primary rat oligodendrocyte progenitors show a more pronounced outward current, but inward currents do not differ. **B.** Mean resting membrane potential in OLN-93 (-79 ± 1 mV (n = 26)) is more negative than in primary progenitors (-64 ± 3 mV (n = 15)), but RMP in primary progenitors shows a larger distribution.

To study voltage-activated currents the same voltage step protocol as in figure 7.2C was used. As shown in figure 7.6A, the density of outward currents, activated from -40 mV is much larger in primary progenitors compared to OLN-93. The reversal potential of the voltage-activated currents, measured by tail current protocols as described earlier, is -81 ± 1 mV (n=28) in primary oligodendrocyte progenitors, which is significantly more negative than the -59 ± 2 mV (n=24) in OLN-93 in 10% FCS (t-test, $p < 0.0001$).

Injection of 250 or 500 pA current induces a single action potential spike in primary oligodendrocyte progenitors. This was however never seen in OLN-93 cells (fig. 7.7). This corresponds with the presence of Na^+-like inward currents in primary oligodendrocyte progenitors, which were not seen in OLN-93 cells (fig. 7.8).

7.3 Discussion

The biochemical and electrophysiological properties of the OLN-93 oligodendroglial cell line were examined in culture conditions with different serum concentrations. Low (0.5%) serum induces differentiation-like morphological changes. Based upon morphological changes, serum deprivated OLN-93 have earlier been used as a model for differentiated oligodendrocytes

(Ernst et al. 2004). Results indicate that the morphological changes do not translate biochemically into a loss or gain of differentiation markers nor functionally into a change in their electrophysiological profile.

7.3.1 Serum-deprived OLN-93 cells undergo differentiation-like morphological changes without altering the expression of oligodendroglial proteins.

When OLN-93 cells are cultured in medium containing 10% FCS, the cells morphologically resemble bipolar O2A progenitor cells and express NG2 mRNA, an early developmental marker. The cells bear the mRNA for other oligodendrocyte proteins like CNP, MBP, PLP/DM20, MAG and MOG. The OLN-93 cell line was also shown to be immunoreactive for NG2, CNP and MAG. The protein expression of CNP and MAG was already described earlier (Richter-Landsberg and Heinrich 1996). Although it was stated that the early developmental marker A2B5 is absent (Richter-Landsberg and Heinrich 1996), the cells do express NG2, typical for early stages of development. The cells do not express MOG or the astrocytic marker GFAP, although mRNAs for these proteins and also for PLP could be detected in this study. This finding is in contrast with the study of van Meeteren *et al.* (2005) who stated that even PLP and MOG mRNA expression were very weak or undetectable in samples of OLN-93 cells after differentiation in serum-free medium supplemented with insulin like growth factor-I (IGF-I). This addition of IGF-I could explain the discrepancy with our results. However, since IGF-I is known to promote oligodendrocyte differentiation *in vitro*, one would rather expect a rise in the differentiation degree upon IGF-I treatment. An alternative explanation could be the difference in the primers used to detect PLP and MOG mRNAs. The absence of GFAP immunostaining could be due to the presence of a GFAP splice variant that is not recognized by the anti-GFAP antibody. There are

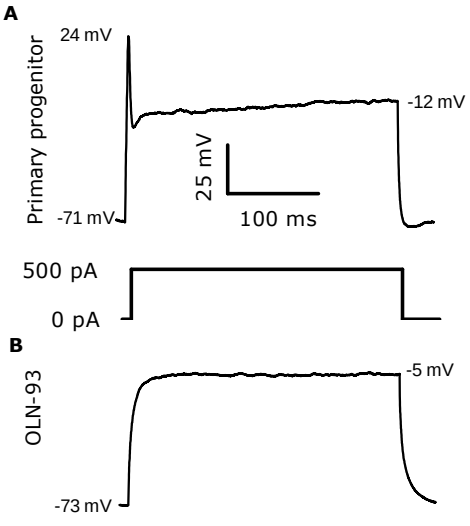

Figure 7.7 *Potential changes evoked by 500 pA current injection in OLN-93 and primary oligodendrocyte progenitors.* **A.** Primary oligodendrocyte progenitors show an action potential-like transient peak. Cells never showed more than one peak. **B.** OLN-93 never showed such a peak when current was injected.

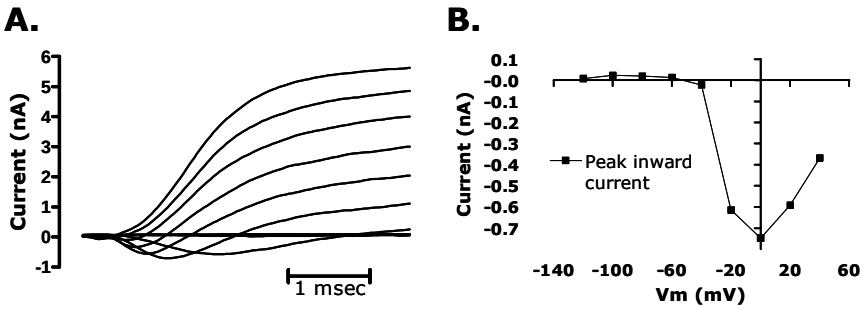

Figure 7.8 *Na^+-like currents in primary oligodendrocytes.* **A.** Primary oligodendrocytes show transient inward currents that are not seen in OLN-93. These representative currents were elicited by the same protocol as shown in fig. 6.2C, in the same cell as fig. 6.7A. **B.** Current-voltage relationship of peak inward currents shown in A has a Na^+ current-like profile. Currents activate at -40 mV.

5 splice variants of GFAP, giving rise to 3 proteins that differ in the C-terminal tail domain (Blechingberg et al. 2007). The exact epitope that is recognized by the anti-GFAP antibody is not known, but it is located in the carboxyterminal of the protein (Debus et al. 1983; Reeves et al. 1989). However, the antibody was raised against GFAP from pig spinal cord. Given that the GFAPα protein is the predominant form in the CNS (Blechingberg et al. 2007), it is very likely that at least this dominant form is recognized by the antibody.

A broad range of markers, including the myelin component MAG, is thus expressed by the OLN-93 cell line. The morphology of OLN-93 cells, cultured in medium containing 10% FCS, would indicate that the cells reside in an early phase of oligodendrocyte development. The cells resemble the bipolar progenitor cells. However, one does not expect in this stage expression of MAG, which is a marker characteristic for more mature oligodendrocytes.

Switching the medium to low serum content (0.5% FCS) does not have an influence on the expression of the developmental markers as shown by the unmodified immunoreactivity of the different markers tested. Again the same broad range of markers is expressed but in contrast to OLN-93 cells grown in high serum medium, the morphology is drastically modified. The cells become flattened and elaborate a more arborized morphology with many fine branches. Serum deprivation evokes a morphology that resembles a more mature phase of oligodendrocytes ranging from pre-oligodendrocytes to the premyelinating mature, characterized by cells bearing multiple branched processes (see fig. 5.3). Since membrane capacitance (C_m) is a measure for membrane surface area, the more branched morphology correlates well with the rise in C_m found in whole-cell patch-clamp experiments, when cells are cultured in 0.5% FCS. Therefore, we can conclude that the observed morphological changes are accompanied by an enlargement of the membrane surface area and are not associated with a change in marker expression.

7.3.2 Serum deprived OLN-93 cells maintain an electrophysiological profile characteristic for immature oligodendroglial cells.

