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GLYCINE AND GLYCINE RECEPTOR SIGNALING IN IMMUNE CELLS

Proefschrift voorgelegd tot het behalen van de graad van Doctor in de Biomedische wetenschappen, te verdedigen door:

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universiteit hasselt

“Everything should be made as simple as possible, but not simpler”

Albert Einstein (1879-1955)

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LIST OF ABBREVIATIONS

(T)M	Transmembrane domain
5-HT	5-Hydroxytryptamine, serotonine
AA	Amino Acid
Ab	Antibody
Ach	Acetylcholine
AIB	2-Amino isobutyric acid
ALX(-5407)	(N-[(3R)-([1,1′-biphenyl]-4-yloxy)-3-(4-fluorophenyl)propyl]-Nmethylglycine)
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ANOVA	Analysis of variance
APC	Antigen presenting cell
APV	D-2-amino-7-phosphonovalerate
asc	Alanine serine cysteine transporter
ASCT	ASC-like transporter
BBB	blood brain barrier
BDNF	Brain derived neurotrophic factor
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CHO	Chinese hamster ovary
CNS	Central nervous system
CRAC	Calcium release activated calcium currents
CSF	Cerebrospinal fluid
DAO	D-amino acid oxidase
DCKA	2,7-Dichlorokynurenic acid
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
EAE	Experimental autoimmune encephalomyelitis
EC	Extracellular
ECD	Extracellular domain
EPSP	Excitatory postsynaptic potential
ER	endoplasmic reticulum
ERK	Extracellular signal regulated kinase
E_x	Equilibrium or Nernst potential of ion x
FCS	Fetal calf serum
fMLP	*N*-formyl-methionyl-leucyl-phenylalanine
GABA	γ-Amino butyric acid
$GABA_A$-R	GABA receptor, typeA
GCS	Glycine cleavage system
GlyR	Glycine receptor
GlyT1/2	Glycine transporter, type 1/2
GPCR	G-protein coupled receptor
GSH	Glutathion (reduced form)
g_x	Conductance of ion x
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IC	Intracellular

IL	Interleukin
IP_3	Inositol 1,4,5-triphosphate
IPSP	Inhibitory postsynaptic potential
KCC	Potassium chloride exchanger
LGIC	Ligand-gated ion channel
LPS	Lipopolysaccharide
LTD	Long term depression
LTP	Long term potentiation
MEM	Minimal essential medium
MHC	Major histocompatiblity complex
MK-801	Dizocilpine
MPT	Mitochondrial permeability transition
MS	Multiple sclerosis
mTOR	Mammalian target of rapamycin
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
nAchR	Nicotinic receptor
NCX	Sodium calcium exchanger
NFAT	Nuclear factor of activated T cells
NFκβ	nuclear factor κβ
NKCC	Sodium potassium chloride exchanger
NKH	Non-ketotic hyperglycinemea
NMDA	N-methyl-D-aspartic acid
NMDAR	NMDA receptor
NO	Nitric oxide
NRSE	Neuron restrictive silencer element
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PIP_2	Phosphatidylinositol bisphosphate
PGA	Prolong gold antifade
PHA	Phytohaemagglutinin
PKA/C	Protein kinase A/C
PM	Plasma Membrane
PMG	Primary microglial cells
PTN	Picrotin
PTX	Picrotoxin
PXN	Picrotoxinin
RT-PCR	Reverse transcriptase polymerase chain reaction
SERCA	Endoplasmic reticulum calcium ATPase
SHMT	Serine hydroxymethyl transferase
SLC	Solute carrier transporter
SNAT	Sodium-coupled neutral amino acid transporter
SOC	Store operated channel
STS	Staurosporine
TCR	T cell receptor
TEA	Tetraethylammonium

TG	Thapsigargin
TGF	Transforming growth factor
THF	Tetrahydrofolic acid
TNFα	Tumour necrosis factor α
VEGF	Vascular endothelial growth factor
VGCC	Voltage gated calcium channel
VIAAT	Vescular Inhibitory amino acid transporter
V_m	Membrane potential

CHAPTER I. GENERAL INTRODUCTION

Part of this chapter is reported in **Van den Eynden J**, SahebAli S, Horwood N, Carmans S, Brone B, Hellings N, Steels P, Harvey RJ, Rigo JM (2009) Glycine and glycine receptor signaling in non-neuronal cells. Front Mol Neurosci 2:9.

It has been over a century since Santiago Ramón Y Cajal (1852-1932), together with Camillo Golgi (1843-1926) received the Nobel Prize in Physiology or Medicine (1906) for demonstrating that the nervous system was composed of individual neurons, a concept known as the **neuron doctrine** (Andres-Barquin, 2002; Lopez-Munoz et al., 2006; De Carlos and Borrell, 2007; Grant, 2007)). The neuron doctrine implied that contacts exist between neurons and it was Sherrington C.S. (1857-1952) who introduced the term **"synapse"** (literally meaning "joining together") to define the special connections between nerve cells (Raju, 1999a). The neuron doctrine and the existence of synapses also implied that neurons had to be able to communicate and some key experiments, performed by Dale H. (1875-1968) and Loewi O. (1873-1961) in the beginning of the 20th century showed that in the peripheral nervous system communication was achieved by chemical substances (**neurotransmitters**), called *Vagusstoff* and *Acceleransstoff* (later called acetylcholine and adrenaline) (Valenstein, 2002). They received a shared Nobel Prize "for their discoveries relating to the chemical transmission of nerve pulses" in 1936 (Raju, 1999c; Todman, 2008). Ulf Von Euler (1905-1983) proved that the sympathetic neurotransmitter was in fact noradrenaline, instead of adrenaline, leading to a shared Nobel Prize winning in 1970 as well (together with Katz B. and Axelrod J.) (Raju, 1999b). Although the award of the Nobel Prize in 1936 is generally considered as the acceptance of chemical substances being responsible for neurotransmission, the controversy of chemical versus electrical neurotransmission in the CNS (central nervous system) continued until the mid 50s, when the introduction of microelectrode techniques by Hodgin A. & Huxley A. allowed Sir John Eccles (1903-1997) - until then in fact a "disbeliever" of chemical neurotransmission - to definitely conclude that chemicals were responsible for neurotransmission. For these discoveries he received the Nobel Prize in 1963, together with Hodgkin & Huxley (Raju, 1999d). The next decades different neurotransmitters were described. The most important excitatory neurotransmitter, glutamate, was first suggested to be a neurotransmitter by the mid 50s (Watkins and Jane, 2006). Dopamine as a neurotransmitter and its role in Parkinson's Disease was described by the end 50s by A. Carlsson (who shared the 2000 Nobel Prize with Greengard P. And Kandel E.) (Fahn, 2008). The role of GABA (γ amino butyric acid), nowadays considered as the most important inhibitory neurotransmitter, was accepted by the end of the 60s (Bowery and Smart, 2006). Besides their classical role in neurotransmission, increasing evidence now indicates a broader role for neurotransmitter molecules, both inside and outside the CNS. In this respect neurotransmitters were shown to be important for CNS development and regulation of astrocyte function (Nguyen et al., 2001; Nedergaard et al., 2002). Different neurotransmitter receptors were described in non-neural tissues as well, where they were suggested to exert a wide array of functions (*e.g.* immunomodulation, modulation of tumour cell proliferation, blood pressure regulation) (Gill and Pulido, 2001; Nedergaard et al., 2002; Lombardi et al., 2004).

In the 60s and early 70s **glycine** was recognized as an inhibitory neurotransmitter in the caudal part of the CNS. Glycine concentrations were first found to be increased in the spinal cord and suggested to be stored by interneurons. It was subsequently shown that glycine could inhibit action potential firing in spinal neurons and it was demonstrated that glycine could be synthesized by neurons and released upon electrical stimulation (Bowery and Smart, 2006). Strychnine, nowadays known as the most important glycine receptor (GlyR) antagonist, was

known long before the discovery of glycine as a neurotransmitter. It was first isolated in 1818 and already in the end of the 19th century it was shown that there is strychnine accumulation in the spinal cord upon strychnine poisoning (Lovett, 1888). In the beginning of the 20th century, Sherrington found that strychnine could reverse reflex inhibition (Sherrington, 1907). It lasted until the beginning of the 70s when strychnine was shown to be a specific GlyR antagonist in low concentrations (Young and Snyder, 1973; Bowery and Smart, 2006). The GlyR was molecularly purified in 1982 and 3 different polypeptides were identified: a 48 kDa strychnine binding polypeptide (later termed the α-subunit), a 58 kDa polypeptide (later termed the β-subunit) and a 93 kDa polypeptide (later found to be the cytoplasmic GlyR-associated protein gephyrin) (Pfeiffer et al., 1982).

In this chapter the general aspects of glycine biochemistry (section 1) and glycinergic neurotransmission (section 2) will be discussed first. As this work focuses on non-synaptic functions of glycine and glycine receptors, we provide an extensive review of non-neuronal glycine signaling in section 3, leading to the aims of this work described in section 4.

1. GLYCINE BIOCHEMISTRY

Glycine (Gly, G) is a simple, protein-coding, amino acid consisting only of a single carbon atom with a carboxyl group and an amino group attached to it (Figure I.1). It is a neutral amino acid, meaning that at physiologic pH it has a zero net charge because no proton is being donated or accepted by the side chain (which only consists of a hydrogen atom in glycine). Glycine belongs to the family of non-essential amino acids, because it can be synthesized by the human body (Champe and Harvey, 1994).
Glycine concentrations in blood plasma were found to be around 200-250 µM, but they may transiently increase up to 1 mM after oral ingestion of glycine (Divino Filho et al., 1998; Gannon et al., 2002). In the cerebrospinal fluid (CSF) much lower concentrations of around 10 µM were measured (Stover et al., 1997).
This chapter discusses the different metabolic pathways involved in synthesis and degradation of glycine and the roles of glycine as a precursor for other metabolic structures. The most important metabolic pathways are summarized in Figure I.1.

1.1 Glycine synthesis

Glycine can be derived from the normal diet, but as it is a non-essential amino acid, cells have the machinery to synthesize glycine by its own. Glycine can be easily converted to L-serine, which is also a non-essential amino acid. This reaction is catalyzed by the enzyme *serine hydroxymethyl transferase (SHMT)* and needs tetrahydrofolic acid (THF) as a cofactor for methylene transfer, forming N^5, N^{10}-Methylene-THF. Two SHMT isoforms are described, one located in the cytosol (cSHMT), the other located in mitochondria (mSHMT) (Heil et al., 2001).
L-serine is synthesized from the glycolytic intermediate 3-P-glycerate, a multistep reaction in which the first step is catalyzed by the enzyme *3-P-glycerate dehydrogenase.* The crucial role this enzyme plays in L-serine and glycine synthesis can be demonstrated by the existence of a metabolic disorder caused by a deficiency in this enzyme, leading to low CSF concentrations of both amino acids and severe neurologic dysfunction. The disease is treated by oral administration of L-serine, leading to increases of CSF concentrations and improvement in neurologic condition (Jaeken et al., 1996).

1.2 Glycine degradation

Although glycine can be converted to L-serine (which can be further degraded to pyruvate by the enzyme serine dehydratase), the primary route of both glycine and L-serine degradation is by means of the *Glycine Cleavage System (GCS)*, a protein complex located in mitochondria (Kikuchi et al., 2008). This complex consists of 4 proteins: The P, T, H and L-protein. The P-protein catalyzes the decarboxylation, while the T-protein further degrades the decarboxylated glycine molecule and transfers the methyl-group to THF. H-protein is a carrier protein, carrying the glycine being degraded, while the L-protein reoxidizes the H-protein after glycine cleavage. The first three enzymes (P, T and H) are specific for the GCS, whereas the T protein is involved in other multi-enzyme reactions as well (*e.g.* pyruvate dehydrogenase).

The enzyme complex can essentially work in reverse (explaining its alternative name *glycine synthase*), but this has probably no physiological meaning as it is only observed under strict anaerobic conditions *in vitro* or in some anaerobic bacteria (Kikuchi et al., 2008).
Mutations in the P (80%), T (15%) and H (rare) proteins lead to a autosomal recessive metabolic disorder called *non-ketotic hyperglycinemia (NKH)*, characterized by high concentrations of glycine in serum and CSF, hypotonia, apnea, convulsions and early neonatal lethality (with the exception of a late onset form) (Applegarth and Toone, 2001; Kikuchi et al., 2008). Mutations in the L-protein lead to a variant of maple syrup urine disease (MSUD or branched-chain ketoaciduria), characterized by an increased concentration of branched-chain amino acids (valine, leucine and isoleucine) and pyruvate as well (Applegarth and Toone, 2001).

1.3 Glycine as a metabolic precursor

Glycine is a precursor for different metabolic substances. The most important ones are listed here (Champe and Harvey, 1994).

Protein synthesis

Glycine is one of the protein coding amino acids. Glycine binds to tRNA, forming glycyl tRNA, which is incorporated into a signal upon an mRNA GGU, GGC, GGA or GGG codon. It is worth mentioning that glycine is abundantly present in collagen in which glycine is found in every third position of the primary protein structure.

Glutathione synthesis

Glutathione is a tripeptide that consists of glycine, glutamate and cysteine. In its reduced form (GSH) this molecule can chemically detoxify hydrogen peroxide and hence is an important anti-oxidant.

Purine synthesis

Glycine is a necessary component in the synthesis of purines. These nucleotides are basic building blocks for RNA and DNA.

Porphyrine synthesis

Porphyrines are compounds that bind metal ions. The most prevalent in the human body is heme, which is mainly found in hemoglobin, myoglobine and cytochrome complexes. Glycine is an important substrate in the rate limiting step of porphyrine synthesis.

Creatine synthesis

Creatine can be phosphorylated by ATP yielding creatine phosphate. This is a high energy compound in skeletal muscle that provides a rapidly mobilizing reserve of high energy phosphates during intense muscular activity. Glycine is a basic substrate for creatine synthesis.

Thymidine synthesis

Both glycine degradation via the GCS and glycine synthesis from L-serine lead to formation of N^5, N^{10}-Methylene-THF. The latter is a necessary cofactor for methylene transfer from dUMP to dTMP and hence thymidine formation.

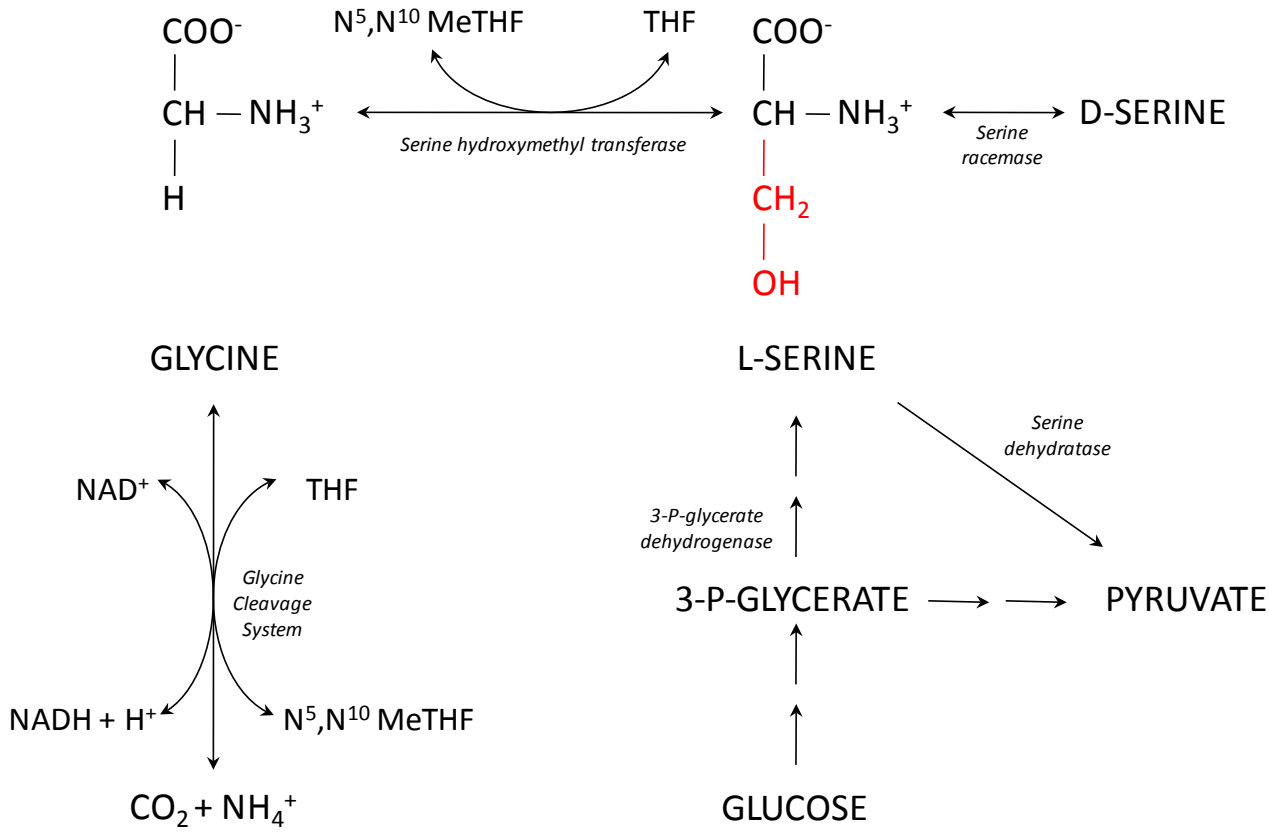

Figure I.1 The most important metabolic pathways involved in glycine synthesis and degradation

2. GLYCINERGIC NEUROTRANSMISSION

This section will first discuss some general aspects of neurotransmitters and their receptors. Next the main aspects of glycinergic inhibitory synapses will be discussed and finally glycine co-agonist functioning on glutamatergic NMDA receptors will be described.

2.1 An introduction to neurotransmitter signaling

As their name implies neurotransmitters transmit signals between neurons. When an action potential is generated in a presynaptic neuron and propagated from the soma along the axon, it will lead to vesicular neurotransmitter release in the synapse. This neurotransmitter then diffuses through the synaptic cleft and binds to its postsynaptic receptors. In the case of excitatory neurotransmission this causes cation influxes and hence depolarizations or excitatory postsynaptic potentials (EPSPs), while in the case of inhibitory neurotransmission this causes anion influxes and hence hyperpolarizations or inhibitory postsynaptic potentials (IPSPs). In some neurons, where the anion equilibrium potential is very close to the membrane potential, anion fluxes do not really change the membrane potential, but rather provide a shunt effect, characterized by a drop in membrane resistance, which tends to clamp the membrane potential near the resting potential end prevent EPSPs from depolarizing the cells (Fain, 1999). If the sum of different EPSPs and IPSPs reaches a certain threshold of depolarization, an axon potential can be generated in the soma or axon hillock of the postsynaptic neuron and the signal can be propagated again. After the action of the neurotransmitter is ended it can be taken up again by the presynaptic neuron by means of neurotransmitter plasma membrane transporters, after which recycling or degradation can occur. An overview of this process is given in Figure I.2 (Kandel et al., 2000).

2.1.1 Different classes of neurotransmitters

Chemically neurotransmitters fall into two main classes: the small molecule transmitters and the neuropeptides. *Small molecule transmitters* can be amino acids (glycine, GABA or glutamate), amines (dopamine, (nor)epinephrine, serotonine or histamine), acetylcholine or ATP (or one of its metabolites, such as adenosine). These small molecules can be taken up or synthesized by neurons and stored in synaptic vesicles. *Neuropeptides* (*e.g.* substance P, enkephalins, …) on the other hand are larger molecules that are stored in secretory granules, which are frequently colocalized with synaptic vesicles in the same axon terminal. The latter will not be further discussed here (Kandel et al., 2000).

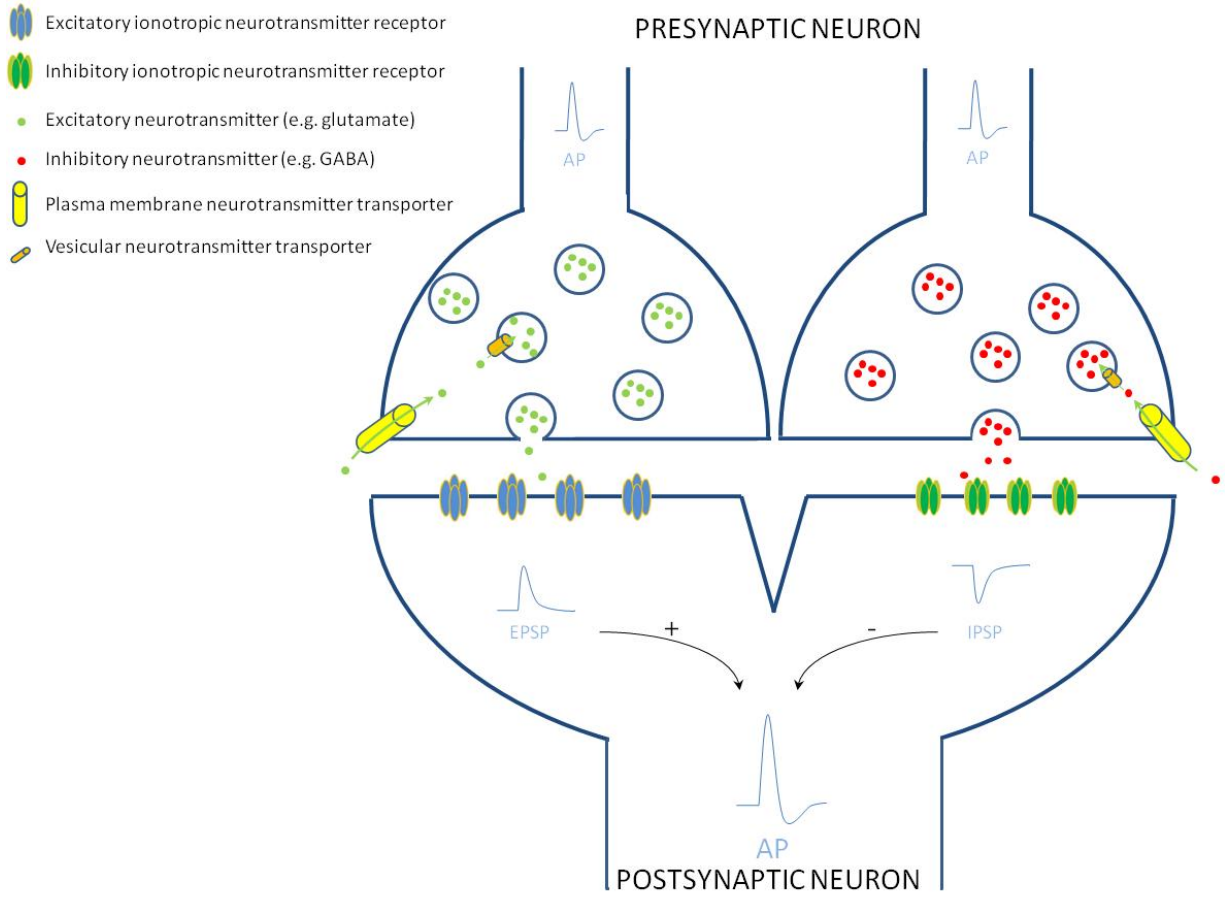

Figure I.2 General process of neurotransmitter signaling.
When a presynaptic neuron is excited, action potentials (AP) will cause vesicular release of neurotransmitters. These neurotransmitters can be excitatory (shown in green on the left), or inhibitory (shown in red on the right). Binding of neurotransmitter molecules to their receptor will lead to ion fluxes, which, depending on the receptor will lead to membrane potential changes in the dendritic membrane of the postsynaptic neuron. An excitatory neurotransmitter (receptor) will lead to EPSPs and an inhibitory neurotransmitter will lead to IPSPs. If the sum of all IPSP and EPSP reaches a threshold, an AP can be generated again in the postsynaptic neuron and the signal can be propagated again. After neurotransmitter binding to its receptor, it can be taken up again by presynaptic membrane transporters after which the molecule can be degraded by neuronal enzymes or recycled again into synaptic vesicles by vesicular transporters.

2.1.2 Different types of synapses

Chemical synapses can be excitatory, inhibitory or modulatory. The most important excitatory synapse is the glutamatergic synapse where glutamate binding to its ionotropic receptor causes cation influx in the postsynaptic neuron and hence EPSPs. The most important inhibitory synapse is the GABAergic synapse where binding of GABA to its ionotropic receptor mostly causes anion influx and hence IPSPs (or shunting). A third kind of synapses are modulatory synapses. Four major modulatory neurotransmitter systems are known in the human brain: the dopaminergic, cholinergic, noradrenergic and serotoninergic. These neurons arise in different nuclei in the brainstem and diffusely project to the cerebral cortex or spinal cord. The function of these systems is to modify signal propagation in neurons, rather than excite or inhibit neurons directly. This can be achieved through binding to metabotropic, rather than ionotropic receptors (see infra), leading to G protein activation, changes in second

messengers and finally an enhancement or an inhibition of neuron excitability (Boron and Boulpaep, 2003). The importance of these systems can be understood by the fact that most clinically available neuropharmacological agents work on these systems.

2.1.3 Neurotransmitter receptors

Neurotransmitter receptors can be divided into two classes: ionotropic and metabotropic receptors.

Ionotropic receptors

Ionotropic receptors are in fact ion channels that are opened upon ligand binding, explaining that they are also known as ligand-gated ion channels (LGICs). Depending on the characteristics of the ion channel, anions or cations will flow through the channel in or outside of cells, depending on their electrochemical gradient. This will lead to membrane depolarization (or EPSPs) or hyperpolarization (or IPSPs), and hence is responsible for the actions of excitatory or inhibitory neurotransmission. The LGICs can be divided into three families: the Cys-loop superfamily, the glutamate receptors and the ATP-gated channels (Figure I.3) (Collingridge et al., 2009) (some consider the transient receptor potential (TRP) channels as a forth family (Connolly and Wafford, 2004)). In mammals the *Cys-loop superfamily* consists of Nicotinic, $GABA_{A/C}$, Glycine and $5HT_3$ (or serotonine) receptors. All these receptors are pentameric structures forming a central ion pore, with each subunit being composed of a large extracellular N-terminus, 4 transmembrane (TM) domains and a short extracellular C-terminus. The *glutamate receptors* consist of α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), *N*-methyl-D-aspartate (NMDA) and Kainate receptors. The receptors are tetrameric structures, with each subunit being composed of 3 TM domains (with a re-entrant helical loop between the first and the second TM domain, which can actually be considered as a fourth TM domain). The *ATP-gated channels* (P2x receptors) are trimeric structures consisting only of two TM domains.

Metabotropic receptors

Metabotropic receptors will lead to activation of G proteins upon ligand binding, explaining their alternative name G protein coupled receptors (GPCRs). G proteins are intracellular membrane bound proteins, which upon activation, lead to the release of second messengers. The most important second messengers are calcium and cyclic adenosine monophosphate (cAMP). Calcium is released from the endoplasmic reticulum (ER) after inositol 1,4,5-triphosphate (IP_3) binding to its receptor. IP_3 is cleaved from the membrane-bound phosphatidyl inositol diphosphate (PIP_2) by phospholipase C, which is activated by a G protein called Gq. cAMP is generated from ATP by the action of adenylcyclase, which is activated by a G protein called Gs (or inhibited by Gi). Molecularly GPCRs are composed of seven membrane spanning domains with an extracellular N-terminal and an intracellular C-terminal. It is remarkable that these receptors constitute the largest superfamily of cell surface receptors in the mammalian genome, making up approximately 1% of the entire human genome (Luttrell, 2006)! For a lot of these GPCR, called orphan GPCRs, the natural ligand is unknown yet (Civelli et al., 2001; Lin and Civelli, 2004).

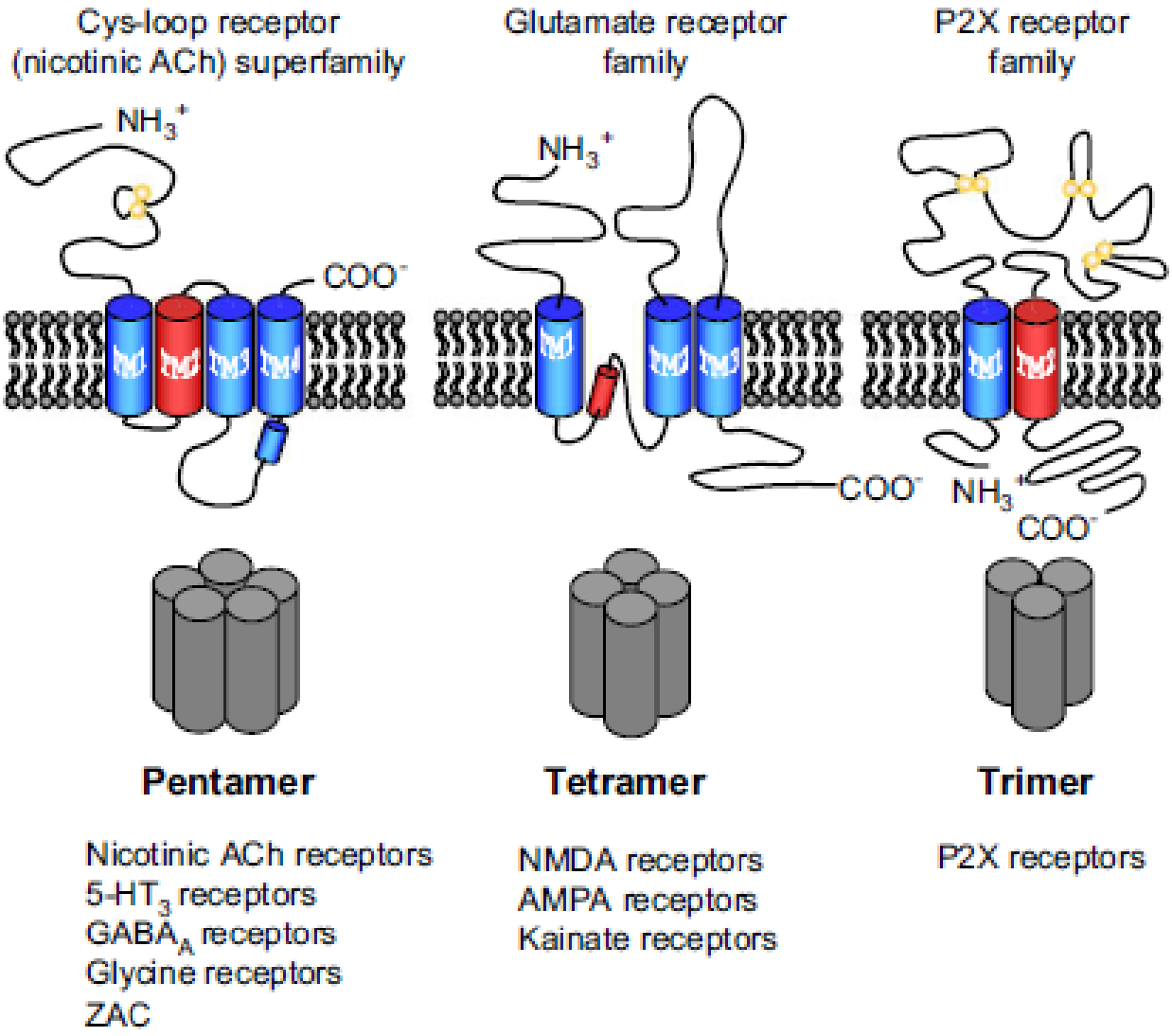

Figure I.3 Schematic representation of the three families of ligand-gated ion channels.
See text for explanation (Collingridge et al., 2009)

2.1.4 Neurotransmitter transporters

Although other mechanisms for inactivation of neurotransmitter substances are known (such as enzymatic degradation, *e.g.* cholinesterase for Ach), the most common mechanism is reuptake (Kandel et al., 2000). Besides terminating the action of the neurotransmitter, this uptake also recaptures the neurotransmitter for possible reuse. In the latter case neurotransmitter substances are taken up again in synaptic vesicles by means of vesicular neurotransmitter transporters.

Two main subclasses of *neurotransmitter plasma membrane transporters* are known: the solute carrier (SLC) 1 transporter family and the SLC 6 family, with the first family mediating glutamate transport and the second family transporting GABA, glycine, dopamine, noradrenaline and 5-HT. Both families are Na^+-dependent cotransporters. However, while the SLC 1 transporters are characterized by K^+-antiport, the SLC6 transporters are characterized by Cl^--cotransport. Structurally the SLC 1 family is composed of trimers with subunits

containing six transmembrane domains, while SLC 6 family is composed of dimers with each subunit composed of 12 TM domains. These transporter systems are of clinical importance as they are the targets of many routinely used drugs and substances of abuse (*e.g.* antidepressants, cocaine) (Gether et al., 2006).
Three subclasses of *vesicular transporters* are known: the vesicular amine transporters (SLC 18 family), the vesicular inhibitory amino acid transports (VIAAT, SLC 32) and the vesicular glutamate transporters (SLC 17) (Gether et al., 2006).

2.2 Glycinergic inhibitory neurotransmission

GABA and glycine are the main components mediating fast inhibitory neurotransmission in the CNS. While GABA, interacting with $GABA_A$ receptors ($GABA_ARs$) acts on the rostral part, glycine-mediated transmission is mainly found in the caudal part (brain stem and spinal cord), although GABA/glycine co-release has been described in the spinal cord and brain stem as well (Seal and Edwards, 2006). In the glycinergic synapse, the molecule interacts with ligand-gated ion channel receptors (GlyRs), leading to a postsynaptic hyperpolarization induced by chloride influx, explaining its inhibitory properties (Figure I.4). Glycine transporters (GlyT) are responsible for regulation of synaptic glycine concentrations, recycling and degradation. The normal physiological function of GlyRs and glycinergic synapses is to provide neuronal inhibition, necessary for a balanced regulation of motor and reflex responses and spinal processing of sensory information (*e.g.* pain). In this section the main components of the glycinergic synapse, the GlyRs and the GlyTs will be discussed.

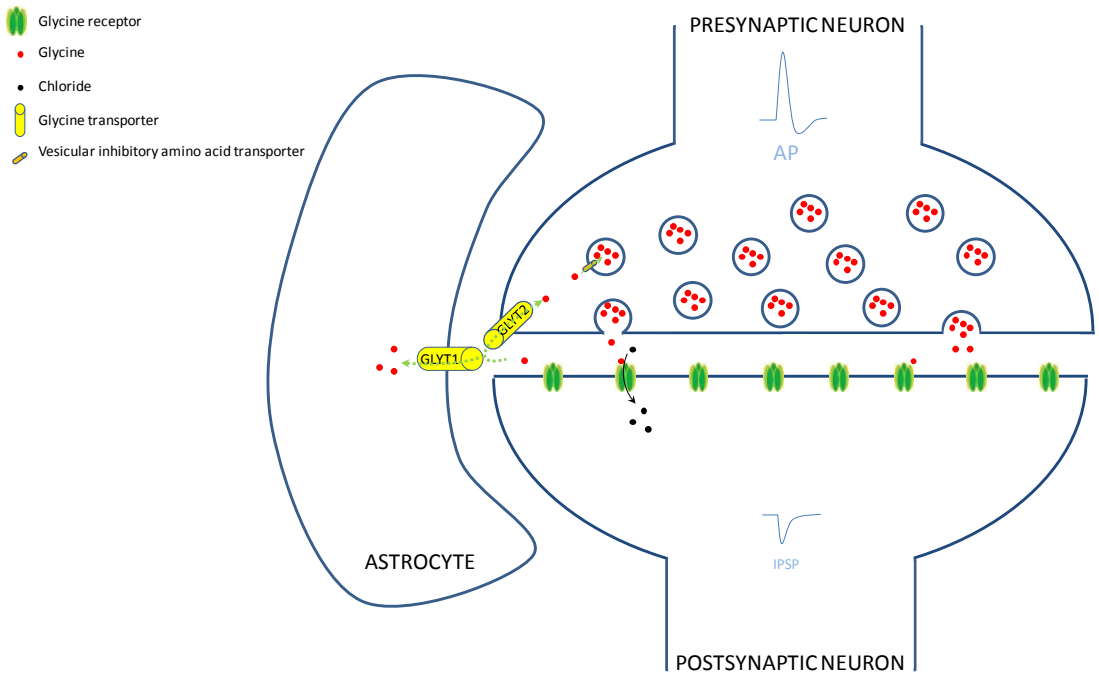

Figure I.4 Glycinergic synapse.
When a glycinergic neuron is excited in the adult CNS, action potentials (AP) will cause vesicular release of glycine. Binding of glycine to its glycine receptor will lead to chloride influx, which leads to IPSPs and hence inhibitory neurotransmission. After glycine binding to its receptor, it can be taken up again by neuronal type 2 glycine transporters or by astrocytic type 1 glycine transporters.

2.2.1 Glycine receptors

2.2.1.1 Molecular structure

Glycine receptors belong to the cys-loop family of LGICs. The prototypic receptor of this superfamily is the nicotinic acetylcholine receptor (nAchR). Recently the crystallographic structure of the ligand binding domain of the nAchR was described, by using the acetylcholine-binding protein (AchBP) as a structural and functional homologue of the N-terminal domain of nAchR α subunits (Brejc et al., 2001).

Like all members of the cys-loop family of LGICs GlyRs are composed of five subunits, with each subunit being composed of a large N-terminal domain, 4 TM domains ((T)M1-4) and a small C-terminal end (Figure I.5A). These five subunits form a central chloride channel, with the second transmembrane domain (M2) lining the channel.

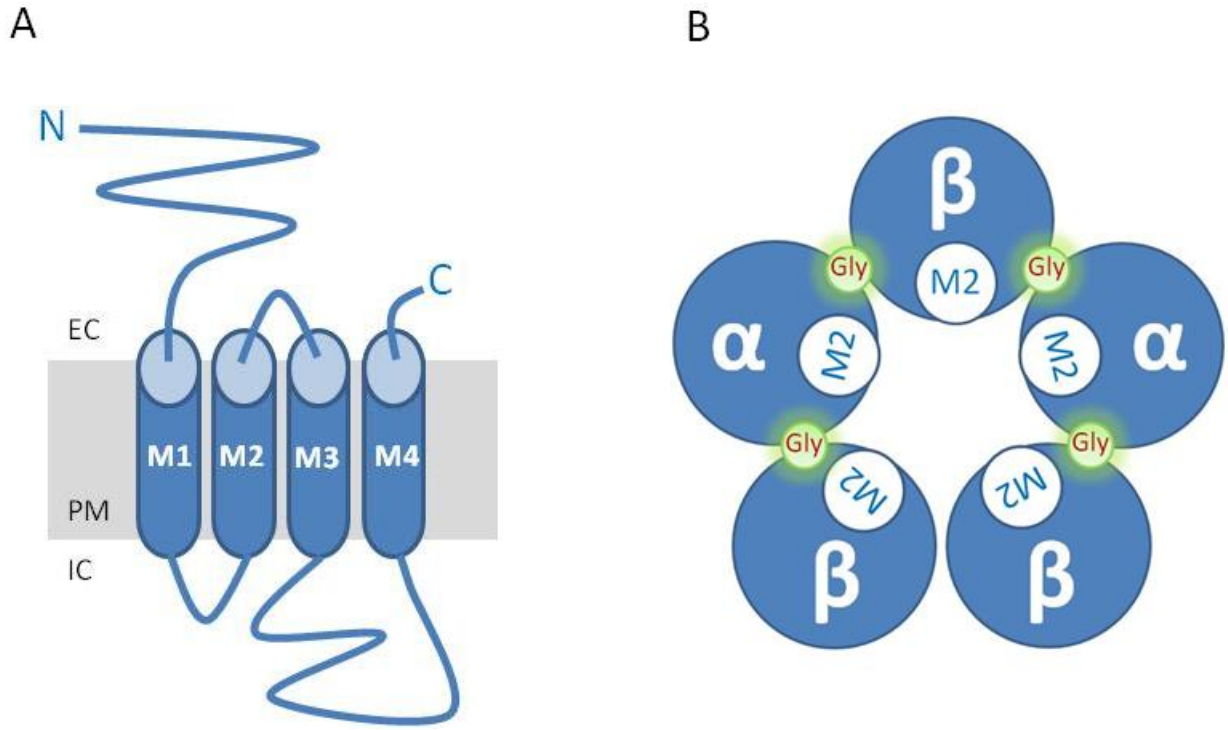

Figure I.5 Schematic representation of glycine receptor molecular structure and amino acid sequences.
A) Transmembrane topology of the GlyR subunit.
B) Pentameric structure of GlyR heteromers. Five GlyR subunits are arranged around a central chloride channel, with the second transmembrane domain (M2) lining the channel. Glycine binding pockets are located in the interface of αβ or βα interfaces in heteromeric receptors (or at αα interfaces in homomeric receptors).
C) (See next page) Amino acid sequences of different human GlyR subunits were aligned with UniProt (www.uniprot.org) (accession numbers are P23415, P23416, O75311, Q5JXX5 and P48167 for α1, α2, α3, α4 and β-subunits respectively) Part of the nicotinic receptor α7 subunit (CHRNA7, accession number P36544) was aligned as well for comparison. Marked in brown is the subunit signal peptide, and in light blue the 4 TM domains. Cystein residues responsible for the cystein loop are underlined and bold. See text for explanation of other colours*.

* Amino acid numbering includes the signal peptide. This may explain differences with numbering in other references where the signal peptide is not included in the numbering.