In response to changes in membrane potential, OLN-93 cells exhibit currents with an outward rectifying profile in 10% FCS as well as in 0.5% FCS. Current density is higher in cells cultured in 10% in comparison to 0.5% FCS (data not shown). The decline in current density in 0.5% FCS can be attributed mainly to the previously mentioned rise in C_m since the amplitude of the absolute currents does not change from 10% to 0.5% FCS. The rise in input resistance in cells cultured in low serum condition also supports the distribution of a stable amount of channels over a larger membrane surface area.

In both culture conditions, voltage-activated currents are predominantly carried by K^+ ions given the tail current reversal potential values which are close to the calculated Nernst potential of potassium (-82.5 mV). The calculated Nernst potentials of Na^+ and Cl^- are respectively +68.5 mV and -4.0 mV. Reversal potential approaches the value of the Nernst potential of the ion with the largest conductance, as described by the Goldmann-Hodgkin-Katz relationship. Relative to K^+, the conductance for Na^+ and Ca^{2+} in response to voltage thus is rather low, but cannot be completely neglected. Further evidence for the important role of K^+ channels is provided by the efficient blocking effect of 25 mM TEA in both culture conditions.

Although the high K^+ conductance relative to other ions does not change with different serum culture concentration, a shift in underlying type of potassium current cannot be excluded. In both 10% and 0.5% FCS, TEA-sensitive currents are clearly outward rectifying, are activated from -40 mV and do not inactivate during the voltage pulse. However, in 0.5% FCS, the TEA-sensitive proportion of the total current was significantly larger. This might be explained by an expression of channel types in 10% FCS which are less sensitive to TEA, but nonetheless are outward rectifying. Although TEA is regarded as a broad K^+ channel blocker, sensitivities to the toxin differ between potassium channel subtypes. The TEA sensitivities of voltage-gated K^+ channels are overviewed in table 7.1. The concentration used was high enough to have a blocking effect on most TEA sensitive K^+ channels, but some channels are TEA insensitive and thus could be responsible for the residual currents. Examples of TEA insensitive K^+ channels are *shaker* channels 1.4, 1.5 and 1.7, the voltage gated Kv4 subfamily (Coetzee et al. 1999) and the K2P channels (Lesage and Lazdunski 2000).

To our knowledge, no research elaborated yet on a possible change in channel type underlying outward rectifying currents during the immature stages of

Table 7.1 TEA sensitivity and half maximal activation potential of the family of voltage gated potassium channels

Subfamily	Subunit	V_{50} (mV)	IC_{50} TEA (mM)	Reference
Shaker (Kv1)	Kv1.1	-30	0.5	(Coetzee et al. 1999)
	Kv1.2	-5 to 5	>10	
	Kv1.3	-30	10 to 50	
	Kv1.4	-22	NB	
	Kv1.5	-10	NB	
	Kv1.6	-20	1.7 to 7	
	Kv1.7	-20	NB	
Shab (Kv2)	Kv2.1	≈ 10	4 to 10	(Coetzee et al. 1999)
	Kv2.2	≈ 10	≈ 8	
Shaw (Kv3)	Kv3.1	≈ 15	≈ 0.2	(Coetzee et al. 1999)
	Kv3.2	≈ 10	0.15	
	Kv3.3	7	0.14	
	Kv3.4	≈ 15	0.09 to 0.3	
Shall (Kv4)	Kv4.1	≈ -10	NB	(Coetzee et al. 1999)
	Kv4.2	-15 to -4	NB	
	Kv4.3	-20	NB	
KCNQ (Kv7)	Kv7.1	-12	5	(Coetzee et al. 1999; Hadley et al. 2000; Tatulian et al. 2001)
	Kv7.2	-11.5	0.3	
	Kv7.3	-29	> 30	
	Kv7.4	-13	3.0	
Eag (Kv10)	Kv10.1	-12 to -4	28	(Coetzee et al. 1999; Saganich et al. 1999)
	Kv10.2	-35.5	≈ 12.5	
Erg (Kv11)	Kv11.1	-21	50	(Coetzee et al. 1999)
	Kv11.2	-3.5	?	
	Kv11.3	-44	?	
Elk (Kv12)	Kv12.1	9.3	NB by 10	(Coetzee et al. 1999; Engeland et al. 1998)
	Kv12.2	25	NB	
	Kv12.3	?	?	

The Kv5, Kv6, Kv8, Kv9 subfamilies do not show detectable current when expressed alone, but can heteromerize with Kv2 (Coetzee et al. 1999; Gutman et al. 2005). Kv nomenclature is as described by the International Union of Pharmacology (Gutman et al. 2003; Gutman et al. 2005). Kv: voltage gated potassium channel, Eag: ether-à-gogo, Erg: eag related gene, Elk: eag-like potassium channel, V_{50}: voltage at which 50% of activation is reached, IC_{50}: concentration at which 50% inhibition is reached, TEA: tetraethylammonium

oligodendrocyte differentiation. Previous studies report an important contribution of the *shaker* family of K^+ channels in immature oligodendrocytes (Attali et al. 1997; Chittajallu et al. 2002; Schmidt et al. 1999) but no time-course of expression of *shaker* subtypes related to separate stages of differentiation has yet been performed.

These studies have described several *shaker* family members (Kv1.1-1.6) being expressed on mRNA level as well as on protein level. The *shaker* channel type seems to play a role in the regulation of the proliferation of immature cells of the oligodendrocyte lineage (see chapter 5). In our results, the V_{50} of TEA sensitive currents is close to -30 mV, regardless of serum concentration. This is in correspondence with reported V_{50} values for the *shaker* family members (Coetzee et al. 1999) (table 7.1).

To check whether *shaker* family members are also expressed in OLN-93 cells, we performed RT-PCR and immunocytochemical experiments. Our results indicate an expression of Kv1.1 and Kv1.3 on mRNA and protein levels. Kv1.2, 1.4, 1.5 and 1.6 could not be detected, regardless of the serum concentration in the cell cultures. These results differ somewhat from earlier studies, but the presence of especially the Kv1.3 subunit is very interesting since it is the main *shaker* family member involved in the regulation of proliferation and differentiation of oligodendroglial cells (Chittajallu et al. 2002; Vautier et al. 2004). Its role in proliferation has not only been investigated in healthy cells, but also in cancer cells (Pardo et al. 2005; Preussat et al. 2003).

To our knowledge, no specific Kv1.1 blockers are currently available, but to better characterize currents mediated by Kv1.3 channels, we applied MgTx, a specific blocker of Kv1.3 channels. MgTx inhibition does not differ between 10% and 0.5% FCS, indicating that Kv1.3 expression does not change when OLN-93 cells are cultured in different serum concentrations. However, MgTx does not block outward rectifying currents in OLN-93 to the same extent as TEA. It remains unclear which other voltage-gated potassium channels are involved in the total TEA-sensitive outward rectifying current. Kv1.1 is an obvious candidate, but although the *shaker* family is important in proliferation regulation, its role is not exclusive. In oligodendrocytes, expression of Kv3.1, a member of the *shaw* family of voltage-gated potassium channels, has also been described. Like members of the *shaker* family, this channel also seems to regulate oligodendrocyte development (Tiwari-Woodruff et al. 2006). Tiwari-Woodruff *et al* (2006) also demonstrate protein expression of Kv2.1, 3.2, 3.3 and 3.4. The *shaw* family (Kv3.x) is more sensitive to TEA than *shaker* channels (Coetzee et al. 1999) and can possibly be upregulated in OLN-93 cells given the increased TEA sensitivity in 0.5% FCS.