C

```
GLRA1_HUMAN       ----MYSFNTLR--LYLWETIVFFSLAASKEAEAARSAPKPMSPSDFLDKLMGRTSG--- 51
GLRA2_HUMAN       MNRQLVNILTALFAFFLETNHFRTAFCKDHDSRSGKQPSQTLSPSDFLDKLMGRTSG--- 57
GLRA3_HUMAN       -MAHVRHFRTLVSGFYFWEAALLLSLVATKETDSARSRSAPMSPSDFLDKLMGRTSG--- 56
GLRA4_HUMAN       MTTLVPATLSFLLLWTLPGPLLSRVALAKEEVKSGTKGSQPMSPSDFLDKLMGRTSG--- 57
GLRB_HUMAN        MKFLLTTAFLILISLWVEEAYSKEKSSKKGKGKKKQYLCPSQQSAEDLARVPANSTSNIL 60

GLRA1_HUMAN       ------YDARIRPNFKGPPVNVSCNIFINSFGSIAETTMDYRVNIFLRQQWNDPRLAY-N 104
GLRA2_HUMAN       ------YDARIRPNFKGPPVNVTCNIFINSFGSVTETTMDYRVNIFLRQQWNDSRLAY-S 110
GLRA3_HUMAN       ------YDARIRPNFKGPPVNVTCNIFINSFGSIAETTMDYRVNIFLRQKWNDPRLAY-S 109
GLRA4_HUMAN       ------YDARIRPNFKGPPVNVTCNIFINSFSSITKTTMDYRVNVFLRQQWNDPRLSY-R 110
GLRB_HUMAN        NRLLVSYDPRIRPNFKGIPVDVVVNIFINSFGSIQETTMDYRVNIFLRQKWNDPRLKLPS 120

GLRA1_HUMAN       EYP-DDSLDLDPSMLDSIWKPDLFFANEKGAHFHEITTDNKLLRISRNGNVLYSIRITLT 163
GLRA2_HUMAN       EYP-DDSLDLDPSMLDSIWKPDLFFANEKGANFHDVTTDNKLLRISKNGKVLYSIRLTLT 169
GLRA3_HUMAN       EYP-DDSLDLDPSMLDSIWKPDLFFANEKGANFHEVTTDNKLLRIFKNGNVLYSIRLTLT 168
GLRA4_HUMAN       EYP-DDSLDLDPSMLDSIWKPDLFFANEKGANFHEVTTDNKLLRIFKNGNVLYSIRLTLI 169
GLRB_HUMAN        DFRGSDALTVDPTMYKCLWKPDLFFANEKSANFHDVTQENILLFIFRDGDVLVSMRLSIT 180

GLRA1_HUMAN       LACPMDLKNFPMDVQTCIMQLFSFGYTMNDLIFEWQEQGA-VQVADGLTLPQFILK-EEK 221
GLRA2_HUMAN       LSCPMDLKNFPMDVQTCTMQLESFGYTMNDLIFEWLSDGP-VQVAEGLTLPQFILK-EEK 227
GLRA3_HUMAN       LSCPMDLKNFPMDVQTCIMQLESFGYTMNDLIFEWQDEAP-VQVAEGLTLPQFLLK-EEK 226
GLRA4_HUMAN       LSCLMDLKNFPMDIQTCTMQLESVGYTMKDLVFEWLEDAPAVQVAEGLTLPQFILR-DEK 228
GLRB_HUMAN        LSCPLDLTLFPMDTQRCKMQLFSFGYTTDDLRFIWQSGDP-VQLEK-IALPQFDIKKEDI 238

GLRA1_HUMAN       DLRYCTKHYN-TGKFTCIEARFHLERQMGYYLIQMYIPSLLIVILSWISFWINMDAAPAR 280
GLRA2_HUMAN       ELGYCTKHYN-TGKFTCIEVKFHLERQMGYYLIQMYIPSLLIVILSWVSFWINMDAAPAR 286
GLRA3_HUMAN       DLRYCTKHYN-TGKFTCIEVRFHLERQMGYYLIQMYIPSLLIVILSWVSFWINMDAAPAR 285
GLRA4_HUMAN       DLGCCTKHYN-TGKFTCIEVKFHLERQMGYYLIQMYIPSLLIVILSWVSFWINMDAAPAR 287
GLRB_HUMAN        EYGNCTKYYKGTGYYTCVEVIFTLRRQVGFYMMGVYAPTLLIVVLSWLSFWINPDASAAR 298
CHRNA7_HUMAN                                                        DSG-EK

GLRA1_HUMAN       VGLGITTVLTMTTQSSGSRASLPKVSYVKAIDIWMAVCLLFVFSALLEYAAVNFVS---- 336
GLRA2_HUMAN       VALGITTVLTMTTQSSGSRASLPKVSYVKAIDIWMAVCLLFVFAALLEYAAVNFVS---- 342
GLRA3_HUMAN       VALGITTVLTMTTQSSGSRASLPKVSYVKAIDIWMAVCLLFVFSALLEYAAVNFVS---- 341
GLRA4_HUMAN       VGLGITTVLTMTTQSSGSRASLPKVSYVKAIDIWMAVCLLFVFAALLEYAAINFVS---- 343
CHRNA7_HUMAN      ISLGITVLLSLTVFMLLVAE

GLRA1_HUMAN       RQHKELLRFRRKRR-HHKSPMLNLFQ-----EDEAGEGRFNFSAYGMGPACLQAKD---- 386
GLRA2_HUMAN       RQHKEFLRLRRRQKRQNKE-----------EDVTRESRFNFSGYGMGH-CLQVKD---- 385
GLRA3_HUMAN       RQHKELLRFRRKRKNKTEAFALEKFYRFSDMDDEVRESRFSFTAYGMGP-CLQAKD---- 396
GLRA4_HUMAN       RQHKEFIRLRRRQRRQRLE-----------EDIIQESRFYFRGYGLGH-CLQARD---- 386
GLRB_HUMAN        RVEAEKARIAKAEQADGKGGNVAKKNTVNGTGTPVHISTLQVGETRCKKVCTSKSDLRSN 418

GLRA1_HUMAN       --------------------GISVKGANNSNTTNPPPAPSKSPEEMRKLFIQRAKKIDKI 426
GLRA2_HUMAN       --------------------GTAVKA-TPAN-PLPQPP--KDGDAIKKKFVDRAKRIDTI 421
GLRA3_HUMAN       --------------------GMTPKGPNHPVQVMP-----KSPDEMRKVFIDRAKKIDTI 431
GLRA4_HUMAN       --------------------GGPMEG-SGIYSPQPPAPLLREGETTRKLYVD-AKRIDTI 424
GLRB_HUMAN        DFSIVGSLPRDFELSNYDCYGKPIEVNNGLGKSQAKNN--KKPPPAKPVIPTAAKRIDLY 476

GLRA1_HUMAN       SRIGFPMAFLIFNMFYWIIYKIVRREDVHNQ-- 457
GLRA2_HUMAN       SRAAFPLAFLIFNIFYWITYKIIRHEDVHKK-- 452
GLRA3_HUMAN       SRACFPLAFLIFNIFYWVIYKILRHEDIHQQQD 464
GLRA4_HUMAN       SRAVFPFTFLIFNIFYWVVYKVLWSEDIHQAL- 456
GLRB_HUMAN        ARALFPFCFLFFNVIYWSIYL------------ 497
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The first purification of the GlyR revealed three different subunits of different molecular weight: 48 kda, 58 kda and 93 kda (Pfeiffer et al., 1982). The first two of these subunits correspond to a 48 kDa α and a 58 kDa β subunit, while the 93 kDa subunit corresponds to the anchoring protein gephyrin. GlyRs can be homomeric, being only composed of α subunits or heteromeric, being composed of both α and β subunits. Homomeric β subunit containing receptors have not been described. Four types of α subunits (called α1 to α4, with a homology

of 75-81% between human GlyR α subunits[*]), but only one type of β subunit has been described so far:

- α1 subunits are mainly found in the spinal cord and the brain stem of the mature CNS where they form heteromeric α1β GlyRs (Malosio et al., 1991). The stoichiometry of this receptor was long thought to be 3α2β, although recent evidence indicates a probable stoichiometry of 2α3β (Grudzinska et al., 2005).
- α2 subunits are expressed more diffusely in the immature CNS with its expression decreasing around birth (Malosio et al., 1991). In the fetal CNS α2-subunits form homomeric glycine receptors. These receptors are suggested to have slower kinetic properties, suggesting non-synaptic functioning (Mangin et al., 2003).
- α3 subunits are only expressed very weakly in the mature CNS, with a main location in the cerebellum, olfactory bulb and hippocampus (Malosio et al., 1991), but also in the dorsal horn of the spinal cord where they may be implicated in pain modulation (Harvey et al., 2004).
- α4 subunits were first described in 1996 and were later found to be mainly expressed in the embryonic spinal cord and developing genital ridge of mice and chicken (Matzenbach et al., 1994; Harvey et al., 2000). In human, a genome search showed that this subunit has a premature stopcodon in exon nine, just upstream of the 4th TM domain, thus deleting this domain, making it unlikely that this subunit can be functional in human (Simon et al., 2004).
- β subunits share only 44-46% homology with α-subunits[*]. They were found to be far more diffusely expressed in the CNS, compared to α subunits. As these subunits cannot form functional channels by themselves, and there is no evidence so far it can assemble with subunits other than those of GlyRs, the physiological significance of this diffuse expression remains unknown. They are involved in gephyrin binding and in ligand binding in heteromeric receptors as will be discussed further.

The originally purified 93 kDa protein was later identified as gephyrin (Greek, meaning "bridge"), an intracellular scaffold protein that anchors GlyRs to subsynaptic microtubules (Prior et al., 1992). Gephyrin is primarily responsible for GlyR clustering at synapses. Molecularly the protein consists of 3 domains: an N-terminal G-domain, a C-terminal E-domain and a central C-domain. The G- and E-domains are named after their bacterial homologs mogA and moeA respectively, enzymes involved in the synthesis of a molybdenum-containing co-factor, essential for the activity of molybdoenzymes. The C-domain is a linker region with no bacterial homolog. Gephyrin binds to short sequence motif of 18 amino acids in the intracellular loop between M3 en M4 of the β-subunit (Meyer et al., 1995) (see yellow marking in Figure I.5C). The current view is that gephyrin forms trimers that bind a single GlyR at each of its β-loops (Fritschy et al., 2008). This is in agreement with the recently suggested stoichiometry of 2α3β for glycine heteromeric receptors (Grudzinska et al., 2005). Besides this anchoring role, also in eukaryotes, gephyrin is involved in the synthesis of a molybdenum-containing cofactor, essential for the activity of molybdoenzymes (*e.g.* sulfite oxidase and xanthine dehydrogenase) (Feng et al., 1998).

[*] Based on a protein sequence blast on www.uniprot.org, with accession numbers indicated in the legend of Figure I.5

2.2.1.2 Functional properties

The chloride electrochemical gradient

In the mature nervous system glycine acts as an inhibitory neurotransmitter. This can be easily understood by the fact that chloride is flowing inside of cells upon GlyR activation, causing a cellular hyperpolarization and hence inhibition of neuronal firing. However, in neurons of the fetal CNS and in some non-neuronal cells (see section 3) GlyR activation causes chloride efflux and hence cellular depolarization. To understand this difference, some fundamental aspects of electrochemical gradients are considered first.

The diffusion of an ion depends on both its concentration and its electrical gradient. For chloride this means that when the ion is flowing from a high chloride to a low chloride hypothetical compartment, this will cause the low chloride compartment to have a negative potential and the high chloride one to have a positive potential (because cations stay behind). This increasing potential difference will counterforce the concentration gradient until no net chloride is flowing anymore. The chloride is said to be in equilibrium. The potential difference that is reached in equilibrium across cellular membranes at is called the equilibrium potential or the Nernst potential and is given by the following equation:

$$(1)\quad E_{Cl} = \frac{RT}{ZF} mV \ln \frac{[Cl]_o}{[Cl]_i}$$

Where $[Cl]_o$ stands for the extracellular chloride concentration and $[Cl]_i$ stands for the intracellular chloride concentration. R is the universal gas constant, T is the absolute temperature, z is the charge of the ion (-1 in the case of chloride) and F is Faraday's number.

According to Ohm's law a current is caused by a potential difference and a conductance (g). For chloride the potential difference is given by the difference of the total membrane potential (V_m) and the chloride equilibrium potential (E_{Cl}). This means that the chloride current (i_{Cl}) is determined by the following equation, derived from Ohm's law:

$$(2)\quad i_{Cl} = g_{Cl} \times (V_m - E_{Cl})$$

Upon GlyR activation the chloride conductance increases. According to the latter equation and depending on the chloride Nernst potential three situations can arise (Figure I.6):

- $V_m < E_{cl}$: In this case the chloride current becomes negative, by convention meaning that chloride starts flowing outside of cells, causing the cells to depolarize. This is the case in neurons of the fetal CNS.
- $V_m = E_{cl}$: In this case there is no net chloride current. However, because of the increase in total membrane conductance, it will be harder for other ions (*e.g.* sodium) to shift the membrane potential. The membrane potential is said to be "shunted".
- $V_m > E_{cl}$: In this case the chloride current becomes positive, by convention meaning that chloride starts flowing inside of cells, causing the cells to hyperpolarize. This is the case in neurons of the mature CNS.

This means that with a relative high chloride Nernst potential, cells will depolarize and with a low Nernst potential they will hyperpolarize. According to equation (1) and assuming that the extracellular chloride concentration remains constant, this means that a relative high

intracellular chloride concentration corresponds to a higher (or less negative) Nernst potential and vice versa. Now we know that the intracellular chloride concentration determines the consequences of GlyR activation, and hence explains differences in membrane potential changes in neurons of the mature and the fetal CNS, an important question is how this intracellular chloride is regulated and where the differences between the mature and the immature CNS come from. In the mature CNS the intracellular chloride concentration is kept relatively low, because of the activity of potassium-chloride cotransporters (KCC2), transporting chloride out of cells. The expression of these transporters is upregulated after birth (Lu et al., 1999). On the other hand in the immature CNS, the chloride concentration is rather determined by the activity of sodium-potassium-chloride cotransporters (NKCC1), transporting chloride inside of cells and hence increasing intracellular chloride concentrations. The expression of these transporters is downregulated around birth (Plotkin et al., 1997). So apparently around birth some developmental switch occurs, causing the expression of NKCC1 to be downregulated and KCC2 to be upregulated (Stein and Nicoll, 2003).
A summary of these events is given in Figure I.6.

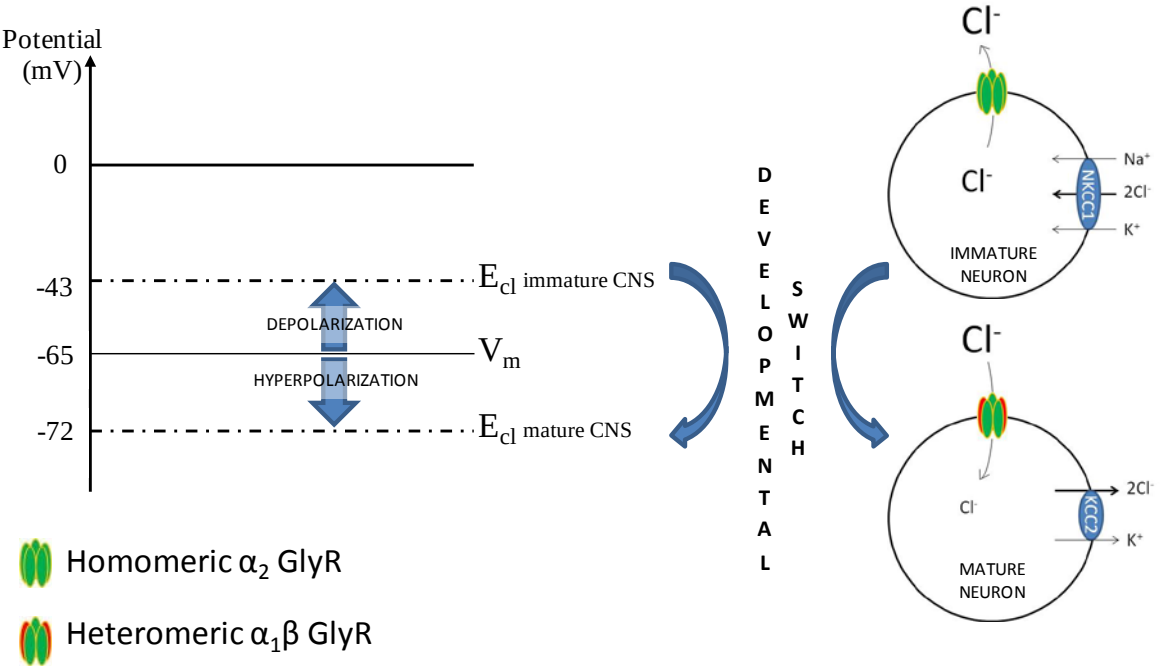

Figure I.6 Chloride gradient dependence of membrane potential changes upon GlyR activation.
In the immature neuron NKCC1 expression leads to intracellular chloride accumulation up to 30 mM (Owens et al., 1996). Assuming an extracellular chloride concentration of 150 mM, this yields a chloride Nernst potential of -43 mV, which, when taken into account a typical neuronal membrane potential of -65 mV, leads to membrane depolarization. Around birth some developmental switch occurs, leading to downregulation of NKCC1 and upregulation of KCC2, leading to decreasing intracellular chloride concentrations to around 10 mM. This yields a chloride Nernst potential of -72mV, which leads to membrane hyperpolarization. Note that there's also a developmental switch in GlyR expression, changing from homomeric α_2 GlyRs to heteromeric $\alpha_1\beta$ GlyRs.

The dependence of the consequences of GlyR activation upon chloride gradients also has clinical importance. It has been shown that alterations in chloride gradients may be important in some neurological disorders and their treatment (De Koninck, 2007). In this context it was shown that some therapy resistant forms of *temporal lobe epilepsy* may be the result of an altered chloride gradient because of KCC2 downregulation and NKCC1 upregulation (Palma et al., 2006). This is a very important finding, as this completely changes the therapeutic

approach for these patients. Most anti-epileptics (*e.g.* benzodiazepines) aim at increasing GABA transmission in the CNS. In patients with altered chloride gradients, this would rather have excitatory effects, exactly the opposite of the therapeutic aim! This mechanism may also be of importance in the pathophysiology of *neuropathic pain*, where it was found that there was a shift in the chloride gradient. Interestingly this shift was caused by microglial brain derived neurotrophic factor (BDNF). BDNF was found to decrease KCC2 expression in neurons and blocking of its Trkβ receptor, but also blocking ATP induced microglial activation, could reverse the effects (Coull et al., 2005). These findings may open a completely new field of therapeutic approaches (*e.g.* aimed at TrkB receptors or microglial purinergic receptors) in these conditions.

GlyR ligand binding

Glycine binding occurs in the N-terminal extra-cellular domain of the receptor subunit. The longstanding view was that binding occurs exclusively at the α-subunit and that β-subunits are not involved in ligand binding (Legendre, 2001). It was demonstrated that different amino acid residues of the α-subunit ECD were essential for ligand binding, shown for α1 in light green on Figure I.5C. Based on site-directed mutagenesis, combined with homology modeling based on the crystal structure of the AChBP, Grudzinska and co-authors recently showed that glycine binding occurs in the interface of different subunits, as could be expected based on the crystallographic data on the homologous soluble AChBP (Brejc et al., 2001). According to this view, ligand binding occurs in a binding pocket at the subunit interface between identical α-subunits in a homomeric receptor or between an αβ or βα interface in a heteromeric receptor (Figure I.5C)[*]. It was shown that some highly conserved amino acids of both α- and β-subunits participate in ligand binding via strong ionic interactions. The authors found that specific arginine residues (R shown in dark green on Figure I.5C) ionically interact with glycine α-carboxyl group, while glutamate residues (E shown in dark green on Figure I.5C) interact with its α-amino group (Grudzinska et al., 2005). The amino acids that were previously already described to be involved in ligand binding (in light green on Figure I.5C) were shown to be important for further stabilization of ligand binding by means of hydrogen bonds or cation-π interactions or for stabilization of arginine guanidium groups.

Functional properties of the anion pore

GlyR are strongly selective for anions. Besides chloride they also conduct bicarbonate, although with a 10 times lower permeability. It was estimated that the smallest diameter of the pore is between 5 and 6 Å. As noted previously the second TM domain (M2) is the area of the subunit lining the pore. This domain is arranged as an α-helical structure. By means of the substituted cysteine accessibility method (SCAM) certain amino acid residues were identified as pore-lining residues, forming a hydrophilic strip along one side of an otherwise hydrophobic α-helix (shown as red fonts in the M2 domain of α1 in Figure I.5C) (Fain, 1999; Lynch, 2004).

As GlyRs belong to the family of Cys-loop receptors, but contrary to certain other members (*e.g.* nicotinic or $5HT_3$ receptors) is permeable to anions, one may question what property of

[*] Because both sides of GlyR subunits (*i.e.* the (-) side and the (+) side) contribute to ligand binding, both αβ and βα interfaces are possible in a heteromeric receptor.

the channel determines this selectivity. When comparing M2 region amino acid sequences of GlyR subunits with Nicotinic α7 subunits several differences can be noticed. In particular at *position 278* of the α1 subunit (and homolog positions of other GlyR subunits), a proline (or alanine for the β-subunit) is present, while absent at the homolog position of the nicotinic receptor subunit (shown in purple on Figure I.5C). As this position is just upstream of the α-helical M2 region, this extra amino acid twists the helix, making other amino acids exposed to the channel wall. By means of mutational analysis it could indeed be shown that nicotinic receptors changed their selectivity from cation to anions upon insertion of a proline at this position and vice versa for GlyRs (Lynch, 2004). Another amino acid that may be important is the neutral alanine residue at *position 279* of the α1 subunit, corresponding to a negatively charged glutamate at the homolog position of the NicR (shown in purple on Figure I.5C). It was suggested that neutral or positively charged amino acids at this position are responsible for anion selectivity, while negative charged ones are responsible for cation selectivity (Lynch, 2004).

GlyR Agonists

Glycine is the main agonist acting on synaptic inhibitory GlyRs in the CNS. Electrophysiological experiments with recombinant α1-GlyRs expressed on *Xenopus* oocytes have given an EC_{50}-value of 0.2mM. It has to be noted however that in other expression systems (*e.g.* HEK293 cells) these values were found to be lower. β-alanine and taurine are 2 partial agonists acting at GlyRs (although taurine is less potent compared to β-alanine). These agonists were in fact shown to be endogenous agonists on non-synaptic GlyRs in the hippocampus where they sustain a tonic background chloride current, which was suggested to change the kinetics of fast synaptic potentials (Mori et al., 2002). In *Xenopus* oocytes EC_{50}-values for β-alanine and taurine were 0.6 mM and 1.7 mM respectively. Several other amino acids (*e.g.* L-alanine and L-serine) are known to act as weak agonists at GlyRs, as shown in Figure I.7 (Schmieden et al., 1992; Schmieden and Betz, 1995). α2-, α3- and α4-subunit containing GlyRs were reported to exhibit similar agonist sensitivities to the α1-subunits described above. Also incorporation of the β-subunit had little effect on receptor sensitivity to glycine (Lynch, 2004).

Ligand	EC_{50} (mM)	n	I_{max} (%)
Glycine	0,2	2,4	100
β-alanine	0,6	1,7	64
Taurine	1,7	1,7	32
L-alanine	3,1	2,0	81
D-alanine	9,0	1,8	69
L-serine	5,1	1,9	87

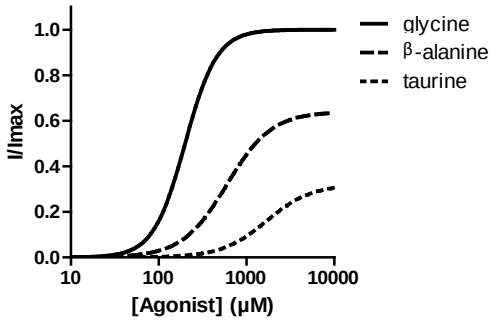

Figure I.7 Comparison of different GlyR agonists.
In the upper table EC_{50}, Hill coefficients (n) and relative I_{max} values are given for agonists that are relevant for this thesis. No agonist activity was found for D-serine. Values were reported on α1-GlyRs expressed on *Xenopus* oocytes, according to (Schmieden et al., 1992; Schmieden and Betz, 1995).
In the lower figure the relative currents were simulated with the Hill equation ($I/I_{max}=1/1+(EC_{50}/A)^n$) for glycine and the 2 endogenous agonists, according to the values given in the upper table.

Phosphorylation of GlyRs

Phosphorylation of LGICs is crucial in the regulation of the activity and synaptic location of these proteins, as exemplified by the role of phosphorylation of AMPA-receptors and its role in long term potentiation, one of the neurophysiological correlates of memory (Santos et al., 2009). Most of the functional phosphorylation sites on LGICs have been mapped to the major intracellular loop (between the third and fourth TM loop in the cys-loop family). In this region, they exhibit a low degree of sequence homology, allowing subunit specific distribution of phosphorylation sites (Lynch, 2004). GlyRs can be phosphorylated by different types of protein kinases:

- *Protein Kinase A (PKA):* Consensus sequences for this cAMP-dependent kinase can be found on a splice variant of α1-subunits, containing an 8 amino acid insert ($\alpha1^{ins}$) or β-subunits. Contradicting effects were however described concerning the effects of PKA on current magnitudes in various parts of the CNS. It was suggested that these contradicting results were explained by the differential distribution of β and $\alpha1^{ins}$ -subunits throughout the nervous system, the latter representing about 30% of total α-subunit expression in the spinal cord (Legendre, 2001; Lynch, 2004). At position 366-369 of the α3 subunit a consensus sequence (RESR) was found for PKA-dependent phosphorylation (shown in grey on Figure I.5C). It was shown that the inflammatory mediator PGE_2 could PKA-dependently phosphorylate the serine residue of this sequence, leading to a reduction of glycine activated currents, a mechanism that may be implicated in inflammatory pain sensitization (Harvey et al., 2004). Interestingly this sequence is not present on the homolog part of the α1 subunit. The same sequence was however present in the α2-subunit and it was later shown that the same modulation could also be found here (Heindl et al., 2007).
- *Protein Kinase C (PKC):* Both α1- and β-subunits contain consensus sequences for PKC phosphorylation. Functional consequences are again conflicting as glycine-evoked responses were described to be either enhanced or to be suppressed (Legendre, 2001; Lynch, 2004).
- *Calcium Calmoduline dependent kinase II (CaCMKII):* Although no consensus sequences of this kinase are known on GlyR α- or β-subunits, it has been shown that an increase in intracellular calcium may activate CaCMKII, leading to an enhancement of glycine-evoked I_{max}. Interestingly this calcium entry and response can be evoked by glutamate receptor activation. The absence of a consensus sequence may be explained by phosphorylation of a not yet identified GlyR splice variant or alternatively the effects of CaCMKII on GlyR function are indirect via phosphorylation of other proteins (Legendre, 2001; Lynch, 2004).

As, like AMPA-receptors, protein kinases were also shown to regulate plasma membrane recycling in certain types of $GABA_A$ receptors (Chapell et al., 1998; Connolly et al., 1999), another member of the cys-loop receptor family, it is tempting to speculate that part of the above mentioned findings can be explained by GlyR plasma membrane recycling. Although not much is known about GlyR recycling, it could be shown in the retina that ^{3}H-glycine and ^{3}H-strychnine binding was reduced upon PKA and PKC activation, suggesting that these kinases were indeed causing receptor internalization

(Salceda and Aguirre-Ramirez, 2005). Also in auditory nuclei it was shown that ^{3}H-binding was reduced upon cochlear ablation and hence deprivation of excitatory input. Interestingly this reduction could be reversed by activating PKA or PKC or by inhibiting CaCMKII (Yan et al., 2007).

GlyRs and tonic inhibition.