Regardless of the underlying channel type, functional differentiation upon serum deprivation should change the electrophysiological profile. Outward rectifying

currents should decrease, while inward rectifying voltage-gated potassium currents should increase (Kettenmann et al. 1991). The inward rectifying potassium channels of mature glial cells are important in buffering the extracellular K^+ accumulation due to neural activity, as was first described by Newman for Müller cells (Butt and Kalsi 2006; Horio 2001; Kofuji and Newman 2004; Newman 1985). Our experiments clearly show that the outward component of voltage-activated currents does not decrease. There is an inward component in the voltage-elicited currents, but its magnitude does not change with different serum concentrations. This indicates that the morphological changes of OLN-93 under low serum conditions do not truly represent classical oligodendrocyte differentiation.

When compared to primary rat oligodendrocyte progenitors with bipolar morphology, current density of outward currents is much smaller in OLN-93 cells while inward currents have the same current density. Primary cells are smaller, as reflected by their lower membrane capacitance. C_m is in the same range as in earlier reports (Barres et al. 1990; Williamson et al. 1997). RMP of primary progenitors was more depolarized than that of OLN-93 cells. This could reflect a higher relative K^+ conductance at rest in OLN-93, but it should be mentioned that the RMP of primary cells displays a higher variability. The mean RMP of -64 ± 3 mV is in good accordance with earlier reported mean values (Sontheimer et al. 1989), but most studies do not elaborate on the heterogeneity of the values amongst cells. Williamson *et al.* (1997) however do report two populations in the distribution of RMP in rat oligodendrocyte progenitors, which is in close resemblance to our observations. It is hypothesized that these differences in RMP are related to cell cycle stage, suggesting that the relative K^+ conductance at rest is stage dependent. The RMP of OLN-93 did not show this heterogeneity. Because of this variability, it would be precarious to state that "the" primary oligodendrocyte progenitor displays a lower resting K^+ conductance than OLN-93. It is certainly the case for a subpopulation of the primary progenitors, but not for all. Like Williamson *et al.* (1997), we could not see any important differences between other electrophysiological properties of the RMP-subpopulations of progenitor cells. The higher input resistance of primary oligodendrocytes indicates a lower overall membrane conductance at rest in these cells than in OLN-93, in accordance with the speculation about a lower K^+ conductance because of more depolarized RMP in primary cells. When investigating tail currents, thus currents after activation of the voltage activated channels present, we see that the reversal potential of these currents is -81 ± 1 mV in primary progenitors, which reflects a very large relative conductance for K^+ (E_K = -82,5 mV) in these cells, larger than observed in OLN-93 cells which had a tail current reversal potential of -59 ± 2 mV in 10% FCS.

Current injection in primary oligodendrocyte progenitors elicited a single depolarizing spike, followed by a repolarization. This was never observed in OLN-93 cells. Although we did not investigate other ion channels than potassium in detail, given the link between differentiation and potassium currents, this hints a more complex ion channel complement in primary progenitors than in OLN-93 cells. It seems contradictory to the tail current data, which show the relative K^+ conductance to be higher in primary cells. However, tail currents reversal potential is a measurement of relative ion conductances, in a state that all voltage gated channels are activated. The values we observed show that the K^+ conductance is very large compared to other ions in primary cells, but this does not exclude presence of other ion channels. The appearance of spikes upon current injection is caused by voltage-gated depolarizing cation channels, followed by hyperpolarizing action of mainly voltage-gated K^+ channels. In the case of primary oligodendrocyte progenitors, the depolarizing spike was suggested to be mainly Na^+ dependent (Barres et al. 1990; Chittajallu et al. 2004; Williamson et al. 1997). The absence of this transient depolarization thus suggests a more important role of Na^+ currents in the primary cells than in the OLN-93 cell line. Indeed, Na^+-like inward currents were observed in primary cells, but not in OLN-93 cells.

In conclusion, our findings strongly support the fact that switching the OLN-93 cell line to a medium containing low serum (0.5% FCS) does not evoke differentiation as suggested by the morphological changes. The expression of markers and the potassium current profile of OLN-93 are not altered by culture serum conditions. OLN-93 show a profoundly lower current density of outward rectifying potassium currents compared to primary oligodendrocyte progenitors. In spite of the expression of NG2, the cell line certainly does not qualify as a model for oligodendrocyte progenitors. This was already hinted by the observed differences in neurotransmitter expression (see chapter 6). Therefore, we conclude that the developmental stage of the OLN-93 cell line mostly resembles the range of a late pre-oligodendrocyte to premyelinating oligodendrocyte, regardless of culture serum concentrations.

Chapter 8 | Summary & general conclusion

The central nervous system is a communication network integrating signals coming from and going to the periphery, thereby regulating and fine-tuning the functioning of the whole body. These communication processes require fast signalling between neurons. The propagation of an action potential through axons is greatly improved by the presence of myelin sheaths around these axons. In the central nervous system, these sheaths are formed by oligodendrocytes, a glial cell type. Once an action potential reaches the axon terminal, the signal has to be quickly transmitted to the receiving cell. This is accomplished by synaptic transmission. The soluble factors released to transmit the signal from the presynaptic to the postsynaptic cell are the neurotransmitters. The binding of these neurotransmitters on their receptors elicits inhibitory or excitatory responses in the receiving cell.

These are in a nutshell the best known facts about oligodendrocytes on one hand and neurotransmitters on the other. Oligodendrocytes myelinate axons to improve action potential propagation and neurotransmitters transmit synaptic signals between neurons. However, research has come a long way from the original view that glial cells are nothing more than "glue" between neurons (hence their name) and evidence is growing that neurotransmitters are also involved in extrasynaptic signalling. In this context it is exciting that oligodendrocytes are not the electrophysiologically silent cells they were once thought to be. In fact, oligodendrocytes express a diversity of ion channels during their development (Belachew and Gallo 2004; Karadottir and Attwell 2007; Kettenmann et al. 1991).

The changes in ion channel expression in oligodendrocytes during development are no coincidence. Proliferating oligodendrocyte progenitors show outward voltage-gated K^+ currents, which diminish during development. The voltage-gated *shaker* K^+ channel family has now been proven to be directly linked to regulation of cell cycle exit in the oligodendroglial lineage (Vautier et al. 2004). The expression of neurotransmitter receptors is also developmentally regulated in oligodendrocytes, with primarily a downregulation during maturation (see table 5.2). Again it is believed that this can influence their development. Neurotransmitters are a likely candidate for communication between developing oligodendrocytes on the one hand and neurons on the other hand (Karadottir and Attwell 2007). For instance, the excitatory glutamate receptors are found to stimulate migration of oligodendrocytes (Gudz et al. 2006; Wang et al. 1996), but to inhibit cell proliferation and lineage progression (Yuan et al. 1998), a mechanism that may be involved in directing the oligodendrocyte to its target axon (see **chapter 5**).

Inhibitory neurotransmitters have also been shown to influence the cell cycle and differentiation of oligodendrocytes. However, the name "inhibitory" might be misleading in this case. Indeed, the ionotropic GABA and glycine receptors (respectively GABAR and GlyR) are permeable for Cl^- and have an inhibiting effect in adult synaptic transmission due to Cl^- influx. But in the developing CNS, the intracellular Cl^- concentration is higher due to activity of the NKCC1 transporter, altering the Cl^- equilibrium potential (Ben-Ari 2002). When GABAR's

or GlyR's are activated by their agonists, this results in a depolarizing action from these "inhibitory" neurotransmitters. Most research concerning the influence of inhibitory neurotransmitters on development has focussed on GABA and neurons (see **chapter 2**), but there are also indications that the activation of GABAR's inhibits the proliferation of oligodendroglial cells (Karadottir and Attwell 2007; Pastor et al. 1995; Yuan et al. 1998). Like GABA, glycine has also a depolarizing effect during development, increasing intracellular Ca^{2+} when GlyR's are activated (Belachew et al. 2000; Pastor et al. 1995).
These data open up several intriguing research questions. What is the exact role of inhibitory neurotransmitters in oligodendrocyte development? Are these neurotransmitter channels then functionally adapted to extrasynaptic signalling? Can modulation of neurotransmitter receptors in the long run provide ways to influence oligodendrocyte development *in vivo* and, hence, be a tool to promote remyelination in demyelinating disease?

In this work, we first investigate the functional properties of GlyR's containing α2 subunits. The α2 subunit is mainly expressed prenatally (see **chapter 2**). This makes the α2 GlyR a good candidate in light of the future study of the influence of the GlyR on developmental processes. Moreover, earlier experiments have shown that homomeric α2 GlyR's have kinetic properties that are not compatible with synaptic signalling, but may be more adapted to paracrine signalling (Mangin et al. 2003).

In **chapter 3**, we investigate the blocking mechanism of the picrotoxin components picrotin (PTN) and picrotoxinin (PXN) on the α2 homomeric GlyR, transfected into CHO cells. Toxins are valuable tools in ion channel research. They provide researchers with means to selectively block channels of interest. Moreover, their interaction with an ion channel can provide useful information about its structure and its translational processes. To make optimal use of toxins, a good characterisation of their mode of action is needed.
Our data show that, unlike in homomeric α1 GlyR (Lynch et al. 1995), PTN and PXN do not have equal potencies in α2 GlyR. Homomeric α2 receptors are more sensitive to PXN than to PTN, with the IC_{50} for PXN being 30 times lower than that for PTN. Such a difference in potency has also been observed for GABA receptors (Qian et al. 2005), but these receptors are completely insensitive to PTN, which is not the case for α2 glycine receptors. In GABAR's, an alanine on the 2' pore lining position is linked with the PTN resistance. α2 GlyR also have this alanine, in which they differ from α1 GlyR, confirming its likely role in PTN resistance. Nevertheless, α2 GlyR still are inhibited by PTN, albeit to a lesser extent, so that the presence of this alanine in α2 GlyR is not sufficient for PTN resistance.
We further show that PXN and PTN are channel blockers that bind to the fully liganded closed state or open state of the α2 GlyR. Neither PXN nor PTN can bind to the unliganded closed state. Differences in voltage dependency and association rate between PXN and PTN suggest PTN binds deeper within the

channel pore than PXN. It is likely that both compounds work through different allosteric mechanisms, given that their kinetic properties depend on the conformational status of the GlyR. A question that remains open is the exact location of the binding site for PTN and PXN on α2 homomeric GlyR's, although this work indicates that there are separate binding sites, with the PTN-site located deeper within the channel pore.

Given the high expression of α2 GlyR during development, we hypothesize that these receptors can influence or regulate developmental processes. A characterization of its functional properties will provide further insight into the physiological role of this GlyR. Mangin *et al.* (2003) have shown previously that α2 GlyR kinetics is not adapted to synaptic signalling. We have investigated in **chapter 4** whether the incorporation of the β subunit changes these kinetic properties. The β subunit is expressed at low levels during development, but increases perinatally, creating a window of co-expression of high levels of α2 and β.
Our results indicate a faster activation in α2β expressing CHO cells. Furthermore, inactivation of the currents elicited in α2β containing patches is faster than inactivation in patches containing α2 GlyR's only. This faster inactivation is consistent with the shorter mean open time observed in single channel measurements. To test whether these changes imply a higher open probability in synaptic signalling, we used an ultrafast application system to mimic synaptic transmission. Our analysis indicates that there is indeed an increase in open probability when a β subunit is incorporated. Open probability in homomeric α2 channels was less than 10% (Mangin et al. 2003). In α2β GlyR's raised the open probability to 40 to 70%, which however remains less than the 90% of α1β heteromeres (Legendre 1998).
It should be noted that the expression system, a permanent transfection of α2 and β subunits in CHO cells, did not result in pure α2β channels. Patches generally consisted of a mixture of homomeric α2 and heteromeric α2β channels. The effects observed in macroscopic currents thus could be an underestimation of the real influence of β subunits due to "contamination" by α2. Transiently transfecting the β subunit in α2 expressing CHO cells did not resolve this issue. A future strategy might be to increase the concentration of β subunit DNA used in transfection, but this remains to be demonstrated.
The fastening of kinetics by the β subunit might be important for the first synaptic glycinergic transmission in the developing CNS, but can also have an influence on paracrine signalling before synapse formation. It could be hypothesized that the steady increase of β expression, and the concomitant changes in response, could be of influence in slowly adapting the developing cell to other signalling modes. These are all interesting questions that warrant further investigation into the role of GlyR in development.

In that respect, our research group is mainly interested in the involvement of the glycine receptor in oligodendrocyte development. Research into the

involvement of inhibitory neurotransmitters in CNS development is mainly limited to role of GABA receptors in neurons (see **chapter 2**). Research into neurotransmitter influence on oligodendrocyte development has mainly focussed on glutamate. Information from the literature regarding the involvement of inhibitory neurotransmitters in oligodendrocyte development is very scarce. Therefore part of this work was aimed at validating an oligodendrocyte model, the OLN-93 cell line, for future work in the area of neurotransmitter involvement in oligodendrocyte development.

In **chapter 6** we have investigated the expression by OLN-93 of several proteins involved in glycine signalling. We looked at glycine receptors, and also at glycine transporters and at the gephyrin protein, which is needed for the interaction between GlyR's and the cytoskeleton. mRNA expression of α1, α2 and β GlyR subunits was found in OLN-93. Immunocytochemistry showed that those cells also express GlyR proteins, but the currently available antibodies do not distinguish between the different subunits. Concerning glycine transporters, only glycine transporter 1 (GlyT1) could be detected both at mRNA and at protein levels. GlyT1 is known to be the isoform expressed by glial cells. The anchoring protein gephyrin was present on mRNA levels as well as on protein level.
Given this expression pattern, it was quite a surprise to observe that glycine did not evoke GlyR-mediated responses in patch-clamp experiments. A small depolarizing current evoked by glycine was however observed, but it was not sensitive to strychnine, did not reverse and was Na^+-dependent. This means that the observed current is likely mediated by GlyT1. The reason for the absence of GlyR activity, despite its expression, remains speculative. The most likely reason is an incomplete trafficking of the receptor proteins to the membrane of OLN-93.
In fact, when glutamate, acetylcholine, GABA, Na_2ATP or 5-HT were applied, neither of these neurotransmitters elicited a response in OLN-93 cells. When the same array of neurotransmitters was applied to primary rat oligodendrocyte progenitors, the results were quite different. Primary cells showed responses to GABA, 5-HT, glutamate and Na_2ATP, but not to acetylcholine or glycine. Responses of primary cells to GABA and glutamate corresponded to activation of the respective ionotropic receptors. Responses to 5-HT and Na_2ATP were not receptor-like and are probably mediated by other proteins, which remain to be characterized. These obvious differences between primary oligodendrocyte progenitors and the OLN-93 cell line, made us wonder whether or not the OLN-93 is actually a suitable oligodendrocyte (progenitor) model.