From the classic point of view glycine is mostly considered to act via phasic inhibition, *i.e.* short synaptic pulses of glycine release induce fast transient GlyR-mediated currents in postsynaptic neurons, leading to neuronal inhibition. Accumulating evidence now points to an additional role for glycine in tonic, non-synaptic, inhibition as well. Tonic inhibition was first described for GABA, pointing to the more sustained parasynaptic inhibition that is provided by GABA spillover from GABAergic synapses (Belelli et al., 2009). The GABA receptor subunits responsible for this type of inhibition were found to have a higher affinity for agonists and slower desensitization kinetics, making them more suitable for non-synaptic functions. These properties were found for the rare GABAR subunits α3, α6 and δ. For GlyRs it was suggested that the functional properties of the GlyR α2 subunit make this GlyR more suitable for a non-synaptic function (Mangin et al., 2003). Furthermore a splice variant of the GlyR α3 subunit (called α3L) was described that exhibited slower desensitization kinetics, and was the most abundant α3 subunit present in the hippocampus, again pointing to an extrasynaptic role glycine (or agonists) in the hippocampus (Eichler et al., 2009). Interestingly Mori et al. suggested that taurine or β-alanine, rather than glycine, where the endogenous agonists mediating non-synaptic activation of GlyRs in the hippocampus (Mori et al., 2002). Recently RNA editing was shown to alter functional properties of GlyRs, making them more suitable for tonic inhibition. Meier et al. found that a single nucleotide and subsequent amino acid substitution in the GlyR α3 subunit ($\alpha3^{P185L}$) leads to a higher agonist sensitivity (with EC_{50} values shifting from 70 to 5 μM) and less desensitization (Meier et al., 2005). A similar editing at the homolog position of the α2 subunit ($\alpha2^{P192L}$) was found to lead to similar properties in this subunit as well (Eichler et al., 2008; Legendre et al., 2009). Interestingly at homolog positions of the GABAR subunits involved in tonic inhibition (*e.g.* α3, α6 and δ), leucine or structurally similar amino acids, rather than proline was found. These high affinity GlyRs, together with an altered chloride homeostasis were suggested to play a role in the pathology and pathogenesis of temporal lobe epilepsy (Meier et al., 2005; Eichler et al., 2008; Eichler et al., 2009; Legendre et al., 2009).

2.2.1.3 Glycine receptor pharmacology

In contrast to most other neurotransmitter systems (*e.g.* glutamate, acetylcholine, serotonine) no agents primarily focused to act on glycinergic transmission are currently used clinically. Two GlyR antagonists will be discussed here because of their importance in *in vitro* experiments when studying GlyR functioning. Other GlyR modulators (*e.g.* ethanol, cyanotriphenylborate) have been described as well (Lynch, 2004), but are not mentioned here because of their limited relevance to this work.

Strychnine

Strychnine is a plant alkaloid, derived from the dried seeds of the Indian tree *Strychnos nux-vomica.* Although now generally known as a GlyR antagonist, it was first isolated in 1818, a long time before any neurotransmitter was described. It is very toxic to man and has been used as a rodenticide for a long time, although its usage is restricted now because of its toxicity to man (Makarovsky et al., 2008). Interestingly in the end of the 19th century this agent was one of the first doping agents used in sports, with the most famous case being the Olympic marathon winner Thomas Hicks in 1904, who was given strychnine and brandy by his coach. He collapsed on the finish line, but was lucky to survive (McCrory, 2005). Even nowadays, strychnine is one of the components that can be found on the 2009 prohibited list of the world anti-doping agency (WADA). The clinical manifestation of strychnine poisoning is illustrating for its antagonistic function on glycine (and GABA) receptors. Patients become hypersensitive to sensory stimuli, get stiff muscles and hyperreflexia at early stages, after which repetitive convulsions with full consciousness start occurring, finally leading to death because of asphyxia or hyperthermia (Makarovsky et al., 2008).

Strychnine is a highly selective and potent GlyR antagonist. Its K_i and IC_{50} were described to be in the low nanomolar range (Legendre, 2001; Lynch, 2004). Although strychnine is highly selective for GlyRs in low concentrations, in higher micromolar concentrations it becomes less selective and $GABA_A$ receptors can be blocked as well (Shirasaki et al., 1991). Strychnine is considered a competitive antagonist of glycine, although the binding places of glycine and strychnine are overlapping but not the same (Legendre, 2001). Because the high affinity and specificity of this component, ^{3}H-strychnine is widely used for binding experiments. With the initial purification of GlyRs, strychnine was described to bind specifically to the 48 kDa α-subunits (Pfeiffer et al., 1982). Recently it was shown however that strychnine, just as glycine, is in fact binding to pockets in the interface of different subunits. It could be shown that some crucial amino acid residues were indeed also involved in glycine binding, while others were unique for strychnine binding. Contrary to glycine-binding, strychnine could only bind to βα interfaces in heteromeric receptors and not αβ interfaces (Grudzinska et al., 2005).

Picrotoxine

The plant alkaloid picrotoxine (PTX) is commonly known as a $GABA_AR$ antagonist. It inhibits these receptors at 1-10 µM concentrations. PTX is an equimolar mixture of picrotoxinin (PXN) and picrotin (PTN). $GABA_A$ receptors are potently blocked by PXN but not PTN. In these receptors PXN binding occurs in the pore at M2, is non-competitive and is considered a use-dependent allosteric inhibitor. Besides $GABA_AR$ PTX also blocks homomeric GlyRs at similar concentrations (IC_{50} = 5-10 µM). Heteromeric GlyRs are not blocked however (an IC_{50} value of 300 µM was found, corresponding to a decrease in sensitivity of 50- to 200 fold compared to homomeric receptors) , as a consequence of an atypical amino acid sequence in the M2 part of the β-subunit (Pribilla et al., 1992). This property allows the toxin to be used to discriminate between the two types of GlyRs. Contrary to $GABA_ARs$, both PXN and PTN seem to be active at α1 homomeric GlyRs, and PTX acts as a non-use dependent competitive antagonist (Legendre, 2001; Lynch, 2004). Recent evidence

indicates however that in α2 homomeric GlyRs PXN is far more potent than PTN and PXN acts as a use-dependent channel blockers (Wang et al., 2006; Wang et al., 2007).

2.2.1.4 Glycine receptor malfunctioning

The physiological role of GlyRs in normal physiological conditions is to provide inhibition, necessary for precise regulation of spinal reflexes, motor responses and processing of sensory stimuli (*e.g.* pain). As was already noted for strychnine poisoning, blocking these receptors leads to hypersensitivity to sensory stimuli, muscular hypertonia and convulsions with unaltered consciousness. These symptoms can also be found in some congenital mutations in GlyR subunits or related proteins, a condition known as hyperekplexia, and in some mouse models resulting from spontaneous mutations in these subunits. Glycine receptor dysfunctioning may also play a role in some forms of an auto-immune (paraneoplastic) neurological disorder, called stiff person syndrome.

Hyperekplexia

Hyperekplexia, also known as “hereditary startle disease” or “hereditary stiff-baby syndrome” is a genetic disorder characterized by neonatal hypertonia, hyperreflexia and an exaggerated startle response. The startle response can easily be elicited by acoustic or tactile stimuli, leading to a non-epileptic seizure-like clinical picture characterized by muscle stiffness and apnea. Most cases are autosomal dominant with most mutations found in the GLRA1 gene on chromosome 5, causing changes in the M1-M2 and M2-M3 loops and leading functionally to uncoupling of ligand binding and chloride channel function. The most common mutations are located in exon 7, ultimately leading to the substitution of Arg299 for an uncharged amino acid (see Figure I.5C, shown in red) (Zhou et al., 2002). Although most mutations were found on the GLRA1 gene, and no mutations are so far found on the GLRA2-4 genes, mutations in the GLRB or GlyR clustering proteins gephyrin (GPHN gene) and its associated protein collybistin (ARHGEF9 gene) have been described (Harvey et al., 2008).
Besides changes in GlyR proteins, it was recently found that also changes in the presynaptic type 2 glycine transporter (see paragraph 3.2.2) can lead to hyperekplexia. Indeed a loss of function of this transporter is expected to lead to deficiencies in glycine reuptake and hence synaptic release, ultimately having the same neurophysiological consequences (Harvey et al., 2008). Undoubtly mutations in other proteins (*e.g.* GlyT1 transporter, VIAAT) will be described in the future.

Hyperekplexia mouse models

Some spontaneous mutations in mouse GlyR genes, leading to hyperekplexia have been described as well. However, unlike human hyperekplexia, these mouse models display autosomal recessive inheritance.
The *Spasmoid (spd)* mouse has a point mutation in the GLRA1 gene (Ala80Ser), leading to a lower glycine affinity. The *Spastic (spa)* mouse has an intronic insertion in the GLRB gene, leading to GLRB downregulation. Both mice bear striking phenotypical similarities to human hyperekplexia (*e.g.* exaggerated startle response and hypertonia). The third mouse model is the *oscillator* mouse, caused by a frameshift mutation in GLRA1, resulting in loss of function of the M3-M4 loop and the M4 TM domain. These mice usually dye within 10 days after

birth, contrary to the human GLRA1 null mutation, which is not lethal, indicating a better compensatory mechanism in human.
Besides these spontaneous mutations, it is worth mentioning that a transgenic mouse with exogenous expression of GLRA1 with Arg299Gln, the most common human mutation, was developed as well. This mouse model caused a similar phenotype to human hyperekplexia, confirming the dominant negative role of this mutation (Legendre, 2001; Zhou et al., 2002; Lynch, 2004).

Stiff person syndrome

Stiff person syndrome (SPS) is an autoimmune disorder, characterized by muscle stiffness and trigger-induced spasm. Auto-antibodies are found, mainly directed against the GABA synthesizing enzyme glutamate decarboxylase (GAD), indicating a pathophysiological role for decreased GABAergic transmission (Raju and Hampe, 2008). However also auto-antibodies to gephyrin were found to lead to SPS, as part of a paraneoplastic syndrome (Butler et al., 2000).

2.2.2 Glycine transporters

Glycine transporters (GlyT) belong to the SLC6 family of neurotransmitter transporters. With other members of this family they share a common membrane topology of 12 transmembrane domains and intracellular N- and C-terminal regions. Neurotransmitters are cotransported with sodium and chloride. Contrary to the other members of the SLC 6 family it was long suggested that the transporters exist as monomers in the plasma membrane (Aragon and Lopez-Corcuera, 2003), although a recent study provided evidence for their dimeric occurrence (Bartholomaus et al., 2008).

2.2.2.1 Type 1 glycine transporter

Type 1 glycine transporters (GlyT1) transport 2 sodium ions together with glycine (and chloride) and, depending on glycine and sodium concentrations gradients, are able to reverse (Huang et al., 2004). GlyT1 are high affinity transporters (Km values reported to be 17 µM (Guastella et al., 1992)) which are mainly found on glial cells near glycinergic or glutamatergic synapses, where they have been suggested to be important modulators of glycine concentrations and hence regulators of synaptic activity (Aragon and Lopez-Corcuera, 2003). Important agonists are sarcosine (N-methyl-glycine) and its more potent derivative ALX 5407 (N-[(3R)-([1,1′-biphenyl]-4-yloxy)-3-(4-fluorophenyl)propyl]-Nmethylglycine), the latter having an IC_{50} of only 3 nM (Atkinson et al., 2001). Knock-out mice have been developed, showing a phenotype characterized by hypotonia, hyporesponsivity and apnea, leading to postnatal lethality (Gomeza et al., 2003a). These symptoms are very similar to the clinical presentation of non-ketotic hyperglycinemia described earlier and are functional consequences of increased glycine concentrations in glycinergic synapses, caused by deficient glial reuptake (KO mice) or deficient glycine degradation (NKH).

2.2.2.2 Type 2 glycine transporter

Type 2 glycine transporters (GlyT2) transport 3 sodium ions together with glycine (and chloride) and are not described to be able to reverse. GlyT2 transporters are high affinity transporters (Km values reported to be 33 µM (Liu et al., 1993)) and are mainly found on presynaptic axon terminals of glycinergic neurons, where they recycle glycine for reuse in synaptic terminals. These transporters are mainly located in the spinal cord, brainstem and cerebellum, corresponding to the location of glycinergic synapses. An important specific antagonist is amoxapine (Aragon and Lopez-Corcuera, 2003). Knock-out mice have been developed, showing a phenotype characterized by hypertonia, tremor and postnatal lethality at the end of the second week (corresponding to the period of developmental α1 upregulation) (Gomeza et al., 2003b). These symptoms are similar to the clinical presentation of hyperekplexia described earlier and are functional consequences of decreased glycine concentrations in glycinergic synapses, caused by deficient glycine recycling. It has indeed been shown that some forms of human hyperekplexia can be caused by GlyT2 mutations, as described earlier.

2.2.2.3 Aspecific plasma membrane glycine transporters

Besides the high affinity, glycine specific GlyTs, other, less specific, amino acid transporter systems have been demonstrated to transport glycine, together with several other (small) amino acids (*e.g.* glutamine, alanine or cysteine). The **sodium-coupled neutral amino acid transporters (SNAT)** of the SLC38 gene family are low affinity transporters (Km values higher than 1 mM), which transport neutral amino acids together with sodium (Mackenzie and Erickson, 2004). SNAT1, SNAT2 and SNAT4, formerly known as *system A neutral amino acid transporters*, transport glycine, together with other neutral amino acids. Among their broad selectivity for neutral amino acids, they also show substrate specificity for 2-amino isobutyric acid (AIB). SNAT1 is expressed in the brain (mainly on neurons), while SNAT2 shows a more widespread expression. The expression of SNAT4 is restricted to the liver. In Bergmann and Müller glia a functional co-expression of the high affinity GlyT1 (reported Km of 25 and 27 µM respectively), together with the low affinity System A neutral amino acid transporter (reported Km of 1.1 and 1.7 mM respectively) was found (Gadea et al., 1999; Lopez et al., 2005). SNAT3 and SNAT5, formerly known as *system N neutral amino acid transporters* have more narrow substrate profiles (glutamine, histidine and asparagine), although glycine transport has been reported for SNAT5 as well. Both are expressed by astrocytes, where a role in the glutamate-glutamine cycle has been suggested (Mackenzie and Erickson, 2004). Glycine can also be transported by the AIB-insensitive, sodium-coupled **ASC-like transporters** of the SLC1 gene family. These transporters, which have a preference for alanine, serine and cysteine (hence their name) are actually amino acid exchangers. Two types have been described: ASCT-1 and ASCT-2, of which only ASCT-2 was reported to transport glycine with Km values measured to be 361 µM (Utsunomiya-Tate et al., 1996). Another exchange system, capable of transporting glycine, that has been described are the sodium-independent **asc transporters** (SLC7 family). Both types that have been described (asc-1 and asc-2) transport glycine with high affinity (Km values of 7.8 and 2.8 µM respectively) (Fukasawa et al., 2000; Chairoungdua et al., 2001). **System L amino acid transporters (LAT)** also belong to the SLC7 family (Verrey et al., 2004). This sodium-

independent transporter exchanges larger hydrophobic amino acids (*e.g.* leucine, explaining its name). It has been shown that the low affinity LAT-2, which is mainly located in the kidney, but to a smaller extend also in the brain, can transport smaller amino acids, including glycine, with Km values reported to be around 1 mM (Pineda et al., 1999).

2.3 Glycine as a co-agonist on glutamatergic NMDA receptors

NMDA receptors (NMDARs) belong to the glutamate receptor family of LGICs (see paragraph 3.1.3). NMDARs are assembled from combinations of NR1, NR2 or in some cases NR3 subunits, and are typically composed of tetramers of NR1 and NR2 subunits (Figure I.8). Each subunit is composed of an N-terminal extracellular domain, a C-terminal intracellular domain and 4 TM domains (M1-M4), where M2 actually forms a re-entrant helical loop (Figure I.3) (Albensi, 2007).

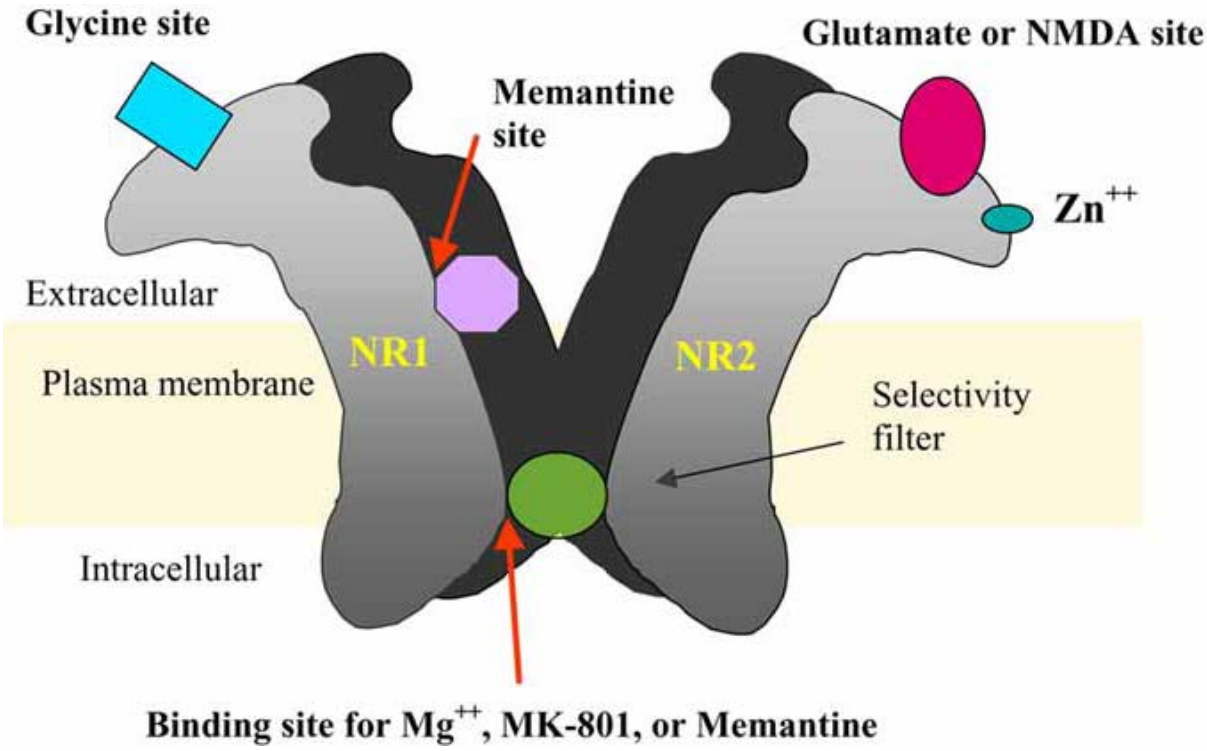

Figure I.8 The NMDA receptor structure.
NMDARs are tetrameric proteins, with the prototype composed of 2 NR1 and 2 NR2 subunits. While the NR2 subunit is responsible for glutamate binding, the NR1 subunit contains the glycine (or D-serine) binding site (Albensi, 2007).

The NR2 subunit is considered as the glutamate (or NMDA) binding subunit. However, under normal physiological conditions these receptors are only activated when the cellular membrane is depolarized and when glycine (or D-serine) is present as a co-agonist. At rest the NMDAR channel is blocked by magnesium. This magnesium block is alleviated in a voltage-dependent manner, for instance as a consequence of AMPA receptor activation and the resulting membrane depolarization. Indeed AMPA receptors were found to be colocalized with NMDARs in postsynaptic neurons. As this means that receptor activation only occurs when there is simultaneous presynaptic glutamate release and postsynaptic membrane

depolarization, the NMDAR has been termed a *synaptic co-incidence detector (Albensi, 2007)*. Already since 1987 glycine, binding at the NR1 subunit, is known as a co-agonist at the NMDAR, meaning that it potentiates the glutamate response (Johnson and Ascher, 1987). Later also D-serine, synthesized by astrocytes, was shown to be an endogenous and even more potent co-agonist, acting at the same site (Matsui et al., 1995; Mothet et al., 2000).
Activation of the NMDAR induces cellular calcium entry, responsible for key neurophysiological processes as LTP and LTD (long term depression), the potential substrates for memory processing. NMDAR overactivation, as occurs in several pathological processes (*e.g.* epilepsy or stroke, but also neurodegenerative diseases such as Alzheimer's disease) causes excitotoxicity and hence neuronal cell death (Albensi, 2007). It was shown that both co-agonists glycine and D-serine could again modulate this excitotoxicity (Katsuki et al., 2007).
Several important antagonists are described, belonging to different classes. Competitive antagonists (*e.g.* D-2-amino-7-phosphonovalerate (APV)) compete with glutamate for its binding site. Noncompetitive antagonists (*e.g.* dizocilpine (MK-801)) work allosterically at a binding place other than the binding place, while glycine site antagonists (*e.g.* 2,7-Dichlorokynurenic acid (DCKA)) are competitive glycine antagonists (Baron et al., 1990). Memantine is an antagonist used in the treatment of Alzheimer's disease. This antagonist interacts with the receptor similar to magnesium, but contrary to magnesium does not leave the NMDA channel during pathologic hyperactivation. It does however leave the channel for short time periods during normal physiologic processes, limiting its side effects and allowing its clinical use.
One study demonstrated primary glycine gated NMDARs ("the excitatory glycine receptor"), when NR1 subunits where co-expressed with NR3 subunits in *Xenopus oocytes*. These receptors were shown to be activated by glycine, unaffected by glutamate (or NMDA), insensitive to the antagonists MK-801 or APV, and D-serine was shown to act as an antagonist (Chatterton et al., 2002). Although these findings are of utmost interest, to our knowledge, to date no studies were published confirming or extending these data.

3. GLYCINE AND GLYCINE RECEPTOR SIGNALING IN NON-NEURONAL CELLS

In this section we focus on non-synaptic roles of glycine and GlyR-mediated signalling in non-neuronal cell types and organs, describing the potential mechanisms of action and biological roles of GlyRs in these cell types.

3.1 Neuroglial cells

Ontogenetically, neuroglial cells in the CNS can be divided into ectodermal-derived macroglial cells (oligodendrocytes and astrocytes) and mesodermal-derived microglial cells. ***Macroglial cells*** have the same embryonic origin as neurons. The first reports of neurotransmitter receptors in oligodendrocytes and astrocytes suggested that no functional GlyRs were present on these cell types (Gilbert et al., 1984; Kettenmann et al., 1984a; Kettenmann et al., 1984b). However, ionotropic $GABA_A$ receptors were detected, which mediate depolarizing currents upon ligand binding. This depolarization is explained by GABA-induced chloride efflux, due to the expression of the Na^+-K^+-Cl^- co-transporter (NKCC1) leading to a high intracellular chloride concentration and, hence, to a less negative Nernst potential for chloride (Kettenmann et al., 1987; Hoppe and Kettenmann, 1989). Around ten years later, molecular and functional studies demonstrated that macroglial cells harbour functional α1β GlyRs when studied *ex vivo* in spinal cord slices (Pastor et al., 1995; Kirchhoff et al., 1996). The apparent contradiction with previous *in vitro* studies may be related to cell culture conditions, which might lead to GlyR down regulation. In this context, excess glycine in certain types of cell culture medium has previously been linked to cell death in heterologous GlyR expression experiments (Groot-Kormelink et al., 2002). Nguyen and Belachew further demonstrated, in their oligodendrocyte and neurosphere models, that both GlyRs and glycine transporters (GlyTs) were molecularly and functionally present at different stages of macroglial development (Belachew et al., 1998a; Belachew et al., 1998b; Belachew et al., 2000; Nguyen et al., 2002). In oligodendrocyte progenitors, glycine was found to depolarize the cell membrane, via the activation of both GlyRs and GlyTs. This depolarization led to the activation of voltage-gated calcium channels (VGCCs) and, hence, calcium influx. This calcium influx may be one of the crucial signals in the development of oligodendroglial cells. GlyT1 and GlyRs containing the α1 and β subunits were also described in retinal Müller cells. In these cells, glycine was shown to have a depolarizing effect, suggesting an important signaling role in potassium siphoning, or in the regulation of synaptic glycine concentrations (Du et al., 2002; Lee et al., 2005). Our group also found molecular evidence for GlyR expression in different oligodendroglial cell lines (MO3.13, OLN-93, HOG), although the receptors appear to show a cytoplasmic location, which might explain why we could not detect any GlyR-mediated ionic currents (Sahebali et al., 2007). In this respect, Nguyen et al. also found a discrepancy between the number of cells that express GlyRs (80%) and those that exhibit glycine-induced currents (33%) (Nguyen et al., 2002). A cytoplasmic location for GlyRs has previously been described in spinal cord neurons (Bechade et al., 1996) and although little is known about GlyR trafficking, it was shown that GlyRs can be ubiquitinated, causing receptor internalization and proteolysis (Buttner et al.,

2001). Also chronically blocking GlyR activity with strychnine (1-10 µM) was demonstrated to cause receptor internalization (Levi et al., 1998). Since glycine-gated currents are detected in tissue slices while they are absent in individual cell cultures, one could speculate that GlyRs are present in macroglial cells, but that their plasma membrane location and, hence, their functionality is dependent upon cell culture conditions. It seems indeed likely that some unknown factor(s) in the normal physiological environment of slices might be necessary for plasma membrane expression of GlyRs. Further research is however necessary to identify this (these) factor(s). Downstream events of such factor(s) could at least imply protein kinase activity. In that respect, it has been shown that, in the retina and in auditory nuclei, protein kinases modulate strychnine binding and, hence, GlyR expression (Salceda and Aguirre-Ramirez, 2005; Yan et al., 2007). Figure I.9 summarizes the current findings on glycine and GlyR signaling in macroglial cells. Glycine transporters, astrocytic GlyT1 as well as presynaptic neuronal GlyT2 are of utmost importance for correct glycine recycling both at glutamatergic and at glycinergic synapses, but fall beyond the scope of the review presented in this section (see (Aragon and Lopez-Corcuera, 2003).

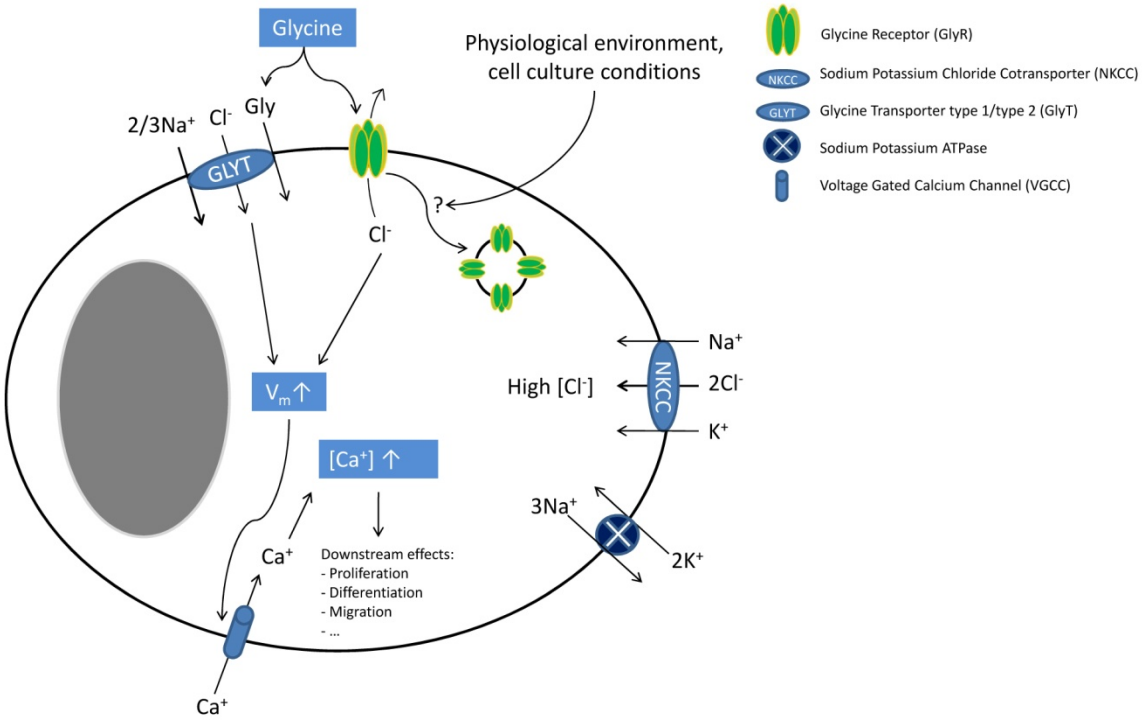

Figure I.9 Glycine signaling in macroglial cells.
In macroglial cells, NKCC activity leads to intracellular chloride accumulation. Upon ligand binding, GlyR activation causes chloride efflux leading to cellular depolarization (which is further enhanced by electrogenic GlyT activity). This depolarization then activates VGCC. The resulting calcium influx increases the intracellular calcium concentration inducing several downstream effects (*e.g.* cell proliferation, migration and differentiation). Endocytosis of GlyRs might inactivate these receptors, explaining discrepancies between molecular and functional findings. Although cell culture conditions appear to have an influence on GlyR functionality and possibly endocytosis, the molecular mechanisms regulating of GlyR endocytosis are unknown.