We therefore conducted a morphological, biochemical and functional characterization of the OLN-93 cell line, described in **chapter 7**. Serum deprivation has earlier been reported to evoke morphological changes reflecting differentiation in OLN-93 (Ernst et al. 2004). Indeed, our observations confirm that OLN-93 show a more arborized morphology when cultured in low serum conditions. However, this morphological differentiation could not be confirmed

by changes in differentiation marker expression profile. Regardless of serum concentration, cells were immunoreactive for NG2, CNP and MAG. Protein expression of the astrocyte marker GFAP or the myelin components MOG and PLP was not observed.
Functionally, we determined differentiation by the study of voltage-gated potassium currents in OLN-93 cells. Both *in vitro* and *in vivo*, oligodendrocyte progenitors mainly express outward rectifying potassium currents, which diminish during development and are progressively replaced by an inward rectifying potassium current profile. The outward currents in immature oligodendrocytes are mainly mediated by the *shaker* family of potassium channels. Therefore, we compared the current profiles and the *shaker* expression in OLN-93 cultured in 10% *versus* 0.5% FCS. Unlike what would be expected if OLN-93 was really differentiating under the influence of culture serum conditions, no change in current profile of OLN-93 was observed. Voltage-activated currents were outward rectifying and did not decrease in OLN-93 cells cultured in 0.5% FCS. Expression of the *shaker* family subunits also did not change, with Kv1.1 and Kv1.3 being expressed in OLN-93, regardless of culture serum concentration. Experiments specifically looking at currents mediated by Kv1.3 also did not show any difference between cells cultured in 0.5 or 10% FCS. However, the proportion of TEA-sensitive currents in the total current increased in cells cultured in 0.5% FCS. TEA is a broad K^+ channel blocker, although some TEA-insensitive K^+ channel subtypes exist. Given this observation and although Kv1.3 expression seems stable, a change in expression levels of other K^+ channel subtypes cannot be ruled out. Nevertheless, we still can likely conclude that the profile of voltage-activated currents in OLN-93 does not change in culture conditions presumed to induce differentiation.
As with the study of the neurotransmitter receptors in **chapter 6**, electrophysiological properties of OLN-93 were compared to those of primary rat oligodendrocyte progenitors. Despite the fact that the primary cells are much smaller, as confirmed by their lower membrane capacitance, they show a much higher current density of outward currents. Another striking difference was the presence of Na^+-like currents in primary oligodendrocyte progenitors, which were able to generate a single potential spike in response to current injection. Na^+-like currents, or potential spikes, were not observed in OLN-93 cells.
These observations support the conclusion that the OLN-93 cell line does not differentiate under the influence of low serum conditions, despite its morphological changes. The marker expression profile and the comparison with the primary oligodendrocyte progenitors lead to the conclusion that the OLN-93 cell line is not a suitable model for oligodendrocyte progenitors and probably is more resembling to a (late) immature oligodendrocyte, regardless of serum concentration.

In conclusion, this study provides new data concerning the function and properties of α2-containing glycine receptors. It was shown that incorporation of the β subunit enables the α2/β channels to respond better to synaptic

transmission. The influence of β on kinetics might also have an influence on effects of extrasynaptic signalling. The findings of this work open up pathways for new explorations into the functioning of these glycine receptors in CNS development, and more particularly in the development of the oligodendroglial lineage. However, the originally proposed model, the OLN-93 cell line, does not qualify as a progenitor model and since differentiation in culture could not be shown, it is not suited to study the influence of neurotransmitter receptors on differentiation. This does not however mean that the OLN-93 model should not be used at all. It is probably more resemblant to a late immature oligodendrocyte and can still be used to study for instance processes such as membrane trafficking in oligodendrocytes. Nevertheless, this work shows again the need for clear characterization of cell line models before using them in research and comparing results from these models with *e.g.* primary cultures.
In the future it would certainly be interesting to investigate the effects of glycine signalling on oligodendrocyte development. Therefore one could make use of cell line models, provided they are well characterized, but more definite conclusions should come from experiments on primary cultures or *ex vivo* preparations. Three broad steps will be needed in such a study, regardless of the model used. Firstly an evaluation of the presence of proteins involved in the glycine signalling in oligodendroglial cells, such as GlyR's and GlyT's, should be conducted. From the ligand point-of-view one should also pay attention to other endogenous molecules such as taurine, which also interacts with GlyR's. Secondly an investigation should be performed on the effects of glycine on oligodendrocytes, both in normal and in pathological conditions, for instance by using the experimental autoimmune encephalomyelitis animal model for multiple sclerosis. And finally the pathways involved in observed effects should be researched. Hopefully, this will provide further insight in mechanisms governing oligodendrocyte survival and development. This insight is of importance to understand and maybe influence processes of remyelination in demyelinating diseases such as multiple sclerosis.

Samenvatting & algemene conclusie

Het centraal zenuwstelsel vormt een communicatienetwerk dat signalen integreert komende van en gaande naar de periferie. Hierdoor worden processen doorheen het gehele lichaam gereguleerd en op mekaar afgestemd. Deze communicatie vereist snelle signaaloverdracht tussen neuronen. De voortgang van een actiepotentiaal doorheen axonen wordt sterk versneld door de aanwezigheid van een myelineschede rond deze axonen. De myeline in het centraal zenuwstelsel wordt gevormd door oligodendrocyten, een gliaal celtype. Wanneer een actiepotentiaal aankomt bij het uiteinde van een axon, moet het signaal snel doorgegeven worden aan de ontvangende cel. Dit proces heet synaptische transmissie. De oplosbare factoren die het signaal doorgeven van de presynaptische naar de postsynaptische cel zijn de neurotransmitters. De binding van deze neurotransmitters op hun receptoren heeft inhiberende of exciterende effecten in de postsynaptische cel.

Dit zijn in een notendop de best gekende feiten over enerzijds oligodendrocyten en anderzijds neurotransmitters. Oligodendrocyten myeliniseren axonen om de axongeleiding te verbeteren en neurotransmitters mediëren synaptische signaaloverdracht tussen neuronen. De wetenschap heeft echter een lange weg afgelegd van de oorspronkelijke visie dat gliale cellen niets meer zijn dan "lijm" tussen neuronen (daar hebben ze ook hun naam aan te danken) en meer en meer studies wijzen op een rol voor neurotransmitters als extrasynaptische signalen. Oligodendrocyten zijn niet langer de elektrofysiologisch inactieve cellen die ze ooit geacht werden te zijn. In werkelijkheid brengen oligodendrocyten een scala aan ionenkanalen tot expressie tijdens hun ontwikkeling (Belachew and Gallo 2004; Karadottir and Attwell 2007; Kettenmann et al. 1991).

De veranderingen in ionkanaal expressie van oligodendrocyten tijdens ontwikkeling zijn geen toeval. Prolifererende oligodendrocytprogenitoren vertonen uitwaartse voltage geactiveerde K^+ stromen, die afnemen tijdens de differentiatie. Studies hebben uitgewezen dat de voltage geactiveerde *"shaker"* K^+ kanaal familie rechtstreeks betrokken is bij de regulatie van de cel cyclus in de oligodendrocyt (Vautier et al. 2004). De expressie van neurotransmitterreceptoren is ook afhankelijk van ontwikkelingsstadium in oligodendrocyten, met voornamelijk een afname in expressie tijdens differentiatie (zie tabel 1.3). Ook hiervan wordt gedacht dat het de ontwikkeling beïnvloedt. Neurotransmitters zijn bovendien uitstekende kandidaten voor communicatie tussen ontwikkelende oligodendrocyten en neuronen (Karadottir and Attwell 2007). De exciterende neurotransmitter glutamaat bijvoorbeeld stimuleert de migratie van oligodendrocyten (Gudz et al. 2006; Wang et al. 1996), maar inhibeert hun proliferatie en maturatie (Yuan et al. 1998). Dit mechanisme kan van belang zijn om de oligodendrocyt naar zijn bestemming te leiden (zie **hoofdstuk 5**).

Ook inhiberende neurotransmitters beïnvloeden mogelijk de celcyclus en differentiatie van oligodendrocyten. De naam "inhiberend" is echter misleidend in dit geval. De ionotrope GABA- en glycinereceptoren (respectievelijk GABAR en GlyR) zijn permeabel voor Cl^- en hebben daardoor een inhiberend effect in

synaptische transmissie in het volwassen zenuwstelsel. In het ontwikkelende centraal zenuwstelsel is de intracellulaire Cl^- concentratie echter hoger door de activiteit van de NKCC1 transporter (Ben-Ari 2002). Wanneer GABAR's of GlyR's dan geactiveerd worden door hun agonisten, geeft dit een depolarisatie door deze "inhiberende" neurotransmitters. Het meeste onderzoek aangaande de invloed van inhiberende neurotransmitters op ontwikkeling heeft zich gericht op GABA en neuronen (zie **hoofdstuk 2**) maar er zijn ook indicaties dat de activatie van GABAR's de proliferatie van oligodendrogliale cellen inhibeert (Karadottir and Attwell 2007; Pastor et al. 1995; Yuan et al. 1998). Net zoals GABA, heeft glycine ook een depolarizerend effect tijdens de ontwikkeling van oligodendrocyten, wat de intracellulaire Ca^{2+} concentratie verhoogt bij GlyR activatie (Belachew et al. 2000; Pastor et al. 1995).
Met deze data in het achterhoofd kunnen verschillende vragen gesteld worden. Wat is de exacte rol van inhiberende neurotransmitters in de ontwikkeling van oligodendrocyten? Zijn deze neurotransmitterreceptoren functioneel aangepast aan extrasynaptische activatie? Kan modulatie van neurotransmitterreceptoren op lange termijn een manier zijn om oligodendrocyt ontwikkeling *in vivo* te beïnvloeden en daardoor een manier zijn om remyelinisatie in demyeliniserende ziektes te promoten?

In deze thesis onderzoeken we in het eerste deel de functionele eigenschappen van GlyR met α2 subeenheden. De α2 subeenheid komt vooral prenataal tot expressie (zie **hoofdstuk 2**). Dit maakt van de α2 GlyR een goede kandidaat voor toekomstig onderzoek naar de invloed van GlyR in ontwikkelingsprocessen. Bovendien hebben eerdere experimenten al bewezen dat de kinetiek van homomere α2 GlyR's niet compatibel is met synaptische signaaloverdracht, maar meer aangepast is aan paracriene signaaloverdracht (Mangin et al. 2003).

In **hoofdstuk 3** onderzoeken we de blokkeringsmechanismen van de picrotoxine componenten picrotine (PTN) en picrotoxinine (PXN) op de α2 homomere GlyR, getransfecteerd in CHO cellen. Toxines zijn belangrijke hulpmiddelen in het onderzoek naar ionkanalen. Met behulp van toxines kunnen onderzoekers bepaalde kanaaltypes selectief blokkeren. Bovendien kan de interactie tussen een toxine en een ionkanaal interessante informatie opleveren over de structuur en de translationele processen van dat kanaal. Om optimaal gebruik te maken van toxines, is een goede karakterisatie van hun werkingsmechanisme nodig.
Onze data tonen aan dat PTN en PXN de α2 GlyR niet even sterk blokkeren, in tegenstelling tot de situatie in α1 GlyR (Lynch et al. 1995). Homomere α2 receptoren zijn gevoeliger voor PXN dan voor PTN, met een IC_{50} voor PXN die 30 keer hoger ligt dan voor PTN. Een dergelijk verschil in sterkte is ook beschreven voor GABA receptoren (Qian et al. 2005), die zelfs helemaal ongevoelig bleken voor PTN. In GABAR's wordt een alanine op de 2' positie in de porie van het kanaal in verband gebracht met hun PTN resistentie. Dit alanine is ook aanwezig in α2 GlyR, een verschil met α1 GlyR, wat het belang voor weerstand voor PTN lijkt te bevestigen. Toch worden α2 GlyR nog steeds geïnhibeerd door PTN, maar

in mindere mate, waaruit blijkt dat het alanine in α2 GlyR niet volstaat voor een volledige ongevoeligheid voor PTN.
Onze data tonen verder aan dat PXN en PTN kanaal blokkers zijn die binden op de volledig gebonden gesloten status van de α2 GlyR. Noch PXN, noch PTN kan binden aan de ongebonden gesloten conformatie. Verschillen in voltage-afhankelijkheid en associatiesnelheid tussen PXN en PTN suggereren dat PTN dieper in de porie van het kanaal bindt dan PXN. Waarschijnlijk werken beide stoffen via verschillende allosterische mechanismen, gezien hun kinetische eigenschappen afhankelijk zijn van de conformatie van de GlyR. De exacte bindingsplaats van PTN of PXN op α2 homomere GlyR blijft onbekend, maar dit werk geeft aan dat er verschillende bindingsplaatsen zijn, met de PTN bindingsplaats dieper in de kanaalporie.

Gezien de hoge expressie van α2 GlyR tijdens de ontwikkeling, is het mogelijk dat deze receptoren de ontwikkeling kunnen beïnvloeden. Een karakterisatie van hun functionele eigenschappen zal verder inzicht geven in de fysiologische rol van deze GlyR. Mangin en collega's (2003) toonden eerder al aan dat de kinetische eigenschappen van de α2 GlyR niet aangepast zijn aan synaptische communicatie. Wij hebben in **hoofdstuk 4** onderzocht of de aanwezigheid van de β subeenheid deze kinetische eigenschappen verandert. De β subeenheid vertoont tijdens de ontwikkeling lage expressieniveaus maar de expressie neemt toe rond de geboorte, waardoor er een periode ontstaat waar er veel expressie is van zowel α2 als β subeenheden.
Onze resultaten geven aan dat er een snellere activatie optreedt in CHO cellen die α2β tot expressie brengen. Ook de inactivatie van de stromen in patches met α2β is sneller dan wanneer enkel α2 aanwezig is. Deze snellere inactivatie komt overeen met de kortere gemiddelde open tijd in "single channel" metingen. Om na te gaan of deze veranderingen voldoende zijn om de kans op opening bij synaptische transmissie te vergroten, gebruikten we een ultrasnel toedieningssysteem om synaptische transmissie na te bootsen. Onze analyse geeft aan dat er inderdaad een toename is in de kans op opening wanneer de β subeenheid aanwezig is. Die kans steeg van minder dan 10% in homomere α2 (Mangin et al. 2003) kanalen naar 40 à 70 % in α2β GlyR. Dit blijft echter minder dan de 90% die gemeten werd in α1β heteromeren (Legendre 1998).
Het gebruikte expressie system, een permanente transfectie van α2 en β subeenheden in CHO cellen, bracht wel niet enkel α2β kanalen tot expressie. Membraan patches bevatten over het algemeen een mengeling van homomere α2 en heteromere α2β kanalen. De vermeldde effecten, vastgesteld in macroscopische stromen, kunnen dus een onderschatting zijn van de echte invloed van de β subeenheid door de "contaminatie" met α2. Een transiënte transfectie van de β subeenheid in CHO cellen die α2 tot expressie brengen kon dit probleem niet verhelpen. Een mogelijke toekomstige strategie om de slaagkans van experimenten te verhogen is het gebruik van hogere concentraties β subunit DNA tijdens transfectie, al moet het nut hiervan nog nagegaan worden.