Microglial cells, although of mesodermal origin, are considered here because of their intimate spatial relationships to other glial cells and neurons. Over ten years ago, key studies showed that astrocyte-derived glycine and L-serine (between 10 and 100 µM) modulate microglial activity. Micromolar concentrations of glycine induced morphological changes in microglial

cells and enhanced lipopolysaccharide (LPS)-induced secretion of nitrogen oxide (NO), superoxide, acid phosphatase as well as metabolic activity. These studies, however, did not focus on the mechanism of this modulatory activity (Tanaka et al., 1998; Yang et al., 1998). Since glycine effects could be mimicked by L-serine, a metabolic precursor of glycine, and since microglial cells were shown to have a low expression of 3-phosphoglycerate dehydrogenase, a key enzyme in L-serine and, hence, glycine biosynthesis, it was suggested that glycine and L-serine could be essential amino acids for microglial cells (Sugishita et al., 2001). This would have implied that the above-mentioned effects could be explained by a metabolic mechanism. This is however contradicted by the high glycine and L-serine synthesis capacities of microglial cells, which seem able to produce glycine and L-serine at concentrations of 88 and 144 µM, respectively (Hayashi et al., 2006). Another study by Schilling and Eder demonstrated that glycine can depolarize BV-2 microglial cells. Glycine-induced currents were insensitive to strychnine (1µM) or GlyT-antagonists, were chloride-independent and could not be mimicked by the GlyR agonist taurine. Based upon the sodium dependency of glycine currents, their blockade by MeAIB (α-(methylamino)isobutyric acid), the agonist effect of glutamine and their low amplitude, the authors concluded that system A neutral amino acid transporters (SNATs) were responsible for these currents. Moreover, they suggested that these currents, although small, could have a signaling role via membrane depolarization (Schilling and Eder, 2004). Indeed, glycine-induced depolarization was substantial (up to 30 mV increase in membrane potential was measured) despite the low amplitude of the currents (around 10pA), and could be explained by the high membrane resistance of microglial cells (Newell and Schlichter, 2005). Although these data suggest that functional GlyRs are not present on microglial cells, our group recently provided molecular evidence for α and β GlyR subunit and gephyrin expression in these cells (Van den Eynden J, unpublished results[*]). However, the role of these GlyRs remains elusive. Indeed, we also confirmed that the above-mentioned findings in microglial cells were GlyR-independent (Van den Eynden et al., 2008). By analogy with macroglial cells, the discrepancy between molecular and functional data might be explained by cell culture conditions and/or GlyR down-regulation. Future research on brain or spinal cord slices and GlyR trafficking will be necessary to examine this hypothesis.

Conclusion: Astrocytes, oligodendrocytes and microglial cells are intimate partners of neurons in the neuronal microenvironment. GlyRs (and GlyTs) have been described on different developmental stages of macroglial cells where they may have a developmental role in the immature CNS, as well as a communication function in the mature CNS. Glycine signaling in microglial cells may be mediated by non-specific amino acid transporters. However, the biological and functional role(s) of microglial GlyRs remain unknown.

3.2 Immune cells

In recent years, it has become obvious that glycine has immunomodulatory effects on different immune cells. As a common feature, inhibition of immune function was found in most *in vitro* studies, explaining the beneficial effects of glycine that were found in animal studies. For reviews on glycine immunomodulatory effects, the reader can also consult

[*] Results are discussed in CHAPTER III

(Wheeler et al., 1999; Zhong et al., 2003). Glycine effects on immune cells have been mainly studied in ***macrophages***, in particular using *Kupffer cells*, the resident macrophages of the liver. When rat were fed with a diet containing 5% glycine for 3 days and then subjected to intravenous LPS injection (an animal model of endotoxic shock), a significant decrease in mortality, liver necrosis, lung damage and serum tumour necrosis factor α (TNFα) levels were found (Ikejima et al., 1996). These beneficial effects were mainly attributed to the inhibitory effects of glycine on Kupffer cells. It was also shown that glycine (0.1-1 mM) blunted LPS-induced calcium fluxes in Kupffer cells, an effect that could be reversed by low concentrations of strychnine (1 µM) and that was not present when a chloride-free buffer was used. Furthermore, glycine was also able to induce uptake of $^{36}Cl^-$ by Kupffer cells (Ikejima et al., 1996; Ikejima et al., 1997; Froh et al., 2002), an effect that could be mimicked by taurine and β-alanine, two GlyR agonists (Seabra et al., 1998). These findings clearly suggest a role for GlyRs in modulating Kupffer cell functions. Since fluorimetric measurements showed that LPS depolarized Kupffer cells while glycine hyperpolarized them, it was suggested that GlyR-dependent hyperpolarization leads to an inhibition of functional VGCC, which were also found in Kupffer cells (Hijioka et al., 1992). More recently, glycine was shown to inhibit Toll-like receptor 4 upregulation after LPS exposure in Kupffer cells, showing that other signaling pathways may operate in these cells (Xu et al., 2008). Finally, GlyR α1, α4 and β subunit transcripts and proteins were detected in Kupffer cells (Froh et al., 2002). All the above-described glycine effects on Kupffer cells may explain why glycine is beneficial when co-administered in D-galactosamine and ethanol-induced hepatotoxicity models (Stachlewitz et al., 1999; Bruck et al., 2003; Yamashina et al., 2005). It was indeed shown that ethanol increases gut permeability and hence endotoxine uptake, leading to Kupffer cell activation and secretion of cytokines (*e.g.* TNFα), responsible for liver toxicity (Wheeler, 2003). In *alveolar macrophages*, glycine was also found to blunt calcium fluxes, to increase $^{36}Cl^-$ uptake and to decrease superoxide and TNFα secretion. The sensitivity of alveolar macrophages to glycine was higher than for Kupffer cells ($IC_{50} \approx 10$ µM, versus ≈ 300 µM in Kupffer cells). One suggestion for this finding was that, due to the lower alveolar extracellular glycine concentration, there might be less GlyR down-regulation and, hence, a higher GlyR density on alveolar macrophages (Wheeler and Thurman, 1999). This hypothesis was confirmed by animal studies where rats were fed on a glycine-rich diet for 4 weeks. After this period, Kupffer cells had lost their glycine responsiveness, while alveolar macrophages had not, further suggesting GlyR down-regulation after chronic exposure to glycine (Wheeler et al., 2000b). In *splenic macrophages* similar effects on calcium fluxes and chloride-uptake were observed (Li et al., 2001). Finally, molecular evidence for GlyR α2, α4 and β subunits was found in alveolar and splenic macrophages (Froh et al., 2002). Next to macrophages, also studies concerning glycine effects on other immune cells were conducted. In ***T lymphocytes***, there is conflicting evidence regarding the modulatory role of glycine. Glycine was suggested to have anti-apoptotic effects on MOLT4 cells, a human T lymphoblastic leukaemia cell line, at millimolar concentrations (Franek et al., 2002). Stachlewitz found that glycine dose-dependently inhibited proliferation of rat lymphocytes between 0.1 and 1 mM and blunted concanavalin A induced calcium fluxes. This latter effect could be blocked by strychnine (1 µM) or by replacing chloride by gluconate, suggesting the involvement of GlyRs (Stachlewitz et al., 2000). The authors hypothesized that GlyR activation hyperpolarized the cells, leading

to inhibition of VGCCs. However, this is in contradiction with the high chloride concentration (30-60 mM) reported in lymphocytes, which, together with the reported membrane potential of -50 to -70 mV, should lead to depolarization upon GlyR activation (Pilas and Durack, 1997; Tian et al., 1999). The putative implication of VGCCs in lymphocytes is also controversial since calcium-release-activated calcium channels (CRACs) seem to be far more important for lymphocyte activation (for review see (Lewis, 2001)). Alternatively, we suggest that these inhibiting effects on calcium fluxes could arise from membrane depolarization induced by a GlyR-mediated chloride efflux. This depolarization would cause both a decrease in the calcium electrical gradient and a decrease in CRAC channel conductance, leading to lower CRAC-mediated calcium influx. This mechanism was already suggested to explain $GABA_AR$-mediated inhibitory effects on calcium fluxes in T lymphocytes (Tian et al., 1999; Alam et al., 2006). The inhibitory effects of glycine on rat lymphocyte proliferation (Stachlewitz et al., 2000) are in contradiction with other studies suggesting that i) glycine does not influence mitogen-induced proliferation of human peripheral blood mononuclear cells (PBMCs) (Sommer et al., 1994) and ii) that glycine does not modulate *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)- or LPS-induced calcium fluxes in human PBMCs (Alam et al., 2006). A feasible explanation for these apparently conflicting results may be interspecies differences in GlyR expression. In this respect, it is interesting to note that most immune cells studied so far, including T-lymphocytes (Van den Eynden J, unpublished results[*]) appear to express the GlyR α4 subunit (Froh et al., 2002; Alam et al., 2006). In human, *GLRA4* is a pseudogene due to the presence of a premature stop codon in exon 9, which could explain the absence of functional GlyRs in human studies (Simon et al., 2004). Finally, ***neutrophils*** also seem to express functional GlyRs. In these cells, glycine inhibits LPS- and fMLP-induced calcium fluxes, increases $^{36}Cl^-$ uptake and decreases superoxide production, all effects that were blocked by 1 µM strychnine (Wheeler et al., 2000a). Rat neutrophils also appear to express GlyR α2, α4 and β subunits (Froh et al., 2002). In human ***monocytes***, glycine was found to inhibit LPS-induced TNFα and interleukin (IL)-1 secretion and to enhance IL10 secretion, effects that were strychnine sensitive (1 µM) (Spittler et al., 1999). Using flow cytometry, we have also found evidence for GlyR α2 subunit expression in human monocytes and in ***natural killer cells***, a cell population where glycine effects were not studied to date (Harvey RJ and Horwood NJ, Figure I.10).

Conclusion: Effects of glycine were demonstrated on most immune cells. On monocytes, macrophages and neutrophils, glycine inhibits calcium fluxes leading to a decreased secretion of cytokines (*e.g.* TNFα). These effects are likely to be mediated by functional GlyRs, although there may be important species differences in subunit composition. Glycine also seems to exert immunomodulatory effects in T lymphocytes, although, in this case, the results are less clear cut.

[*] Results are discussed in CHAPTER II

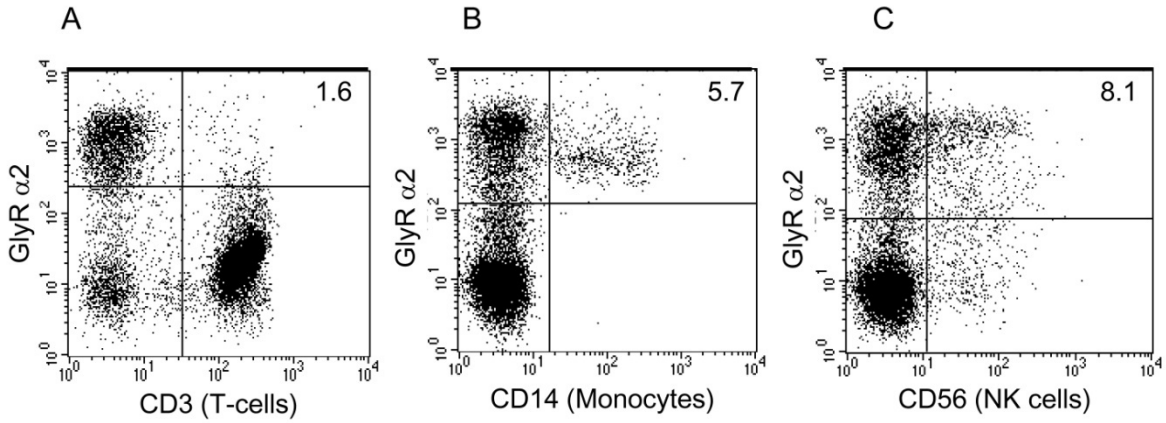

Figure I.10 Human monocytes and NK cells express GlyR α2 subunits.[*]
Human peripheral blood mononuclear cell subsets were evaluated for the expression of GlyR α2 using a rabbit anti human GlyR α2 antibody (FITC) and either mouse anti-human CD3 (APC), mouse anti- human CD14 (PE) or mouse anti-human CD56 (PE) [BD Pharmingen]. Cells were stained according to the manufacturers protocols. GlyR α2 positive cells make up 30% of the total PBMC population. The T cell population (CD3+) accounts for more than 60% of the PBMC population and was mostly negative for GlyR α2 expression (A), whilst all of the monocyte population (CD14+) was positive for GlyR α2 (B). More than half of the natural killer (NK) population (CD56+) was positive for GlyR α2 (C). However, since the T cell population was negative, it is likely that the NK T cells are also negative. Cells that were positive for both GlyR α2 and for the lineage specific cell surface marker appear in the top right hand side quadrant.

3.3 Renal cells

In the kidney, several *in vitro* studies point to cytoprotective effects of glycine against ischemia. The first evidence of such a protective role of glycine came from a study showing that glycine, produced by the metabolism of glutathione (rather than glutathione itself, as previously thought (Paller, 1986)), protects isolated proximal tubules against ischemia in a concentration range of 0.25 – 2 mM (Weinberg et al., 1987). Since hypoxic conditions lead to a decrease of β-oxidation and, hence, to an increase in acyl-coenzyme A which, in turn, may lead to detergent-like membrane damage, it was initially suggested that glycine effects could be explained by acylglycine formation and, hence, metabolic detoxification. However, it was subsequently shown that this metabolic mechanism plays no major role in glycine-induced cytoprotection (Weinberg et al., 1991a). It was shown that this protection could be mimicked by structurally related amino acids such as L-alanine (Weinberg et al., 1990a), that it was independent of ATP (Weinberg et al., 1997) or calcium (Weinberg et al., 1991b; Venkatachalam et al., 1995) and that it does not protect either against swelling of ischemic cells (Weinberg et al., 1990b) or against plasma membrane breakdown (Venkatachalam et al., 1995). In comparable experimental paradigms, strychnine, in high concentrations (1 mM), was shown to act as an agonist, rather than an antagonist (Aleo and Schnellmann, 1992; Dong et al., 2001). Other GlyR antagonists and chloride channel blockers (*e.g.* brucine, bicuculline, avermectin B1a, and cyanotriphenylborate) were also demonstrated to exert glycine-like activity (Miller and Schnellmann, 1994; Venkatachalam et al., 1996). Dong and colleagues investigated whether this strychnine protection was due to an (aspecific) intracellular interaction or to an interaction at the cellular membrane, as would be expected for ligand-gated channel-mediated actions. They showed that membrane-impermeable strychnine

[*] Data are from the laboratory of Dr. Harvey RJ.

derivatives maintained their cytoprotective properties in MDCK (Madin–Darby canine kidney) cells, a distal tubular cell line. Moreover, they showed that small modifications to the molecule near the interacting centre for receptor binding abolished the effect (Dong et al., 2001). These results clearly prove specific strychnine interactions with surface-oriented extracellular domains. Although the effects of these compounds are typically receptor dependent, in renal cells these effects do not seem to depend on chloride ion gradients, since replacement of chloride by gluconate could not abolish the effects. In an attempt to explain this apparent contradiction, it has been suggested that under ischemic conditions, dysregulation of GlyRs leads to the development of a pathological pore, allowing the penetration of macromolecules and, hence, cell death (Venkatachalam et al., 1996). This pore formation can be prevented by adding agonists as well as antagonists, which somehow stabilize the GlyR. This hypothesis was supported by Dong and co-authors, who showed that, in ischemic conditions, there is a size-dependent membrane penetration of macromolecular dextrans, with a progressive increase in permeability for larger dextran molecules during the period of ischemia. This permeability defect could be prevented by a cross-linking agent (3,3' dithiobis(sulfosuccinimidylpropionate)) or by adding millimolar glycine (Dong et al., 1998). Direct evidence for GlyR involvement was recently provided by a study that showed that inhibition of endogenous GlyR expression by RNA interference attenuates cytoprotection in MDCK cells (Pan et al., 2005). They also found that HEK (human embryonic kidney) cells were not protected from ischemic cell death by glycine, unless they were transfected with plasmid constructs expressing the GlyR α1 subunit. GlyR α1 mutants of Y202F or Y202L, which are shown to decrease glycine and strychnine binding, also specifically abolished the effects of glycine and strychnine, respectively, further suggesting the involvement of glycine receptor subunits in cytoprotection At the molecular level, most studies have only found evidence for GlyR β subunit and gephyrin expression at the basolateral membrane of proximal tubule cells (Grenningloh et al., 1987; Miller and Schnellmann, 1994; Sarang et al., 1999). There is also *in vivo* evidence for a protective role of glycine against ischemic conditions in the kidney. For example, when rats were fed with a diet containing 5% glycine, their renal function was improved (higher glomerular filtration rate (GFR) and lower plasma creatinine) after renal ischemia (Yin et al., 2002). These effects might also be relevant under normal physiological conditions since when normal rats are infused with glycine, a decrease in renal vascular resistance is observed, leading to an increase in renal plasma flow and, hence, in GFR. In addition, glycine also decreases proximal tubular reabsorption (Thomsen et al., 2002).

Conclusion: Numerous *in vitro* and *in vivo* studies provide evidence for a cytoprotective role of glycine against kidney ischemia . This effect seems to be mediated by GlyRs with unusual properties: i) there is little evidence that GlyR α subunits are expressed in kidney cell types, ii) glycine effects can be mimicked by both GlyR agonists and antagonists and iii) these effects do not depend upon the chloride gradient. Currently, porous defects are thought to develop in plasma membranes of ischemic cells as a consequence of a molecular perturbation of GlyR channels, that lead to leakage of macromolecules and, hence, to cell death. Glycine and GlyR antagonists might stabilize this unusual GlyR of unknown subunit composition accounting for cytoprotective effects.

3.4 Hepatocytes

In the liver, ischemia-reperfusion injury is a serious problem after liver transplantation that often leads to primary graft dysfunction. Thus, glycine is a potential cytoprotective agent of particular interest for the liver. The specific role of glycine in hepatic ischemia-reperfusion injury was recently reviewed (Habib et al., 2006). A few years after the first report of a cytoprotective role of glycine in renal ischemia, Marsh and colleagues showed that isolated hepatocytes exposed to cold ischemia in the presence of glycine (3 mM) experienced a significant improvement of cellular injury compared to controls (Marsh et al., 1991). This effect was shown to be independent of glutathione synthesis (Marsh et al., 1991; Ozaki et al., 1994). Comparable effects were observed with high concentrations of alanine (10 mM) and strychnine (1 mM) as well as under conditions of metabolic inhibition (chemical hypoxia) (Dickson et al., 1992; Marsh et al., 1993; Sakaida et al., 1996; Nagatomi et al., 1997). Also for hepatocytes, the strychnine effects were shown to be specific (Dong et al., 2001). Glycine hepatoprotective effects were independent of ATP levels or intracellular acidosis, and glycine could not protect against injury caused by calcium ionophores or by oxidative stress (Marsh et al., 1993; Sakaida et al., 1996). It was also shown that glycine could inhibit calcium-dependent non-lysosomal protease activity during ischemia (Ferguson et al., 1993; Nichols et al., 1994). High concentrations of glycine (2-10 mM), but also L-alanine (10 mM) or strychnine (1 mM), were shown to be protective and to non-specifically block influx of different ions (*e.g.* sodium, cobalt or nickel) (Carini et al., 1997; Frank et al., 2000). Although it was shown that blocking sodium influx by itself was cytoprotective and was dependent upon chloride influx, glycine was still protective in a sodium- or chloride-free buffer. Glycine was hypothesized to block non-specific 'leak' channels, which might increase in number and permeability with time (Frank et al., 2000). Glycine protects hepatocytes form ischemic cell death, but it does not prevent the mitochondrial permeability transition (MPT), contrary to cellular acidosis or sodium-free culture conditions, both preventing the MPT (Qian et al., 1997). In a review on the role of the MPT in ischemic cell death of hepatocytes, it was argued that glycine acted downstream of the MPT, at a time point where cells are completely deprived of ATP and destined for necrotic cell death (Kim et al., 2003). Glycine was also shown to protect against hepatotoxicity induced by different agents (*e.g.* valproate, paracetamol, cadmium chloride or copper chloride), an effect that can only partly be explained by metabolic detoxification of acyl-coenzyme A by means of acylglycine formation (Vance et al., 1994; Deters et al., 1998). To date, only one study has described clear GlyR-related modulatory effects in hepatocytes. Qu and colleagues demonstrated that glycine blocks prostaglandin E_2- and epinephrine-induced calcium fluxes in hepatocytes, an effect that is chloride-dependent and antagonized by low concentrations of strychnine (10 µM). However, no direct link between calcium modulation and cytoprotective effects could be made (Qu et al., 2002). Molecular evidence for GlyRs in hepatocytes is scarce. A GlyR β subunit splice variant was described in liver homogenates (Oertel et al., 2007), but its cellular origin is unclear (hepatocytes, Kupffer cells or endothelial cells). To date, no GlyR α subunit gene expression has been described (Grenningloh et al., 1987; Froh et al., 2002). *In vivo* evidence also supports a protective role of glycine against liver ischemia and other pathologies. Several animal studies have shown that glycine has beneficial effects during liver

transplantation. When given intravenously to donors or when given to acceptors (den Butter et al., 1993; Schemmer et al., 1999; Ito et al., 2008), glycine improved survival and decreased liver enzyme concentrations. It is however noteworthy that besides a direct cytoprotective effect on hepatocytes, there is also an important immunomodulatory contribution via the suppression of Kupffer cell activity *in vivo*. Indeed, a positive correlation was found between the protective effects of glycine and the decrease in Kupffer cell-derived TNFα and proteases (Schemmer et al., 1999; Duenschede et al., 2006; Yamanouchi et al., 2007). Further evidence for Kupffer cell involvement in beneficial glycine effects came from an animal study that showed that glycine, when given to donors, decreases levels of TNFα and liver enzymes, an effect that can be blocked by adding low levels of strychnine (5 µM) (Zhang et al., 2005). As strychnine antagonism is only established for immunomodulatory effects on Kupffer cells, but not for cytoprotective effects on hepatocytes, these findings strongly support a crucial role for Kupffer cells. As mentioned earlier, beneficial effects of glycine in alcoholic liver disease were mainly due to Kupffer cells as well. Also in animal models of endotoxine and hemorrhagic shock, glycine modulation of Kupffer cells and inhibition of TNFα-secretion seem to be crucial for its beneficial effects (Zhong et al., 1999; Mauriz et al., 2001; Yang et al., 2001; Wang et al., 2004; Neyrinck et al., 2005). On the other hand, it was shown that destruction of Kupffer cells by gadolinium chloride (GdCl) was far less potent than glycine in animal liver transplantation studies, suggesting that not all *in vivo* findings can be explained by modulation of Kupffer cells (Rentsch et al., 2002; Rentsch et al., 2005). Also, in animal models of cholestasis, where bile cannot flow from the liver to the duodenum it was shown that beneficial glycine effects (lowering of TNFα levels, liver necrosis and liver enzymes) remained when Kupffer cells were destructed by GdCl (Fang et al., 2003; Froh et al., 2008). Finally, some of the cytoprotective effects of glycine could also be attributed to glycine interacting with (sinusoidal) endothelial cells (see following section). The above-mentioned cytoprotective effects of glycine against liver ischemia were considered solid enough to trigger clinical trials. Preliminary studies with few patients were undertaken showing, in one first study, that 2 mM glycine added to the perfusion solution during transplantation induced a significant decrease in liver enzymes and evoked less complications (Arora et al., 1999). A second study with 7 patients confirmed that a one-week long daily infusion of glycine post-operatively (corresponding to ~2 mM plasma concentration) significantly decreased plasma liver enzyme levels (Schemmer et al., 2001; Schemmer et al., 2002). These preliminary results led to a multicenter, randomized, placebo-controlled double blind clinical trial that is currently under investigation (HEGPOL, "Hepatoprotective Effects of Glycine in the Postoperative Phase") (Luntz et al., 2005).

Conclusion. In the liver, glycine protects against ischemic injury what may have clinical applications to reduce complications in liver transplantation with preliminary clinical trials showing promising results. However, the molecular mechanism of the glycine effect remains elusive and nothing is known about the precise role or composition of hepatic GlyRs. As for the kidney, one hypothesis is that glycine could block non-specific pore formation, preventing plasma membrane leakage and, eventually, cell death. Besides direct cytoprotective effects on hepatocytes, glycine modulatory effects on Kupffer and endothelial cells have to be taken into account especially in *in vivo* studies.

3.5 Endothelial cells

By analogy to the situation in the liver and in the kidney, glycine was also suggested to have cytoprotective effects in endothelial cells subjected to hypoxia or challenged with ionomycine, cyanide, hydrogen peroxide or maitotoxin (Weinberg et al., 1992; Nishimura et al., 1998; Nishimura and Lemasters, 2001; Estacion et al., 2003). These effects were independent of pH, ATP levels or calcium and could be mimicked by L-alanine. Estacion and co-workers found that glycine protective effects on cell death were taking place at the late phase of maitotoxin cytotoxicity and could prevent pore-formation in the cytoplasmic membrane, which would normally lead to cell death. Nishimura and co-workers also hypothesized that glycine could block the opening of a "death channel" during hypoxia. They found that sinusoidal endothelial cells were going through different stages during ischemia. During early hypoxia, a cation influx appears as a consequence of a decrease in Na^+/K^+ ATPase activity. However, this is not accompanied by an anion influx and causes only moderate cell swelling. In a next stage, called the metastable state (characterized by the MPT), anions start flowing inside cells through a "death channel" leading to an increase in colloid osmotic pressure, to bleb formation and, eventually, to the rupture of plasma membranes and to cell death. Based on the finding that glycine slows anionic calcein entrance, decreases bleb formation and prevents entrance of propidium iodide and macromolecules into cells, it was suggested that glycine could somehow inhibit this "death channel" (Nishimura and Lemasters, 2001). Besides protecting against ischemic necrosis, glycine also seems to prevent apoptosis of sinusoidal endothelial cells induced by vascular endothelial growth factor (VEGF) deprivation. This protective effect is blocked by strychnine (1 μM) and is possibly mediated by enhanced bcl-2 expression (Zhang et al., 2000). In this context, VEGF-induced cellular calcium influxes can be completely prevented by addition of 1 mM of glycine in CPA cells, a bovine endothelial cell line. This effect is clearly GlyR-dependent since it can be blocked by micromolar concentrations of strychnine and is eliminated in chloride-free buffer conditions. Furthermore, GlyR β subunit expression was found at the mRNA and protein level and glycine could decrease the proliferation and the migration of endothelial cells (Yamashina et al., 2001). This inhibiting effect of glycine on endothelial cells may explain why glycine was found to be tumour suppressive in melanoma and liver tumours. In this respect, it is of interest to mention that glycine inhibited tumour growth in the advanced stages only, which is when neo-angiogenesis has an important role in tumour progression because of oxygen need (Rose et al., 1999b; Rose et al., 1999a). Later, it was demonstrated that glycine could indeed inhibit angiogenesis, leading to a decreased wound healing and tumour growth (Amin et al., 2003), suggesting that glycine effects on endothelial cells may be of future interest for research into atherosclerosis (McCarty et al., 2009). Glycine-mediated vascular effects were also attributed to NMDA receptors (NMDARs) in some studies. For example, Mishra and colleagues showed that glycine lowers the blood pressure in normotensive rats, while it increases it in hypertensive and L-NAME (NO synthase inhibitor)-treated rats. These effects were attributed to NMDAR activation on both endothelial and vascular smooth muscle cells, with the former being NO-dependent and prevailing under normotensive conditions and the latter prevailing under hypertensive conditions (Mishra et al., 2008). NMDAR-mediated vascular effects were also suggested to

mediate glycine vasodilatory effects and, hence, to increase GFR in the kidney (Deng et al., 2002; Slomowitz et al., 2004).

Conclusion: In endothelial cells, some modulating effects of glycine are present that appear to be mediated by GlyRs. Potential effects of glycine on migration and proliferation may explain beneficial glycine effects on tumour growth and wound healing. As in the liver and in the kidney, some of the cytoprotective effects of glycine could be mediated by a non-specific pore formed under ischemic conditions.

3.6 Other cell types

In ***cardiomyocytes***, a few recent studies showed beneficial glycine effects under ischemia-reperfusion conditions. For example, 3 mM glycine increases the cell viability of isolated cardiomyocytes and of isolated rat hearts after ischemia/reperfusion (Ruiz-Meana et al., 2004). Based on the findings that glycine prevented mitochondrial swelling and calcein release, it was suggested that glycine somehow inhibits the MPT. However, this contrasts with other findings in the kidney and in the liver where the general assumption is that glycine acts downstream of the MPT (Kim et al., 2003). In another study, glycine infusion into animal donor hearts was beneficial for right ventricular function after transplantation (Warnecke et al., 2006). Lastly, a recent study showed that 2 mM glycine could protect isolated rat heart function and viability in an experimental LPS-induced decreased cardiac function model. This protective effect of glycine was linked to an attenuation of LPS- and hypoxia-induced calcium influx. Interestingly, the authors again detected the presence of GlyR β subunits, but not GlyR α subunits (Qi et al., 2007). GlyRs are also well described in ***sperm*** in humans, pigs, mice and golden hamsters where they are involved in the zona pellucida-initiated acrosome reaction, an indispensable step in the fertilization process. Glycine induces the acrosome reaction at micromolar concentrations and this effect can be blocked by very low concentrations of strychnine (50 nM). In the *spasmodic* and *spastic* mouse models (characterized by GlyR α1 and β subunit mutations, respectively), deficiencies in the acrosome reaction are found. Molecular studies have detected GlyR α1, α3 and β subunits in the periacrosomal plasma membrane (Melendrez and Meizel, 1995, 1996; Meizel, 1997; Sato et al., 2000a; Sato et al., 2000b; Llanos et al., 2001; Bray et al., 2002; Sato et al., 2002; Llanos et al., 2003; Kumar and Meizel, 2008). Interestingly, GlyR α4 subunit transcripts were also found in the male genital ridge in chicken (Harvey et al., 2000). Finally, cytoprotective effects of glycine in ischemia-reperfusion injury have also been described in ***other organs***, such as in the gastro-intestinal system, in skeletal muscle and the lungs (Mangino et al., 1996; Iijima et al., 1997; Tariq and Al Moutaery, 1997; Ascher et al., 2001; Lee et al., 2001, 2002; Gohrbandt et al., 2006). However, in those models, no clear cellular targets for glycine have been identified, so indirect effects via immune cells or even the nervous system have not been eliminated.

3.7 Conclusion

The effects of glycine, mediated by classical or unconventional GlyRs, have been described in many different non-neuronal cell types and both inside and outside the nervous system. Broadly, glycine effects can be classified into two main types: cytoprotective and modulatory, an overview of which can be found in Table I.1.

Modulatory effects of glycine were mainly found in immune cells such as macrophages and are associated with clear molecular and pharmacological evidence of functional GlyRs. For example, glycine in macrophages induces uptake of $^{36}Cl^-$ and inhibits calcium flux in a dose-dependent manner, in a concentration range of 0.1 and 1 mM. Pharmacological findings also support the involvement of GlyRs in these effects: blockage is observed by low concentrations of strychnine (1 μM), similar effects are elicited by taurine and β-alanine, but not by L-alanine, and these effects are eliminated by replacement of chloride in buffers. At the molecular level, both GlyR α and β subunits have been described on these cells, although there may be important species differences. Besides immune cells, comparable modulatory effects were observed in glial cells, endothelial cells and spermatozoa. Although the GlyRs on these cells are clearly identical in subunit composition to selected neuronal GlyRs, it is remarkable that, to our knowledge, no direct electrophysiological evidence for functional GlyRs exists in immune cells. Although, technically, there is no problem performing patch-clamp experiments on these cells, the only non-neuronal cells where GlyR-mediated ionic currents have been directly measured are glial cells, and even in this case currents could only be recorded from spinal cord slices, as opposed to cultured cells. This suggests a complex regulation of GlyR expression in non-neuronal cells and further research is required to confirm the existence of functional GlyRs on these cell types. In this regard, an emphasis on correct cell culture conditions may represent the best way forward.

Cytoprotective effects were mainly studied in renal cells, hepatocytes and endothelial cells, and have obvious clinical and therapeutic importance in the protection that is provided under ischemic conditions. There is some evidence linking these effects to GlyRs, although these receptors seem to function in an entirely different mode as described for the classic neuronal GlyRs. Glycine effects also seem to be more non-specific in the sense that the observed effects can be mimicked by other structurally related amino acids (*e.g.* L-alanine) and are caused by higher concentrations of glycine (up to 10 mM). Other findings inconsistent with GlyR-mediated effects are the 'agonist-like' effects of strychnine in high concentrations (1 mM) and of non-specific chloride channel blockers, as well as the chloride-independent effects. At the molecular level, mainly GlyR β subunits have been detected, which are not reported to form functional homomeric GlyRs in recombinant systems. It is however noteworthy that the GlyR β subunit distribution in the CNS is far more extended compared to GlyR α subunits, suggesting that β subunits may have some as yet unknown function (Malosio et al., 1991). The current hypothesis concerning the cytoprotective effects of glycine against ischemia is tenuous at best, since it requires the existence of a glycine-sensitive pore that is activated in later stages of ischemic cell death, leading to membrane leakage of macromolecules. Moreover, this pore is stabilized and/or blocked by glycine, GlyR antagonists and some chloride blockers. Molecularly, this pore seems to be some multimeric protein, somehow associated with GlyR subunits which can stabilize the pore upon ligand binding. More research is certainly needed to further explore this intriguing hypothesis. Finally, when considering glycine-related effects in non-neuronal cells, it is important to realize that other mechanisms do exist, some of which maybe also important for the reported modulatory and cytoprotective effects, that are not discussed here. These include the NMDAR-mediated glycine effect in the vascular system, transporter-mediated effects in microglial cells or possible metabolic effects in microglia or the liver.

Table I.1 Mechanisms responsible for glycine effects in non-neuronal cells.

	CYTOPROTECTIVE EFFECT	MODULATORY EFFECT
CELLS MOST STUDIED	renal cells hepatocytes endothelial cells	immune cells macroglial cells endothelial cells
MAIN EFFECT	protection against ischemic necrosis	modulation of proliferation, migration, differentiation, apoptosis, …
ACTIVE CONCENTRATIONS	up to 10 mM	0.1-1 mM
CALCIUM FLUX MODULATION	no	yes
UPTAKE OF 31-CHLORIDE	no	yes
CHLORIDE DEPENDENCY	no	yes
PHARMACOLOGICAL FINDINGS	no strychnine block described mimicked by structurally related amino acids (*e.g.* L-alanine, L-serine) mimicked by 1 mM strychnine and other chloride channel blockers no mimicking by taurine	blocked by 1 µM strychnine mimicked by taurine and β-alanine
MOLECULAR FINDINGS	only β-subunits	both α- and β-subunits
ELECTROPHYSIOLOGY FINDINGS	none	only in slice preparations of macroglial cells
SUGGESTED MECHANISM	glycine sensitive death pathway unknown role for GlyR (subunit)s	glyR dependent modulation of calcium signalling

4. AIMS OF THIS WORK

In the previous section we suggested that glycine effects in non-neuronal cells were mediated by two main mechanisms. The modulatory mechanism seems to be clearly mediated by GlyRs, whereas the cytoprotective mechanism seems to require GlyRs working in a more unusual mode. Concerning the immunomodulatory mechanisms important questions remain regarding the role of glycine and GlyRs in the modulation of T lymphocytes and microglial cells.

T lymphocytes (or T cells) are part of the adaptive immune response, meaning that they are responsible for specific recognition of pathogens. They are derived from bone marrow stem cells and subsequently develop in the Thymus (explaining their name). Broadly there are 2 groups of T cells: helper and cytotoxic T cells. T-helper cells are responsible for crucial interactions with B lymphocytes (responsible for antigen secretion) and macrophages. Cytotoxic T cells on the other hand are able to destroy host cells when infected by viruses or other intracellular pathogens. T cells are normally not present inside the CNS, due to the blood brain barrier. However in pathological conditions (*e.g.* multiple sclerosis) they can infiltrate the brain to mediate the immune response. Under these circumstances local substances, present in the normal neuronal micro-environment, such as glycine and other neurotransmitters, may modulate T cells responses. As discussed in the previous section the present reports on glycine immunomodulatory effects on T cells are contradictory. On the one hand it has been shown that glycine exerts an anti-proliferative effect on T lymphocytes and blunts calcium fluxes, effects suggested to be mediated by strychnine sensitive GlyRs (Stachlewitz et al., 2000). It was also suggested that glycine may have anti-apoptotic effects on T cells (Franek et al., 2002). On the other hand Sommer and colleagues could not find any effect on proliferation of human peripheral blood mononuclear cells (Sommer et al., 1994) and Alam and co-workers could not find any effect of glycine on calcium fluxes in human T lymphocytes (Alam et al., 2006). Furthermore there is no molecular evidence for GlyRs in T lymphocytes and most data are derived from non-human cells. Also the mechanism on which glycine exerts its effects is unclear as discusses previously.

Since their first characterisation by del Rio Hortega at the end of the 20s, **microglia** have always been considered as the macrophages of the brain. After activation they can become phagocytic, present antigens and secrete cytokines (Kim and de Vellis, 2005). Besides this important immune surveillance function, there is increasing evidence for a broader role of these cells. In development they have an important function in determining neuronal fate, differentiation and synaptogenesis (Polazzi and Contestabile, 2002; Bessis et al., 2007). Also their role in different pathologies seems to be more important than originally thought. These cells have been linked to neuro-inflammatory disorders (Muzio et al., 2007), neuropathic pain (Coull et al., 2005; Scholz and Woolf, 2007), neurodegenerative diseases (Gao and Hong, 2008), epilepsy (Aronica et al., 2007) and even psychiatric disorders (Bayer et al., 1999). In these pathologies it is often quite difficult to determine whether they have a beneficial or a harmful role. For better understanding of these roles, insight is needed in the regulating

mechanisms of these cells. Being released from both neurons and macroglia, neurotransmitters may be of interest as potential regulators of microglial function. In microglial cells not much is known about glycine immunomodulation. It has been suggested that astrocytes modulate microglial activity by secreting glycine and L-serine (Tanaka et al., 1998; Yang et al., 1998). This would lead to an increase in microglial activity, contrary to the inhibitory role that is given to glycine in the peripheral immune system (see previous section). However, nothing is known about the mechanism of this modulation. Given the role of microglial cells in different pathologies, the exact mechanism may be of special interest as a focus of potential new therapeutic targets.

The main aim of this work is to obtain better insight in the immunomodulatory properties of glycine and its receptor in T cells and microglia. In *chapter II* our findings on human T cells will be discussed. As GlyR expression was not shown before in T cells, molecular evidence for GlyR expression in these cells is provided and subsequently the functional (electrophysiological) properties of these receptors are examined. Furthermore the potential modulatory effects of glycine on cell viability will be investigated. In *chapter III* the mechanism behind the modulatory effects of glycine on microglial cells is examined. Again the first focus is the potential molecular evidence for GlyR expression in microglia. Next the effects of glycine on the production of immune cell mediators is investigated and these mediators are subsequently used as a parameter for a pharmacological study on the precise mechanism. After examining the electrophysiological consequences of glycine perfusion of microglial cells, the effects of glycine on calcium signals will be examined, which have been correlated to glycine effects in peripheral immune cells. In *chapter IV* it is investigated whether the glycine effects found on microglial cells are specific for microglia or rather represent a more general property in macrophages. As glycine is a neurotransmitter in the CNS, insight in glycine modulatory properties in these cells may gain insight in intercellular communication in the CNS both in normal and pathological conditions. Although this work has a primary fundamental nature, insight in the precise mechanism of glycine immunomodulation may provide new targets for therapeutics for the above-mentioned disorders.

CHAPTER II. GLYCINE SIGNALING IN HUMAN T LYMPHOCYTES

1. INTRODUCTION

T lymphocytes belong to the adaptive immune system, meaning that they can react to specific antigens. These cells are characterized by the presence of the $CD3^+$ T cell receptor (TCR), which is responsible for antigen recognition, once presented by means of the MHC-complex (major histocompatibility complex) of antigen presenting cells (APC). The binding of MHC/antigen complexes to the TCR results in the recruitment and activation of tyrosine kinases, leading to the activation of phospholipase C, the release of inositol 1,4,5-triphosphate (IP_3) and calcium release from the ER. This "store emptying" then results in the activation of calcium release activated calcium channels (CRACs) at the plasma membrane, leading to an additional calcium influx from the extracellular environment. The resulting rise in intracellular calcium concentration then leads to the activation of the calcium-calmodulin-dependent calcineurine, a phosphatase which dephosphorylates the transcription factor nuclear factor of activated T cells (NFAT), leading to nuclear translocation of this factor and transcription of different cytokines such as IL-2, as well as proliferation of T cells (Lewis, 2001). The importance of the calcium signal in determining lymphocyte effector functions means that this signal needs to be tightly regulated. In this context stabilization of the membrane potential is crucial. Indeed the driving force for calcium influx through CRACs is partially determined by the negative membrane potential. Upon CRAC activation, the resulting calcium influx depolarizes cells, tending to decrease further calcium influx. However voltage- ($K_V1.3$) and calcium-activated ($IK_{Ca}1$) K^+ channels, which are activated upon calcium influx, tend to clamp the membrane potential at a hyperpolarized potential, allowing sustained calcium influx. The role of these channels is supported by the findings that K^+ channel blockers are capable of inhibiting T cell activation (Chandy et al., 2004; Panyi et al., 2004). Also, classical immunosuppressant agents (*e.g.* Cyclosporin A, rapamycin or dexamethasone) were shown to reduce K^+ currents in T cells (Panyi et al., 1996; Lampert et al., 2003). The central role of the membrane potential in determining the magnitude of calcium fluxes (and hence T cell effector functions) makes neurotransmitters, acting on ionotropic receptors, interesting targets to study as potential immunomodulators.

T cells are normally unable to penetrate the blood brain barrier (BBB), but in multiple sclerosis (MS) and other neuro-inflammatory conditions, damage of this barrier leads to the infiltration of T cells. MS is the most frequent occurring neurological disease in young adults. A prevalence of 0.1% was reported in Flanders (van Ooteghem et al., 1994). The disease is characterized by immune-mediated damage to the myeline sheet, leading to problems in nerve conduction resulting in a wide array of clinical symptoms (*e.g.* sensory disturbances, visual symptoms, bladder dysfunction, motor deficits), often occurring in a relapsing-remitting pattern. The etiology and pathogenesis is not clarified yet. Probably the cause is multifactorial with both environmental (*e.g.* viral infections) and genetic factors (*e.g.* HLA-DR2 phenotypes) playing an etiological role (Noseworthy et al., 2000). It is now generally accepted that auto-immune (helper) T cells initiate the disease, while macrophages, microglia, antibodies, complement factors as well as intrinsic CNS processes determine disease progression (McFarland and Martin, 2007).

Changes in the concentrations of different amino acids and neurotransmitters have been described in both blood and cerebrospinal fluid (CSF) of MS patients. Glycine concentrations were found to be elevated in blood, while glycine, glutamate, taurine and noradrenaline were found to be elevated in the CSF (Stover et al., 1997; Barkhatova et al., 1998). These alterations may suggest a pathogenic role for these neurotransmitters in neuro-inflammatory conditions like MS. Different studies provided indeed a pathogenic role for glutamate, the most important excitatory neurotransmitter. Some key *Nature Medicine* studies in the beginning of the millennium showed that AMPA-antagonists could indeed ameliorate the disease course in experimental autoimmune encephalomyelitis (EAE), an animal model of MS (Pitt et al., 2000; Smith et al., 2000). The authors suggested a toxic role for glutamate, both on oligodendrocytes and neurons. More-over also T lymphocytes were shown to be modulated by different concentrations of glutamate. Generally an inhibition of mitogen-induced proliferation was found and both ionotropic and metabotropic receptors as well as metabolic mechanisms were suggested to mediate the effect, possibly via modulation of voltage gated potassium channels (Lombardi et al., 2001; Lombardi et al., 2004; Pacheco et al., 2004; Poulopoulou et al., 2005). Remarkably the inhibition of proliferation was found to be less pronounced in T cells, derived from MS patients (Lombardi et al., 2003). Also GABA, considered as the most important inhibitory neurotransmitter in the cerebral cortex, was shown to modulate T cell function. GABA was found to activate $GABA_A$-Rs, leading to an inhibition of mitogen induced proliferation, IL-2 production and increase in intracellular calcium concentration (Tian et al., 1999; Alam et al., 2006). The authors hypothesized that $GABA_A$-R activation leads to a chloride efflux in T lymphocytes (as discusses in chapter II), a membrane depolarization and hence an inhibition of calcium fluxes through CRACs. Besides glutamate and GABA, other neurotransmitters (*e.g.* acetylcholine, serotonine) and even neuropeptides (*e.g.* Neuropeptide Y, Substance P) were shown to modulate T cell function (Levite, 2001; Stefulj et al., 2001; Razani-Boroujerdi et al., 2008; De Rosa et al., 2009).