De veranderingen in de kinetiek onder invloed van de β subeenheid kan belangrijk zijn voor de eerste synaptische glycinerge transmissie in het ontwikkelend centraal zenuwstelsel, maar kan ook een invloed hebben op paracriene signalering voordat synapsen ontstaan. Misschien is de geleidelijke toename in β expressie, en de bijhorende verandering in respons, van belang voor het aanpassen van de ontwikkelende cel aan andere types van signalen. Dit zijn allemaal interessante vragen voor het verder onderzoek naar de rol van GlyR in de ontwikkeling.

Bij die ontwikkelingsvragen is onze onderzoeksgroep vooral geïnteresseerd in de rol van de glycinereceptor in ontwikkeling van oligodendrocyten. Onderzoek naar de betrokkenheid van inhiberende neurotransmitters in de ontwikkeling van het centraal zenuwstelsel is tot nu toe vooral gericht op de rol van GABA receptoren in neuronen (zie **hoofdstuk 2**). Onderzoek naar de invloed van neurotransmitters op oligodendrocytontwikkeling had vooral aandacht voor glutamaat. Informatie aangaande de invloed van inhiberende neurotransmitters op oligodendrogliale ontwikkeling is dan ook zeer beperkt. Daarom is een deel van dit werk gericht op het valideren van een oligodendrocytmodel, de OLN-93 cellijn, voor toekomstig werk in het gebied van oligodendrocytontwikkeling.

In **hoofdstuk 6** hebben we de expressie van verschillende eiwitten, betrokken in de glycinesignalering, onderzocht in OLN-93. Hierbij werd gekeken naar glycinereceptoren, maar ook glycinetransporters en gephyrine. Gephyrine is nodig voor de interactie tussen GlyR's en het cytoskelet. In OLN-93 was er mRNA expressie van α1, α2 en β GlyR subeenheden. Immunocytochemie toonde ook GlyR proteïne-expressie, maar de momenteel beschikbare antilichamen kunnen geen onderscheid maken tussen de verschillende subeenheden. Wat glycine transporters betreft, kon enkel glycine transporter 1 (GlyT1) worden gedetecteerd en dit op zowel mRNA als op eiwit niveau. GlyT1 staat bekend als de isovorm die tot expressie komt in gliale cellen. Gephyrine was steeds aanwezig op mRNA en eiwit niveau.
Ondanks dit expressiepatroon kon er geen GlyR respons gemeten worden in patch-clamp experimenten. Er werd wel een kleine depolariserende stroom opgemerkt in respons op glycinetoediening, maar die was ongevoelig voor strychnine, keerde niet om en was Na^+-afhankelijk. Deze stroom is dan ook waarschijnlijk gemedieerd door GlyT1. Waarom er geen GlyR activiteit was, ondanks de expressie, blijft onduidelijk. De meest voor de hand liggende reden is een verstoorde inbouw van de receptoreiwitten in het membraan van OLN-93.
Wanneer glutamaat, acetylcholine, GABA, Na_2ATP of serotonine werden toegediend aan OLN-93, kon geen enkel van deze neurotransmitters een respons uitlokken in OLN-93. Die resultaten bleken sterk verschillend van de situatie in primaire rat oligodendrocytprogenitoren. De primaire cellen vertoonden reacties op GABA, glutamaat, serotonine en Na_2ATP maar niet op acetylcholine of glycine. Stromen in respons op GABA en glutamaat kwamen overeen met activatie van de respectievelijke ionotrope receptoren. Stromen

uitgelokt door serotonine en Na_2ATP leken echter niet het resultaat van receptor activatie en zijn waarschijnlijk gemedieerd door andere eiwitten. De duidelijke verschillen tussen primaire oligodendrocyten en de OLN-93 cellijn deed de vraag rijzen of de OLN-93 wel een geschikt oligodendrocyt (progenitor) model is.

Daarom voerden we een morfologische, biochemische en funtionele karakterisatie van de OLN-93 cellijn uit, die beschreven wordt in **hoofdstuk 7**. Morfologische veranderingen gelijkend op differentiatie werden eerder al beschreven wanneer OLN-93 gekweekt werd in lage serumconcentraties (Ernst et al. 2004). Onze observaties bevestigen dat OLN-93 een meer vertakte morfologie vertoont in lage serumconcentraties. Deze morfologische veranderingen gingen echter niet gepaard met veranderingen in het expressieprofiel van differentiatiemerkers. De cellen waren immunoreactief voor NG2, CNP en MAG, ongeacht serumconcentratie. Er was geen eiwitexpressie van de astrocytmerker GFAP of de myelinecomponenten MOG en PLP.
Functionele differentiatie werd onderzocht door de voltage-geactiveerde K^+ stromen in OLN-93 in kaart te brengen. Zowel *in vitro* als *in vivo* vertonen oligodendrocytprogenitoren vooral uitwaarts rectificerende K^+ stromen, die afnemen tijdens de ontwikkeling en die geleidelijk aan vervangen worden door inwaarts rectificerende K^+ stromen. De uitwaartse stromen in immature oligodendrocyten worden vooral gemedieerd door de *shaker* familie van kalium kanalen. We vergeleken daarom de stroomprofielen en de *shaker* expressie van OLN-93 gekweekt in 10% met die in 0.5% FCS. In tegenstelling tot wat men zou verwachten als OLN-93 echt zou differentiëren in lage serumconcentraties, was er geen verandering in stroomprofiel van OLN-93 waarneembaar. Voltage-geactiveerde stromen waren uitwaarts rectificerend en namen niet af in OLN-93 cellen die gekweekt waren in 0.5% FCS. Expressie van de *shaker* familie subeenheden veranderde ook niet. In OLN-93 kwamen Kv1.1 en Kv1.3 tot expressie, ongeacht serumconcentratie. Wanneer specifiek gekeken werd naar Kv1.3 gemedieerde stromen was ook daar geen verschil te zien tussen 10% en 0.5% FCS. Het aandeel van TEA-gevoelige stroom in de totale stroom nam wel toe in cellen gekweekt in 0.5% FCS. TEA is een brede K^+ kanaal blokker, al zijn er enkele TEA-resistente K^+ kanaal subtypes. Dus ondanks dat Kv1.3 expressie stabiel lijkt, kan een verandering in expressieniveaus van andere K^+ kanaal subtypes niet uitgesloten worden. Desondanks kunnen we concluderen dat het uitwaarts rectificerend profiel van voltage-geactiveerde stromen in OLN-93 niet verandert in kweekcondities die geacht worden differentiatie te induceren.
Net zoals bij de experimenten met de neurotransmitterreceptoren in **hoofdstuk 6**, hebben we de elektrofysiologische eigenschappen van OLN-93 vergeleken met die van primaire rat oligodendrocyte progenitoren. Ondanks het feit dat primaire cellen veel kleiner zijn, vandaar ook hun lagere membraancapaciteit, vertonen ze toch een grotere stroomdichtheid van uitwaartse stromen. Een ander opvallend verschil was de aanwezigheid van Na^+-achtige stromen in primaire oligodendrocyten, die in staat waren om een enkele potentiaalpiek te

veroorzaken bij stroominjectie. Na^+-achtige stromen of potentiaalpieken werden niet waargenomen in OLN-93 cellen.
Deze data wijzen uit dat de OLN-93 cellijn niet differentieert onder invloed van lage serumconcentraties, ondanks de morfologische veranderingen. Het expressieprofiel van de merkers en de vergelijking met de primaire oligodendrocytprogenitoren leiden tot de vaststelling dat OLN-93 waarschijnlijk geen geschikt model is voor oligodendrocytprogenitoren en meer lijkt op een (late) immature oligodendrocyt, ongeacht serumconcentratie.