As indicated glycine and taurine, one of the physiological GlyR agonists, are elevated in the blood and/or CSF of MS patients, making this inhibitory neurotransmitter an interesting target to study as well. Only one study examined the direct effects of glycine on rodent T lymphocytes (Stachlewitz et al., 2000). The authors found an inhibition of cell proliferation as well as a blunting of calcium-signaling, effects that were shown to be GlyR-dependent. These results were contradicted however by other studies showing that β-alanine, another GlyR agonist, did not inhibit mitogen-induced proliferation (Tian et al., 1999), glycine did not blunt fMLP-induced calcium fluxes (Alam et al., 2006), nor changed proliferation of human PBMCs (Sommer et al., 1994). An anti-apoptotic effect of glycine and other amino acids was suggested in a human T cell line, but the mechanism was not studied (Franek et al., 2002). Also no molecular evidence for GlyR expression is available, contrary to other immune cells.

The aims of this chapter are to search for molecular evidence for GlyR expression in human T lymphocytes and to determine the functional role of glycine and potential GlyRs both electrophysiologically and in regulating apoptosis. For a lot of these studies we used the human leukemic T-cell line Jurkat. This cell line was first identified in 1980 (Gillis and Watson, 1980) and even nowadays is very popular for studying signal transduction pathways. Besides the general advantages of cellines (low cost, accessibility, homogeneity and purity) these cells have the advantage of being easily transfectable for studying specific signaling

pathways. Some caution is necessary because differences with peripheral blood T cells have been described. Besides differences in cytokine profile, Jurkat cells were found to be deficient in two lipid phosphatases (PTEN and SHIP). The biological implications of these deficiencies are poorly understood, although they may explain the exaggerated proximal signaling that was found in Jurkat T cells. Despite the differences, many of the fundamental insights of the TCR signaling machinery came from studies with normal or mutant Jurkat T cells, and many of the discoveries have been subsequently shown in peripheral blood T lymphocytes (Abraham and Weiss, 2004; Bartelt et al., 2009).

2. MOLECULAR EVIDENCE FOR GLYCINE RECEPTOR SUBUNIT EXPRESSION IN HUMAN T LYMPHOCYTES

2.1 Introduction

In different types of macrophages different GlyR subunits were found, both at the mRNA and the protein level (Froh et al., 2002). However GlyR molecular structure was not studied in T cells so far, at least not directly. In a paper studying NRSE (neuron-restrictive silencer element)-dependent gene silencing in small lung cancer cell lines and their role in paraneoplastic syndromes, mRNA was found to be present for GlyR β-subunits and gephyrin, but not for GlyR α1- nor α3-subunits in Jurkat T-cells. In primary human lymphocytes both GlyR α-subunits as well as the β-subunit were found to be absent, while gephyrin was not studied. The other GlyR α-subunits (α2 and α4) were not studied either (Neumann et al., 2004). To our knowledge no electrophysiological studies were performed examining glycine (receptor)-induced currents in human T cells either. The first aim of this section is to provide molecular evidence for GlyR-expression, both at the mRNA and the protein level, and examine the electrophysiological consequences of glycine perfusion of T cells.
Glycine and to a smaller extent also the GlyR agonist β-alanine (both 1-4 mM) were shown to have anti-apoptotic properties in a starvation model of the human T cell line MOLT-4 (Franek et al., 2002). The precise mechanism was not studied. In a similar model of B lymphocyte hybridoma cells, glycine, as well as the GlyR agonists β-alanine and taurine had anti-apoptotic properties as well (Franek and Sramkova, 1996). Although the authors concluded the most likely explanation is a specific signaling mechanism, rather than a nutrient function, they did not study GlyRs specifically. Therefore the second goal of this chapter is to investigate potential glycine mediated protective effects on T cell viability.

2.2 Materials and methods

2.2.1 Cell culture

Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of healthy humans by Ficoll density gradient centrifugation using Histopaque® 1077 (Sigma-Aldrich, Bornem, Belgium). Freshly collected blood was diluted (½) in RPMI-1640 medium (Invitrogen, Merelbeke, Belgium) and 35 ml of the dilution was carefully poured into a 50 ml-tube containing 15 ml Histopaque® 1077. The mixture was centrifuged for 20 minutes at 1800 rpm and after removal of plasma/ RPMI-1640 medium, the PBMC band was put in a separate tube and washed 3 times in RPMI-1640 medium. Cells were counted, suspended at 500.000 cells/ml in RPMI-1640 medium, supplemented with 10% Fetal Calf Serum (FCS, Invitrogen) and activated with phytohaemagglutinin (PHA) (4µg/ml) and IL-2 (5U/ml) (Sigma-Aldrich). After 7 days of culture purity of $CD3^+$ T cells was checked by means of CD3-staining and flowcytometry and was always found higher than 95%. Cells were washed in ice cold PBS and cell pellets were frozen at -80°C until usage for molecular experiments.
Jurkat T cells, a human leukaemic T cell line, were cultured in RPMI-1640 medium supplemented with 10% FCS, 1% penicilline-streptomycin (Invitrogen) and 5 µg/ml

streptomycin (Invivogen, Toulouse, France). Cells were put on new medium twice a week and were never kept in culture for longer than 6 weeks.
Chinese Hamster Ovary (CHO) cells, stably expressing α2 homomeric GlyRs (Mangin et al., 2003), were used as positive controls for immunolabelling and electrophysiological experiments. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10 % FCS and 2% penicillin and streptomycin and 500 µg/ml Zeocin (Invitrogen). Growth medium was replaced twice a week and the cells were subcultured weekly.

2.2.2 Chemicals

Glycine, strychnine, staurosporine, hydrogen peroxide, MTT (Methylthiazolyldiphenyl-tetrazolium bromide) were all purchased from Sigma Aldrich (Bornem, Belgium).

2.2.3 Reverse transcriptase polymerase chain reaction (RT-PCR)

Cell pellets were prepared as described in *cell culture*. RNA was isolated with an RNeasy Mini Plus kit following the manufacturer's protocol (Qiagen, Venlo, The Netherlands). This kit ensures additional elimination of genomic DNA. cDNA was then prepared from the collected RNA using the Reverse Transcription System (Promega, Madison, WI, USA) after which DNA samples were stored at -20 °C until analysis. 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 (55.7-63.0 °C, dependent on the primers, see Table II.1) for 60 s, and elongation (72 °C for 40 s). Primers were developed using Oligo6 software (Molecular Biology Insights, Cascade, CO, USA) and synthesized by Eurogentec (Seraing, Belgium). PCR products were then separated on a 1% agarose gel, containing Etidium Bromide. The bands of interest were extracted from the gel using the Qiaquick gel extraction kit (Qiagen), purified and sequenced (Qiagen sequencing services, Hilden, Germany) to confirm cDNA identity.

Table II.1 Primers used for RT-PCR of human T lymphocytes

	Gene of interest	Forward primer (5'-3')	Reverse primer (5'-3')	PCR product size	Ta (°C)
GLRA1	GlyR α1 subunit	GCGGCAGATGGGTTACTACCT	CCTTGTGATGTCTCCGCTTCC	326 (1031-1336)	59.3
GLRA2	GlyR α2 subunit	TGGCGTACAGTGAGTACCC	CGCCATCCAGATGTCAAT	647 (850-1479)	56.8
GLRA3	GlyR α3 subunit	TTCATCAACAGTTTCGGCTCT	ATCGGCTTTCCCTTACCTC	913 (649-1543)	55.7
GLRA4	GlyR α4 subunit	CCACGCCTGTCCTACCGAGAA	GTAGCCCATCTGCCGTTCC	459 (440-880)	59.8
GLRB	GlyR β subunit	AGGACCTTGCCCGAGTACCTG	ATGTGTAGTAGCCCGTGCCTT	627 (278-884)	55.7
GPHN	gephyrin	TTCAGAATCGCCTCGTGCTC	GTGACCATCATAGCCGTCCAA	1418(1922-3319)	59.3
GLYT1	GlyT, type 1	CCGGACAGGGCGCTAC	AGCTCAGGGCAGGGTATAAGG	469 (2013-2461)	63.0
GLYT2	GlyT, type 2	CACCCTGGAGCGGAACAA	TAGCGGTAAGAGCCATAGGTC	1713 (698-2390)	58.5
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	GCTCTCCAGAACATCATCCCTGCC	CGTTGTCATACCAGGAAATGAGCTT	347 (706-1028)	61.5

All primers were designed using Oligo6 software. Nucleotide positions are shown between brackets after the PCR product length. Ta annealing temperature.

2.2.4 Western Blotting

Cell pellets were prepared as described in *cell cultures*. The protein content of cell lysates was determined by a Bradford protein assay. Ten µg of cell protein extract was loaded on a SDS-polyacrylamide gel (10%) and subsequently electrophoresed at 200V until the dye front reached the bottom of the gel. The gel proteins were then transferred to a PVDF membrane, by blotting overnight at 90mA. Two primary antibodies were used for western blotting. The GlyR mAb4a antibody (Pfeiffer et al., 1984) and the gephyrin specific 3B11 antibody (both from Synaptic Systems, Goettingen, Germany). After blocking in blocking buffer, membranes were incubated for 1 hour in primary antibody (1/500 for mAb4a and 1/1000 for 3B11), washed and incubated for 1 hour in a peroxidase-conjugated labelled secondary antibody (Rabbit anti-mouse 1/1000, Dakocytomation, Heverlee, Belgium). Proteins were detected by adding the chromomeric substrate DAB (3,3' Diaminobenzidine tetrahydrochloride) and 30% hydrogen peroxide.

2.2.5 Immunolabelling and confocal microscopy

Glass coverslips were coated with poly-L-lysine (PLL, 100 µg/ml) to allow cells to stick to the surface. A cell suspension of 50.000 cells was put on the coverslip and cells were allowed to stick to the surface for 45 minutes at 37 °C. After incubation the medium was removed and cells were fixed with paraformaldehyde (PFA) 4% for 10 minutes and permeabilized with TRITON X-100 (Sigma-Aldrich) 0.2% for 10 minutes. To avoid aspecific binding of antibodies to cells, cells were blocked with Image Signal Enhancer (Invitrogen) for 30 minutes and subsequently with 3% goat serum (Millipore, Brussels, Belgium) for 20 minutes. After washing, primary antibody was added and allowed to incubate for 1 hour. After washing, secondary antibody was added and allowed to incubate for 1 hour as well. After this procedure cells were mounted on a microscope slide using a small amount of prolong gold antifade (PGA, Invitrogen) and allowed to dry in the dark for at least 24 hours. For every labelling blank control labellings were performed using the same procedure, but without labelling with the primary antibody. The following primary antibodies were used: Anti-GlyR mAb4 1/500, Anti-Gephyrin 3B11 1/500 (both from Synaptic Systems), Anti-GlyR N-18 1/100 (Santa Cruz Biotechnology, Heidelberg, Germany) and non-commercial Anti-GlyR α4 1/400 antibody, which was kindly provided by Dr.R.J.Harvey (London, UK) (Heinze et al., 2007). As secondary antibodies we used goat anti-mouse or goat anti-rabbit, both conjugated to Alexa-555 and purchased from invitrogen. The slides were examined with a Zeiss LSM 510 META confocal microscope (Zeiss, Jena, Germany). We used a helium neon laser, with excitation at 543 nm and filters set to detect emission above 560 nm to detect alexa-555 fluorescence. Images were taken at 63x magnification with oil immersion.

2.2.6 Electrophysiology*

Changes in total membrane currents and potentials of Jurkat T cells were studied at room temperature using the whole cell configuration of the patch clamp technique both in voltage clamp and current clamp conditions using standard extracellular and intracellular solutions and continuous bath perfusion. Intracellular solutions contained KCl 125 mM, NaCl 5mM, $CaCl_2$ 2 mM, HEPES 10 mM, EGTA 10 mM, MgATP 2.5 mM and Na_2ATP 2.5 mM (pH = 7.1) and extracellular solutions contained KCl 5 mM, NaCl 145 mM, $MgCl_2$ 1.5 mM, $CaCl_2$ 2 mM and HEPES 10 mM (pH = 7.4). For perfusion experiments intracellular solutions contained CsCl 130 mM, $CaCl_2$ 1 mM, $MgCl_2$ 4 mM , HEPES 10 mM, EGTA 10 mM and Na_2ATP 4 mM (pH = 7.1) and extracellular solutions contained KCl 2 mM, NaCl 125mM, $CaCl_2$ 2 mM, HEPES 2 mM, $MgSO_4$ 2 mM, $NaHCO_3$ 26 mM and KH_2PO_4 1.15 mM (pH = 7.4). Glucose was added to all solutions in order to adjust the osmolality to ~300 mOsm/l. The cells were transferred to a RC-25 perfusion chamber (Warner Instruments – Harvard Apparatus, Holliston, USA) on the stage of an inverted phase contrast microscope (Nikon Diaphot, Japan). The experimental protocols and data acquisition were performed with a personal computer controlled EPC-10 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany). Pipette electrodes were made from 1.5 mm (o.d.) borosilicate glass capillary tubes, and had a resistance between 4 kΩ and 5 MΩ. Liquid junction potentials were calibrated using the Junction Potential Calculator for Windows (JPCalcW, Peter H. Barry, Dept. of Physiology&Pharmacology, Australia & Axon Instruments, Inc., California, USA) and were taken into account at the start of each experiment. Capacitive and leak currents were compensated for automatically by the Pulse program (HEKA Elektronik, Lambrecht, Germany) and residual capacitances and leak currents were eliminated by means of a P/6 protocol. The series resistance was lower than or equal to 10 MΩ after compensation. Data were filtered at 2.9 kHz and sampled at 20 kHz. The recording of whole-cell current began 5 min after rupture of the cell membrane, to allow the cell interior to adequately equilibrate with the pipette solution. Acquired data were stored on a computer hard disk for later analysis and, when quantified, expressed as mean ± S.E.M. Glycine 2 mM was applied by means of a local microperfusion system (SF-77B Perfusion Fast Step, Warner Instrument, Harvard Apparatus, Holliston, USA), positioned laterally above the cell at a distance of approximately 25 – 50 µm. However, in order to investigate the effect on potential sensitive K^+ currents, chemicals were bath applied.

2.2.7 Colorimetric MTT cell viability assay

We used the *colorimetric MTT assay* as a screening test for cell number and viability after cytotoxicity induction of Jurkat T cells (Mosmann, 1983). In this assay the yellow MTT is converted to purple-blue formazan crystals by means of mitochondrial reducing enzymes, present in living cells. Jurkat T cells were cultured for 0-3 days in different (glycine) conditions at 37° and 5% CO_2 after induction of cytotoxicity. At the end of the experiment MTT (0.5 mg/ml) was added and cells were again allowed to incubate for 4 hours. Then

* Electrophysiological experiments were performed by Dr.D.Janssen but are mentioned here for their crucial relevance in this research project.

supernatants were removed and the blue formazancrystals were solubilized with a mixture of DMSO 90% and TRIS 10% (pH 10.5). The absorbance was measured on a microplate reader at a wavelength of 550 nm, with a reference wavelength at 630 nm. The difference A550-A630 was normalized to the value of day 0 and used as a percentage of viable cell number. After determining the optimal cell culture starting number by means of calibrating cell number to A550-A630, three substances were chosen to induce cytotoxicity in Jurkat cells: staurosporin (STS), hydrogen peroxide and starvation by diluting the culture medium in PBS. The lowest dose that was able to signifantly decrease the number of viable cells was determined and used to examine potential cytoprotective effects of glycine.

2.2.8 Caspase-3 assay

Because caspase-3 is known as a key enzyme in apoptotic pathways, we determined caspase-3 activity, with a caspase-3 assay kit with fluorescent marker Z-DEVD-AMC (EnzCheck®, Invitrogen), which yields a bright blue fluorescent product upon proteolytic cleavage by caspase-3. Apoptosis was induced by adding staurosporin for 2 hours, after pre-incubating cells for 1 hour in different glycine concentrations to determine the possible modulation by glycine. The caspase-3 activity was measured in the lysate of Jurkat T cells, according to the instructions of the manufacturer. Fluorescent activity was determined in the Fluostar optima plate reader (Isogen Life Science, De Meern, The Netherlands) with excitation filters set at 355 nm and emission at 450 nm. Fluorescence data were normalized to the control glycine condition (0mM) after STS addition.

2.2.9 Fluorimetric cell viability assay*

For correlating caspase-3 activity with cell viability, we used *the fluorimetric "DHL Cell viability and proliferation assay kit"* (Tebu-Bio, Boechout, Belgium). The basic method of measuring viability, by means of the activity of mitochondrial dehydrogenases in cell cultures as an indicator of total viable cell number, is comparable to the MTT assay. However the fluorimetric assay has several advantages. As there is no need for solubilisation of crystals, it allows faster and kinetic measurements, is very sensitive, less time consuming and less prone to variability. Cellular activity with the fluorimetric method was determined according to the instructions of the manufacturer. Briefly, 20µl of reagent and 100µl culture medium was added to each well and the change in fluorescence was measured during a one hour time interval as an indicator of total dehydrogenase activity.

2.2.10 Statistics

Data are expressed as means ± S.E.M. Statistical significance was evaluated using ANOVA testing or student t-testing when comparing only 2 sets of data. P-values of less than 0.05 were considered significant.

* The reason this assay was not used for timeline screening of cell viability was because of the unavailability of a fluorescent plate reader at the moment of the MTT assays. Caspase-3 activity assays were performed later in time when a fluorescent plate reader was available.

2.3 Results

2.3.1 Molecular evidence for glycine receptor expression in human T lymphocytes

As nothing is known about GlyR expression in human T lymphocytes, we first performed a western blot screening to detect GlyR-subunits. Using the GlyR subunit mAb4a we detected a clear band around 48 kDa in both Jurkat T cells and PBMCs, indicating presence of GlyR α-subunits (Figure II.1A). Remarkably this band was located somewhat higher than the one in the spinal cord. No band could be detected in T cells at 58 kDa corresponding to GlyR β-subunits, as can be seen in the spinal cord positive control. Also the anchoring protein gephyrin could be detected in both Jurkat T cells as PBMCs (Figure II.1B).

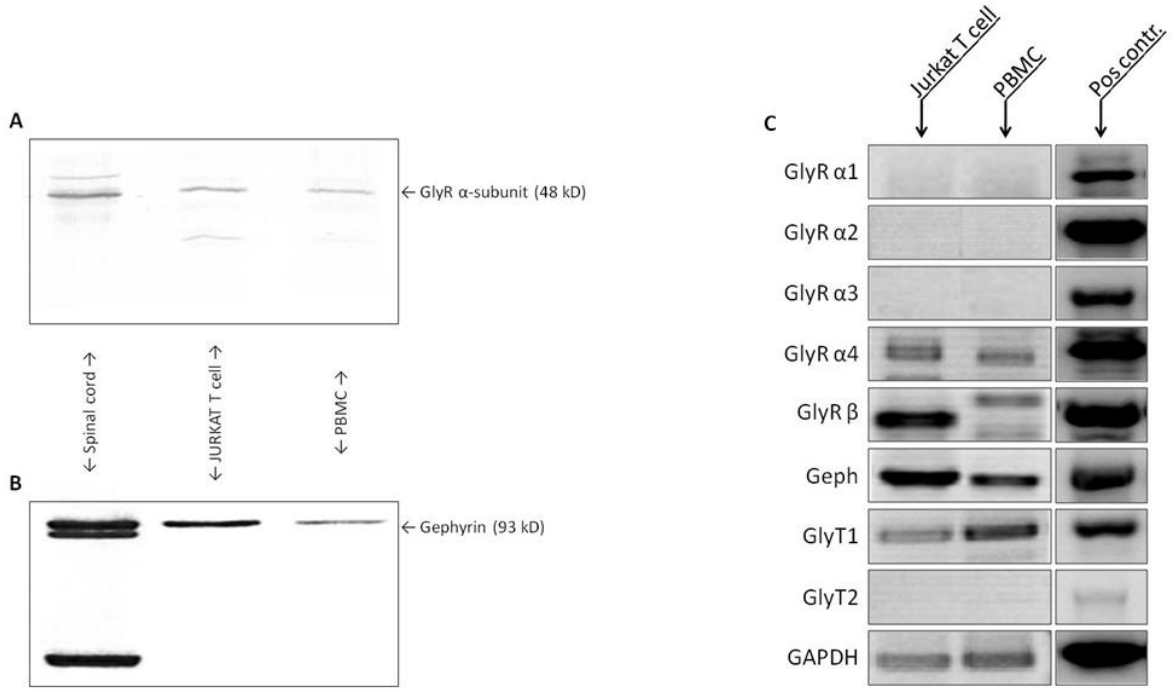

Figure II.1 Molecular evidence for GlyR expression in human T lymphocytes.
A and B. Western blot results. Cell protein extracts from spinal cord (positive control), Jurkat T cells or PBMCs were loaded and blotted as indicated in materials and methods. using anti-GlyR mAb4a (A) a clear 48 kDa band, corresponding to GlyR α-subunits, could be detected. This band was located slightly higher compared to the spinal cord positive control. Using the anti-gephyrin 3B11 Ab (B) a band was detected at 93 kDa in both Jurkat T cells as PBMCs. C. RT-PCR results. In both Jurkat T cells and PMBCs mRNA could be detected for GlyR α4 subunits, gephyrin and GlyT1, while the GlyR β-subunit was only clearly present on Jurkat T cells and not on PMBCs. No mRNA for GlyR α1-3 subunits could be detected in either of both cells. mRNA extracts from a human temporal lobe sample was used as positive control.

Because these immunoblots only point towards the presence of the proteins, but did not tell us anything about the location, our next step was to locate these proteins inside cells. As can be seen in Figure II.2A GlyR subunits could be clearly detected inside Jurkat T cells. These proteins were located in the periphery of cells and had a punctuate appearance, which seemed to be mainly located in the cytoplasm as can been seen in Figure II.2B. CHO cells expressing GlyR α2 subunits, which are known to be functional (Figure II.4), were used as positive controls (Figure II.2C). Also the anchoring protein gephyrin could be clearly detected in Jurkat T cells, again located peripherally in the cytoplasm (data not shown).

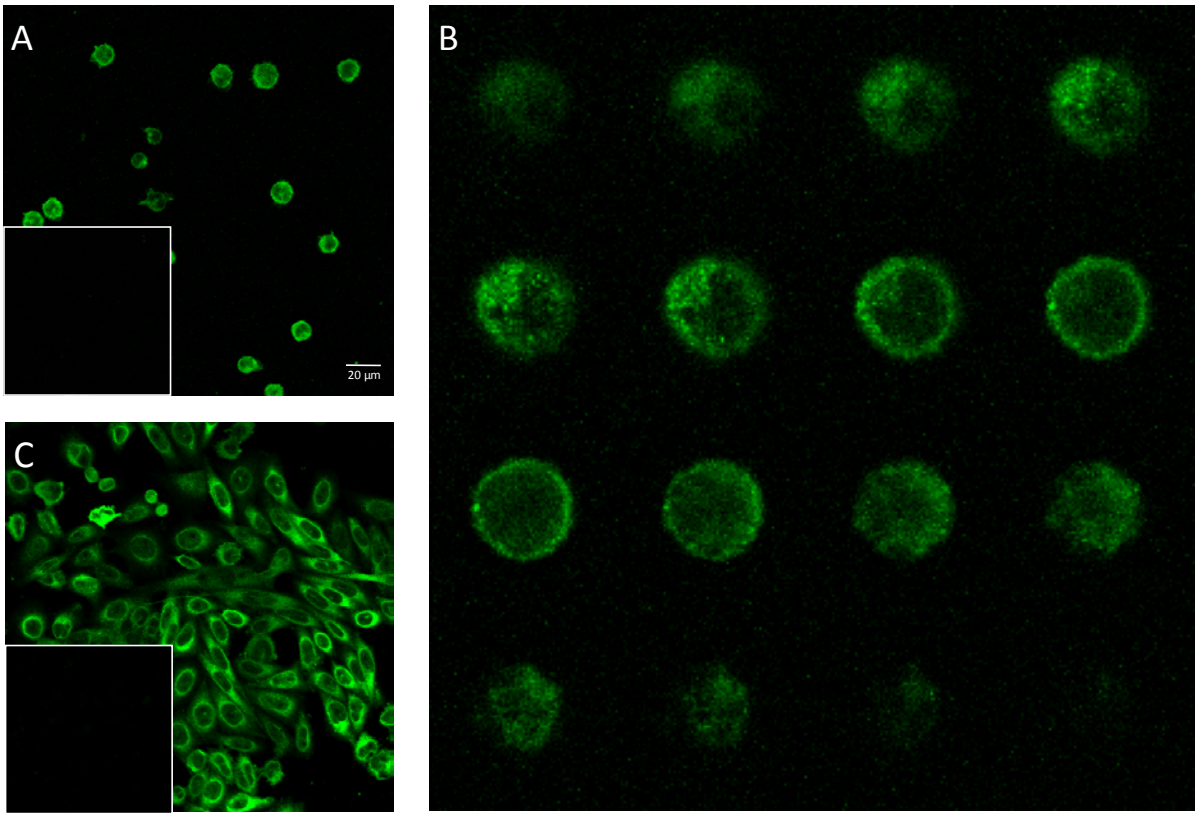

Figure II.2 Confocal imaging of GlyR expression in Jurkat T cells.
Jurkat T cells were labelled for the presence of GlyR subunits (A and B) as explained in materials and methods. GlyRs could be clearly detected at the periphery of the cells (A, 40x magnification). B. Z-stack at 63x magnification of one of the GlyR-labelled cells shows a punctuate appearance of GlyR proteins, located mainly in the cytoplasm. Panel C shows the positive control labeling of CHO cells, stably expressing α2 homomeric receptors (40x magnification). The inserts at figures (A) and (C) show blank negative controls. Scale bars = 20µM.

As the antibody we used for GlyR subunit detection is not specific for one of the different GlyR (α) subunits, we next examined which subunits are expressed at the mRNA level by means of RT-PCR. Surprisingly our RT-PCR results did not show the presence of GlyR α1-3 subunits (Figure II.1). β-subunits were detected, but only in Jurkat T cells, and gephyrin as well as type 1 glycine transporters (GlyT1) could be detected in both Jurkat T cells as PBMCs. Unexpectedly our RT-PCR results also showed weak mRNA expression of the α4 subunit. The sequence was confirmed by sequencing the nucleotides of the PCR product. Because human α4 subunits have not been described in any specific human tissue so far, we compared the homology of the sequenced part with the known mRNA sequence of mice, which was 90% (data not shown). As an analysis of genes in the human genome indicated the GLRA4 gene actually contained a premature stopcodon (TGA) in exon 9, instead of the usual arginine codon (CGA) that is found in mice or other GlyR α-subunits (see Figure I.5C, position 419 of the GlyR α1 subunit), we developed a PCR primer pair located up- and downstream of this stopcodon using Primerdesign 3.0 software. Using the forward primer 5' TACGTGAAGGCAATCGACAT 3' (located at exon 8) and the reverse primer 5' CGGGAGATGGTGTCAAT 3' (located at exon 9, downstream of the presumed stopcodon) we detected an expected band of 341 base pairs at an annealing temperature of 59 °C (Figure II.3A). This PCR product was sequenced and indeed, we could detect a stopcodon (TGA) in exon 9. The functional significance of this stopcodon would be a deletion of the C-terminal

part of the protein, containing the 4th transmembrane domain, making a functional role of these GlyRs doubtful (Figure II.3B). The group of Dr.R.J.Harvey (London, UK) recently developed an antibody directed against the C-terminal part of the GlyR α4-subunit (Haverkamp et al., 2003). We used this antibody to confirm the absence of the C-terminal part. Although an obvious staining was observed of Jurkat T cells with this antibody, this was rather diffuse compared to the pattern of our mAb4a GlyR staining, suggesting aspecific binding (Figure II.3C). A western blot using this antibody confirmed the absence of near-48 kDa proteins, but did show some aspecific heavier bands between 60 and 80 kDa (Figure II.3D), possibly explaining the aspecific staining in Figure II.3C.

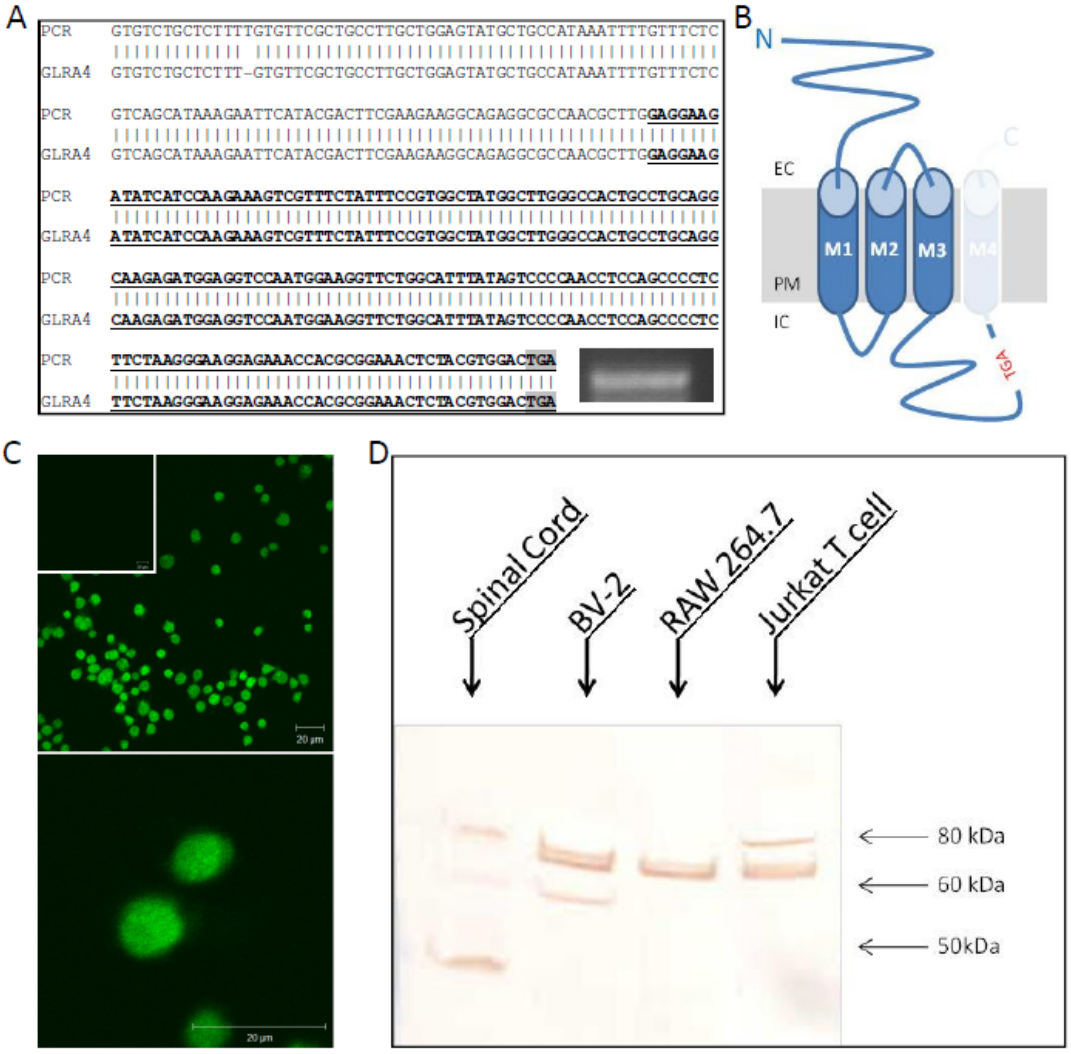

Figure II.3 GlyR α4 subunits contain a premature stopcodon in Jurkat T cells.
A. RT-PCR sequencing results showing an alignment of the PCR product that was found in exon 8 and 9 with the GLRA4 gene (accession number NM_001024452). Exon 9 is shown in bold and underlined. The stopcodon (TGA) is indicated in grey. B. Location of the stopcodon in the GlyR subunit protein structure. The premature stopcodon is located in exon 9, just upstream of M4, leading to a deletion of this part of the protein. C. Confocal images of Jurkat T cells labelled with a specific anti-GlyR α4 subunit antibody. Although a clear staining could be observed with this antibody at 40x magnification (upper figure, with blank control labelling shown in the insert), a 63x magnification demonstrated a diffuse and rather aspecific staining pattern. D. Western blot using the anti-GlyR α4 subunit antibody. No band could be detected near 48 kDa for Jurkat T cells.

2.3.2 Glycine perfusion does not induce any ionic currents*

Electrophysiological measurements were performed on Jurkat T cells as described in materials and methods. Glycine (2 mM) perfusion did not induce any currents (Figure II.4B). Also the resting membrane potential (-58 ± 2 mV, n=15) was unaltered after glycine (2 mM) perfusion as determined by current clamping (data not shown). As a positive control we performed the same experiments in GlyR transfected CHO cells and measured a clear inward current (282 ± 81 pA/pF, n=3) (Figure II.4A). Under current clamp conditions a rise in the resting membrane potential (-63.8 ± 3 mV, n=3) to 0 mV was measured (data not shown).