Samenvattend kunnen we stellen dat deze studie nieuwe data oplevert omtrent de functie en de eigenschappen van α2 bevattende GlyR. Er werd aangetoond dat de inbouw van de β subeenheid het α2β kanaal toelaat om beter te reageren op synaptische transmissie. De invloed van β op de kinetiek kan eveneens een invloed hebben op de effecten van extrasynaptische signalen. De bevindingen van deze thesis bereiden de weg voor naar meer onderzoek naar de functie van deze glycinereceptoren in de ontwikkeling van het centraal zenuwstelsel, en meer in het bijzonder de ontwikkeling van de oligodendrocyt.
Het oorspronkelijk voorgestelde model, de OLN-93 cellijn, voldoet echter niet als progenitormodel en aangezien differentiatie in cultuur niet kon bewezen worden, is het model niet geschikt voor de studie van de invloed van neurotransmitters op differentiatie. Dit betekent echter niet dat het OLN-93 model volledig onbruikbaar is. Het lijkt waarschijnlijk meer op een late immature oligodendrocyt en kan nog altijd gebruikt worden in de studie van bijvoorbeeld membraanbiologie in oligodendrocyten. Dit werk toont nogmaals de noodzaak voor een goede karakterisatie van cellijn modellen voordat ze gebruikt worden in onderzoek en voordat resultaten geëxtrapoleerd worden naar andere modellen, zoals primaire culturen.
In de toekomst zou het zeker interessant zijn om de effecten van glycinesignalen op oligodendrocytontwikkeling verder te evalueren. Daarbij kan men gebruik maken van cellijnmodellen, op voorwaarde dat ze goed gekarakteriseerd zijn, maar definitieve conclusies worden best gebaseerd op experimenten op primaire culturen of *ex vivo* modellen. Drie grote stappen zijn er steeds nodig, ongeacht het gebruikte model. Ten eerste moet de expressie van eiwitten betrokken in de signalering door glycine, zoals GlyR's en GlyT's , worden nagegaan in oligodendrocyten. Betreffende de liganden, moet men ook aandacht hebben voor andere endogene moleculen zoals taurine, dat ook kan binden op GlyR's. Ten tweede zouden de effecten van glycine op oligodendrocyten, in zowel normale als pathologische omstandigheden, onderzocht moeten worden. Men kan hierbij bijvoorbeeld gebruik maken van het experimentele autoimmune encephalomyelitis (EAE) diermodel voor Multipele Sclerose. Tot slot zou men de biochemische cascades moeten onderzoeken die betrokken zijn in de waargenomen effecten. Hopelijk zal dit leiden tot verder inzicht in de mechanismen die oligodendrocytoverleving en -ontwikkeling sturen. Dit inzicht is belangrijk om de remyelinisatie in demyeliniserende ziektes te kunnen begrijpen en misschien te kunnen beïnvloeden.

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Curriculum vitae

Roeland Buckinx was born on the 14th of December, 1981 in Leuven, Belgium. In 1999, he finished his secondary grade education (Latin-Mathematics) at the "Heilig-Hartcollege" in Maasmechelen. In September of that year he started the study of biomedical sciences at the "Limburgs Universitair Centrum" (now Hasselt University) in Diepenbeek. In June 2003 he graduated with honours from the transnational University Limburg, after completing part of his studies at Maastricht University.
In September 2003, he joined the laboratory of cell physiology at Hasselt University, where he already had performed research for his master's thesis under guidance of Prof. Dr. Paul Steels. He successfully applied for an IWT scholarship, allowing him to perform the work described in this thesis. Part of this work was done during a 3 month training period in the laboratory of Dr. Legendre at the Université Pierre et Marie Curie in Paris. During his graduate studies, he completed the doctoral training program in biomedical sciences.

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Dankwoord

September 1999, vers van het Maasmechelse college kom ik binnen in het Limburgs Universitair Centrum. "De universiteit"… wat te verwachten? Zouden de professoren streng zijn? Zouden de "straffe verhalen" kloppen? Gaat me dit wel lukken? Ga ik er iets van snappen? En dan ook nog eens het vak biofysica. Fysica! Konden ze nu echt niks gemakkelijkers kiezen om mee te beginnen? Biofysica, coördinator: Prof. Dr. Marcel Ameloot. Ik zie het nog zo staan in mijn studiegids. "Physics" van Urone was mijn eerste universitaire boek. Helemaal in het Engels dan ook nog. "Dat belooft…"
Juni 2008, het zit erop. Ik studeer af als doctor aan de Universiteit Hasselt. Ik verlaat deze universiteit met goede herinneringen. Mijn eerste bedankje gaat eigenlijk uit naar de "ziel" van deze universiteit, een plaats waar het aangenaam studeren en werken is. En wie of wat vormt dan die "ziel"? Je kunt dat niet terug brengen naar één persoon. Ik heb vele mensen leren kennen, allemaal met een hart voor de universiteit. Ik denk natuurlijk in de eerste plaats aan de onderwijsteams, professoren en assistenten, die dagelijks hard werken, voor en achter de schermen, om hun studenten kwaliteitsvol onderwijs te bieden. Maar daar houdt het niet op. De mensen van het secretariaat, van de catering, van materiële voorzieningen, de wijkagent… allemaal dragen ze hun steentje bij aan de fijne sfeer aan "onze" unief. Daarbij zijn er een aantal mensen die me in het bijzonder zullen bijblijven. Zoals Sali, een toffe vent, altijd supervriendelijk en goedgemutst. Of Gerard, altijd wel in de stemming, zelfs voor onze flauwe mopjes aan de cafetariakassa. Het zijn zo van die figuren die, als je hier een tijdje bent, deel gaan uitmaken van het "meubilair". Merci mensen, voor wat jullie dagelijks doen.

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Na 3 jaar, een tijd die omgevlogen is, moest ik een keuze maken voor een stage-onderwerp in het laatste jaar. Gezien mijn voorkeur voor fysiologie, viel mijn oog op een project met "patch-clamp" van prof. Steels. Een techniek waarbij je die miniscule stroompjes over celmembranen kon meten? Dat sprak me wel aan. Het heeft me wat gesakker gekost, maar ik kreeg die "patch-clamp" onder de knie. Hiervoor moet ik zeker Bert Brône, mijn stagebegeleider destijds, bedanken. Dankzij zijn deskundige en geduldige begeleiding en de wijze raad van Prof. Steels kon ik mijn licentiaatsthesis met succes beëindigen. Het einde van mijn stage bleek echter nog maar een begin, in grote mate dankzij prof. Steels.
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I would like to express my gratitude to the members of the jury for their comments. Thank you for your willingness to evaluate this work, your feedback has certainly improved its quality.

Dit zijn dan nu echt de laatste regels van mijn doctoraatsproefschrift. En die zijn gereserveerd voor het belangrijkste in mijn leven: familie en vrienden. Ik heb het geluk een aantal steengoede vrienden te hebben overgehouden aan zowel mijn middelbare school als mijn universitaire studies. Merci mannen, voor alle plezier en alle zwans. Het doet deugd te weten dat ik vrienden heb waarop ik altijd kan rekenen. Ik ben misschien ook één van de eersten die zijn buren

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Roeland

"Door wetenschap bereikt men veel,
doch slechts de liefde voert tot volmaaktheid."
Rabindranath Tagore
Indisch dichter, 1861-1941