The voltage-activated currents of Jurkat T cells were measured in the interval from -120 mV to + 60mV (Figure II.4C). The records demonstrated an outward current, which activated between -40 and -20 mV and reached a mean peak of 150 +/- 40 pA at +40 mV. We performed a series of tail current protocols, which showed that the recorded currents had a reversal potential of -70 mV (data not shown). This value is close to the calculated potassium reversal potential (E_{K+} = -82.5 mV) for our recording solutions, suggestive for K^+-selective currents. These currents were partially (48 +/- 8%) blocked by TEA chloride, confirming their K^+-selectivity. We examined the effects of glycine on these Kv-channels by applying 2 mM of glycine to the bath solution, and again analysing voltage-gated currents. The recorded currents were identical to the ones measured in the control (no glycine) conditions with mean peak amplitude of 153 +/- 82 pA at +40 mV. TEA blocked the currents in the same magnitude as in control conditions (51 +/- 7%) (data not shown).

* Electrophysiological experiments were performed by Dr.D.Janssen but are mentioned here for their crucial relevance in this research project.

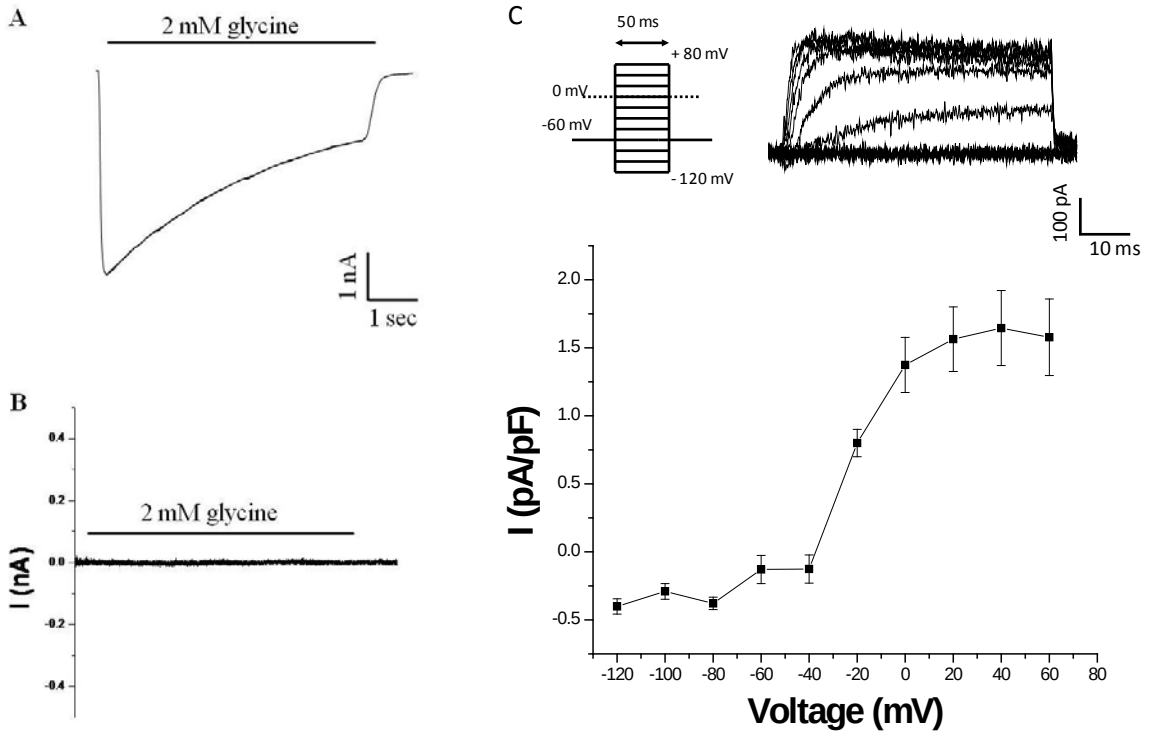

Figure II.4 Glycine does not influence ion currents in Jurkat T cells
Representative examples of current traces after glycine (2 mM) perfusion of GlyR stable transfected CHO-cells (positive controls) (A) and Jurkat T cells (B). Perfusion of cells with 2 mM glycine evoked a clear inward current that reached a peak amplitude of - 3.9 nA in CHO cells (A) but did not induce any currents in Jurkat T cells (B). C. Voltage-activated currents of Jurkat T-cells. Starting from a holding potential of –60 mV, voltage steps of 20 mV were applied and the voltage activated currents were measured in intervals, ranging from –120 to +60 mV. The IV curve in demonstrates the voltage-evoked currents activated between -40 and -20 mV and reached a peak amplitude at +40 mV.

2.3.3 Glycine does not protect against apoptosis.

We first used the MTT assay to screen for glycine effects on Jurkat T cell proliferation or viability. To determine the optimal starting cell number a calibration experiment was performed in which we could find a linear correlation between cell number and absorbance values at 550 nM (with reference absorbance at 630 nM) between 10.000 and 400.000 cells (data not shown). We decided to put the starting cell number (at day 0) at 100.000 cells. This way we would retain reliable data as long as cells did not decrease in number by a factor 10 or increase by a factor 4. We then searched for the lowest dose of three cytotoxic substances that was still able to reduce viable cell number, compared to control proliferation. These concentrations were determined to be 100 nM for STS, 50 µM for H_2O_2 and 10% medium diluted in buffer for the starvation experiments. Figure II.5 shows the percentage of viable cells (compared to day 0) in function of the time course of the experiment. As could be expected there was a linear increase in viable cell number during control conditions (no cytoxicity induction), because of expected cell proliferation. After addition of one of the three cytotoxic substances there was a gradual reduction and significant decrease in viable cell percentage. Under these conditions we screened for potential effects of glycine on the viable

cell percentage. We used a logarithmic range of glycine concentrations between 1 µM and 10 mM. No significant differences could be found in any of the glycine conditions neither after cytotoxic substance addition, nor on the control proliferation (Figure II.4).

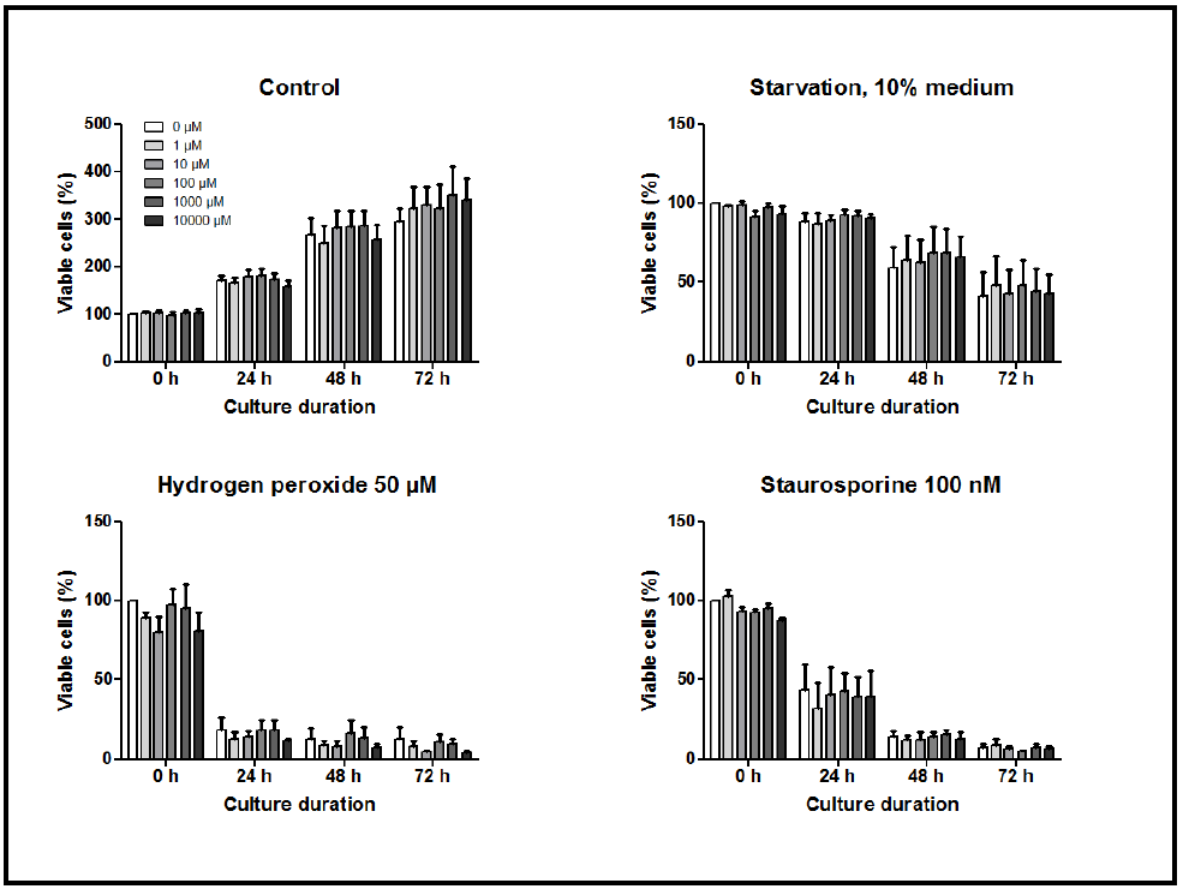

Figure II.5 Glycine does not alter Jurkat T cell viability between 1 µM and 10 mM.
The percentage of viable cells was determined using the MTT assay and normalized to the control experiments (no glycine) at the start of the experiment (0h) as indicated in materials and methods. Glycine did not change the percentage of viable cells in any of the concentrations tested. Data are expressed as means ± S.E.M. of 5 (control) or 3 (other conditions) independent experiments, each performed in triplicate.

To look for more subtile or specific effects of glycine on apoptosis, we looked for changes in caspase-3 activity after STS addition to Jurkat cell cultures. The lowest dose of STS that was still capable of inducing maximal caspase-3 activity after 2 hours of cell culture was first determined to be 1 µM. Without adding STS the caspase 3 activity was always very low (14.4 ± 3.1% of the activity after STS addition, $p<0.001$). When cells were preincubated with glycine (1 mM) for 1 hour before STS addition, there was a decrease of caspase-3 activity to 85.7 ± 12.4%, which was not found to be significant ($p=0.29$) (Figure II.6). Viability control experiments did not indicate any changes in cell viability after STS and/or glycine addition.

2.4 Discussion

In the first part of this study we found **molecular evidence for GlyR subunit expression in human T lymphocytes**. Since no studies have been performed investigating the molecular structure of GlyRs directly in human T lymphocytes, we used RT-PCR, western blotting and confocal microscopy to examine GlyRs in these cells. Confocal microscopy showed a very clear and somewhat punctuate staining of GlyRs using the GlyR mAb4a antibody. This punctuate staining pattern cannot be explained by cross-linking of antibodies, as cells were fixed before the labelling procedure, and might indicate a clustered presence of GlyRs. Although this clustered presence is well known in glycinergic synapses in the mature nervous system (Legendre, 2001), where they increase the efficacy of neurotransmission (Lim et al., 1999), we were surprised to find this pattern in T cells. In the mature nervous system this clustering behaviour is mediated by gephyrine, a submembrane anchoring protein, which anchors GlyRs receptors to the cytoskeleton by means of interacting with GlyR β subunits. In our study we found clear evidence for gephyrin in T-lymphocytes, but β subunit expression was only obvious at the mRNA level of Jurkat T cells and no corresponding 58 kDa band could be detected with the mAb4a antibody. Although the affinity of this antibody for β subunits is rather low, compared to α subunits, this indicates that protein expression is low or absent for β subunits. As the GlyR staining seemed to be mainly located in the cytoplasm, another explanation for the punctuate pattern, apart from clustering of receptors, might be a submembrane vesicular location of GlyRs. Although GlyR (vertical) trafficking is not well studied for GlyRs, protein kinases were suggested to cause internalisation, as discussed in chapter I.

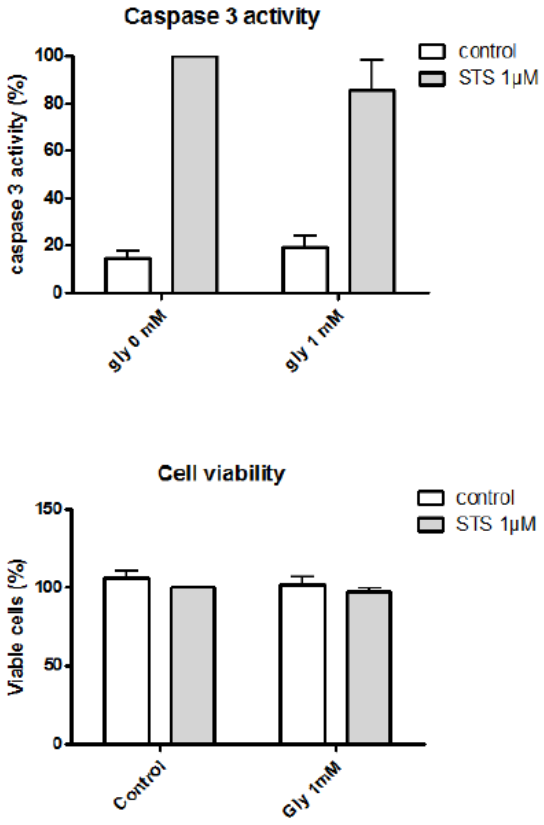

Figure II.6 Glycine effects on caspase-3 activity in Jurkat T cells.
After addition of 1 µM of staurosporin (STS) to cell cultures for 2 hours, caspase-3 activity was measured. Fluorescence values were normalized to the control values (no glycine) in the STS-condition. Without STS there was only a weak caspase-3 activity (14.4 ± 3.1%). When preincubating cells for 1 hour with glycine (1 mM) a small decrease in caspase-3 activity could be found, although this was not significant (p=0.29). Cell viability was unaltered after STS and/or glycine addition to cell cultures for the duration of the experiment. Data are expressed as means ± S.E.M. of 4

We confirmed the presence of the 48 kDa α subunits using western blotting. This protein was actually located slightly higher than the 48 kDa spinal cord positive control. The main GlyR α-subunit that is expressed in spinal cord is α1, which has a molecular weight of 48 kDa. However other α subunits are somewhat heavier and have a molecular weight between 48 and

50 kDa (Deleuze et al., 2005), which may explain the slight difference in position. The findings of a somewhat heavier α-subunit were made in molecular studies on GlyRs in macrophages as well (Wheeler et al., 1999). Our RT-PCR experiments showed a weak presence of the rare α4 subunit, and not of any other α-subunit. Remarkably, this subunit was identified on other immune cells as well (Froh et al., 2002). In our cells GlyR α4 expression could not be confirmed by immunolabelling cells with an α4 specific antibody (Heinze et al., 2007), as only a non-specific diffuse staining was observed, confirmed by western blotting. These findings may be explained by the fact that the α4 antibody binds to the C-terminal tail of the GlyR (Heinze et al., 2007), while mAb4a binds to the N-terminal tail (aa 96-105 of the GlyR α1 subunit). It has been demonstrated that the human α4 subunit has a premature stopcodon, in wich *c(ga)* (arginin) has been substituted to a *t(ga)* (stopcodon)) at amino acid 417 (Simon et al., 2004) (see figure I.5). This stopcodon is located just upstream of TM4 in human cells. This means that the C-terminal tail and the TM4 part of the GlyR would not be translated and may explain our findings. This raises the question whether these aberrant GlyRs could be functional? Theoretically this may be possible, as ligand interaction takes place at the N terminal part and the chloride channel is formed by TM2. Also most known agonists, antagonists and modulators of GlyR bind at upstream parts of amino acid 417 (Laube et al., 2002). Despite this theoretical possibility a functional role is doubtful. The α4 subunit expression raises an important discrepancy with the western blot results. The premature stopcodon and the loss of the downstream located amino acids would reduce the molecular weight to values around 40 kDa, which is contradicted by the +48 kDa band we found in our western blot experiments. As we found no mRNA evidence for α-subunits explaining this latter band, we hypothesize the existence of a yet unknown α-subunit. In this context it is remarkable that the same +48 kDa band could also be observed using a polyclonal antibody (N18), directed against the N-terminal part of the α2 subunit (data not shown), although our RT-PCR results and the results reported in Figure I.10 clearly showed the absence of the GlyR α2 protein. Given the homology between α-subunits (see chapter I), this may point towards aspecific binding at other (unknown?) subunits. Our group is currently examining the molecular structure of these proteins (work performed by Drs.S.Sahebali). Interestingly in supra-optic nucleus axons of the neurohypophysis an unknown 54 kDa subunit was described, which blocked voltage gated calcium fluxes in a picrotoxin sensitive manner, indicating homomeric receptors with a non-synaptic function (Deleuze et al., 2005).
The molecular weight of gephyrin was around 95-100 kDa, which is somewhat different form the original purified GlyR associated 93 kDa gephyrin molecule (Pfeiffer et al., 1982). It has been demonstrated that numerous gephyrin isoforms exist, both within and outside the central nervous system (Kawasaki et al., 1997). This gephyrin may be responsible for the observed clustered presence of GlyRs, although it may also have a function in clustering other receptors, as has been demonstrated for GABA (Sassoe-Pognetto and Fritschy, 2000). Furthermore it has been described that gephyrin has a dual function. Besides being an anchoring protein, it also has molybdoenzyme activity in non-neural tissues (Feng et al., 1998). This may offer an explanation for the broader diffuse staining we observed in the cytoplasm with confocal microscopy, compared to the GlyR staining.

Taken together these results indicate molecular presence of GlyRs although they seem to be mainly located in the cytoplasm and uncertainty remains about the precise subunit composition.

The molecular findings were subsequently linked to **electrophysiological glycine effects** on human T lymphocytes by using the well known leukaemic Jurkat T cell line as a model. Although we found clear evidence for GlyR expression, we could not induce any currents upon perfusion of cells with glycine. Since the microscopical images of immunolabelled GlyRs mainly showed a cytoplasmic location, we conclude that, at least under our cell culture and electrophysiological conditions, no GlyRs are actually expressed at the membrane or the GlyRs that are expressed are not functional (which in the latter case may be due to expression of the non-functional α4 subunit). A remarkable finding, when reviewing the literature, is that, although ionotropic GABA and glutamate receptors have been described at different levels in T lymphocytes (molecular, cell culture, ^{31}chloride uptake or calcium fluorimetry), to our knowledge, no paper was ever published demonstrating these currents electrophysiologically! This may point to some technical difficulty, although patching these cells is perfectly feasible. Poulopoulou and co-authors described a glutamate-induced enhancement of Kv1.3 currents, which are shown to be crucial in T cell signaling (Poulopoulou et al., 2005). Therefore we also investigated the effects of glycine on Kv currents, but again without success.

The goal of our **cell culture experiments** was to clarify glycine effects on apoptosis and investigate a potential role of GlyRs. We used three known inducers of cytotoxicity and determined the lowest effective concentration. STS, which is a highly potent though non-specific inhibitor of protein kinases, is also known as an inducer of apoptosis. In Jurkat T cells STS was found to alter mitochondrial membrane potential, induce release of cytochrome c and activate caspase-3 (Scarlett et al., 2000). When applying 100 nM STS a clear drop in viability was noticed in time, compared to control conditions. For activation of caspase-3 induction we needed a higher concentration (1 µM). The reason is the duration of the experiments and hence STS exposure, which was only 2 hours in the latter experiment. Importantly this higher dose of STS did not alter cell viability in this time span. Hydrogen peroxide (50 µM) was used as another inducer of apoptosis (Cerella et al., 2009).

In our starvation model, which was also used in the studies of Frañek et al., we diluted the cell culture medium in increasing concentrations of PBS. Remarkably a dilution of 10% medium in PBS was needed before cellular viability decreased. This value was comparable to the 15% medium that was found in other starvation studies with lymphocytes (Franek and Sramkova, 1996; Franek et al., 2002). Although these authors described protective effects of glycine 1 mM (up to 60%), we could not confirm this effect in our system. The most reasonable explanation seems to be the absence of any GlyR-mediated modulation. Our starting hypothesis was that GlyRs would mediate the protective effects and therefore we used a standard RPMI medium, without glycine, but with several other amino acids. Although this medium would be perfectly suited to describe GlyR-mediated effects (as none of any known GlyR modulators is present in this medium), it was found that other amino acids (*e.g.* serine, alanine, proline) had similar protective effects (Franek and Sramkova, 1996; Franek et al., 2002). As all these amino acids are actually still present in the used culture medium, these amino acids may mask a potential glycine effect, assuming that the effect is more aspecific. As nothing pointed to an involvement of GlyRs at this point, we decided not to study these

effects on viability further than this. However, if glycine-effects are part of a more general amino-acid mediated protective effect, there may be a possibility that glycine exerts more subtle effects in the apoptosis pathway. As caspase-3 is a crucial enzyme in both the intrinsic and the extrinsic pathway (Hengartner, 2000), we decided to induce apoptosis with STS (1 µM), measure caspase—3 activity and examine the effects of glycine. Although addition of 1mM glycine to cell cultures led to a trend towards a 15% reduction in activity, this was not found to be significant.

We conclude from this study that GlyRs are not active under standard cell culture conditions and not surprisingly they were not found to be responsible for anti-apoptotic effects that were previously described in both T as B lymphocytes.

3. CONCLUSION

In this chapter we provided molecular evidence for GlyR expression in human T lymphocytes. Although some GlyR subunits were already studied previously in the context of NRSE-specific gene regulation in small cell lung cancer (Neumann et al., 2004), this is the first study that performed a complete characterization of the currently known GlyR proteins. GlyR proteins were found to have a predominant cytoplasmic location. This was confirmed by electrophysiological experiments, showing that no active currents could be evoked by glycine perfusion, indicating that no GlyR are functionally present at the membrane. Concerning the subunit composition of GlyRs we unexpectedly found GlyR α4-subunit expression. As this is in fact a pseudogene in human, the functional significance of this finding is unknown. Remarkably GlyR α4-subunit mRNA was detected in every immune cell studied so far (*e.g.* different kind of macrophages and neutrophils (Froh et al., 2002)), suggesting some unknown function.

Concerning the potential functional meaning of these receptors, one study provided evidence for a modulating role of glycine on rodent T lymphocytes (Stachlewitz et al., 2000). The authors found a strychnine- and chloride sensitive inhibition of proliferation when applying glycine (0.1–1 mM) to mouse mixed lymphocyte cultures, pharmacologically suggesting GlyR involvement. However this was not confirmed in human lymphocyte cultures were glycine was not reported to modulate mitogen-induced proliferation (Sommer et al., 1994). In light of our molecular findings a reasonable hypothesis is that α4 containing GlyRs have a signaling role in rodents, but are not functional in humans because of a premature stopcodon.

In human T lymphocytes glycine was reported to have anti-apoptotic properties in millimolar concentrations (Franek et al., 2002). Because the effect could be mimicked by β-alanine, a GlyR partial agonist, we hypothesized the involvement of GlyRs in this anti-apoptotic effects. Under this hypothesis we used a modified glycine-free RPMI to perform cell cultures and induced apoptosis by different agents. With the exception of a limited trend towards a lowering of caspase-3 activity we could not confirm the anti-apoptotic effect, which does indirectly point to GlyR independency. Indeed it was suggested that the anti-apoptotic effects may be attributed to more specific amino acids signaling effects through system A and N neutral amino acid transporters (Franek and Sramkova, 1996), and many of these amino acids were still present in the cell culture medium, masking potential glycine effects in our cell culture system.

CHAPTER III. GLYCINE SIGNALING IN MICROGLIAL CELLS

Part of the results of this chapter will be reported in Van den Eynden J et al. Glycine enhances microglial intracellular calcium signaling. A role for neutral amino acid transporters. J Phys. *Submitted.*

1. INTRODUCTION TO MICROGLIA

The cellular micro-environment of the CNS consists of neurons (10%) and neuroglial cells (90%). The term "neuroglia" was first used by Rudolph Virchow in 1859 who considered these cells as being inactive supportive elements, responsable for holding neurons together in the CNS (explaining their name neuroglia or *nerve glue*). Three types of neuroglial cells are described in the CNS: astrocytes, oligodendrocytes (together called macroglia) and microglia. Macroglia are ectodermal derived cells which have the same embryonic origin as neurons. While oligodendrocytes are mainly responsible for myelination of neurons, the main functions of astrocytes are potassium buffering, provision of energetic substrates to neurons, composition of the blood brain barrier (BBB) and neurotransmitter concentration regulation in the neuronal micro-environment (Boron and Boulpaep, 2003). Microglia and oligogodendrocytes as distinct cell types from astrocytes were first recognized by Franz Nissl in 1899 who named them *Stäbchenzellen* (rod cells). It was del Rio Hortega (a student from Cajal RY) who further identified microglial cells as distinct cell types from oligodendrocytes in 1927. Based upon silver impregnation methods he described a ramified phenotype in the normal adult brain, and observed that these ramified cells could transform into a migratory, ameboid and phagocytic phenotype (Kim and de Vellis, 2005).

Microglia constitute 5-20% of the total cell population in the brain. The prevailing current view is that their myeloid microglial precursor cells invade the brain early during development and colonize the CNS (Kim and de Vellis, 2005; Chan et al., 2007). Each microglial cell occupies a certain territory with branches that are highly dynamic, with permanent retractions and extensions. This property allows microglial cells to permanently scan the neural micro-environment and search for potential abnormalities. When brain damage is detected, microglia transform into an activated ameboid and phagocytic phenotype. The experimental drawback of these properties implies that, when microglial cells are cultured, they are always in some state of activation because of the disturbance of the normal neural micro-environment that takes place during cell isolation or even the slicing procedure when making brain slices. It was suggested that "resting microglia" in culture conditions are in fact in a more activated state than activated microglia in the normal neural micro-environment (Streit et al., 1999). This means that properties of true resting microglia are difficult to study! As most publications on microglia so far report results on cultured cells (or slices more recently) caution is necessary when interpreting findings. Recently, using transcranial two-photon microscopy of GFP-expressing microglial cells through the thinned skulls of anesthesized mice, it was possible to study the behavior of true resting microglial cells and their response to brain damage (Davalos et al., 2005; Nimmerjahn et al., 2005).

Upon activation microglia can become phagocytic, secrete both pro-inflammatory (*e.g.* IL-1, IL-6, IL-15, IL-18, TNFα) and anti-inflammatory cytokines (*e.g.* IL-10, TGF-β), chemokines, prostanoids and cytotoxic molecules (*e.g.* nitric oxide (NO) and oxygen radicals) and present antigens to T lymphocytes (Aloisi, 2001; Kim and de Vellis, 2005). Based on this immune function it can be understood that microglia are involved in different neuro-inflammatory conditions such as MS. However, microglia were also shown to be involved in neuronal development and in regulation of neuronal function. As indicated in chapter I microglial-

derived BDNF was shown to alter anion gradients in neurons of the spinal cord, leading to altered pain sensations and neuropathic pain (Coull et al., 2005). During development microglia are essential for neuronal differentiation, determination of neuronal fate and synaptogenesis (Polazzi and Contestabile, 2002; Bessis et al., 2007). It is obvious from these findings that microglial function is not strictly limited to immune surveillance and hence these cells may have a much broader role than initially thought. In this regard microglia have been suggested to be implicated in the pathophysiology of many other neurological disorders (*e.g.* neuropathic pain, epilepsy or neurodegenerative disorders) (Aronica et al., 2007; Scholz and Woolf, 2007; Gao and Hong, 2008).

Because of the broad range of molecules these cells can secrete, which can either be neurotoxic or neuroprotective (or both), it is often difficult to know whether microglial involvement in these conditions is harmful or beneficial. Better understanding of the precise role of these cells implies better understanding of the interactions of microglia with neurons and other components of the micro-environment. In this context it has been shown that different neurotransmitters can influence microglial function. GABA, catecholamines and acetylcholine all attenuate parameters of microglial activation (Chang and Liu, 2000; Civelli et al., 2001; Kuhn et al., 2004; Pocock and Kettenmann, 2007). The role of glutamate, the most important excitatory neurotransmitters is not clear yet and may depend upon the specific microglial receptor expression. In this context it was shown that metabotropic group III receptor activation reduces microglial toxicity while group II activation triggers neurotoxicity (Bessis et al., 2007; Pocock and Kettenmann, 2007).

It was shown that astrocytes can secrete glycine, which enhances LPS-induced microglial secretion of NO and superoxide, and increases acid phosphatase and metabolic activity (Tanaka et al., 1998; Yang et al., 1998). Nothing is known about the mechanism. This chapter focuses on the mechanism behind the modulating action of glycine on microglia. Two *in vitro* cell systems will be used: primary microglia (PMG), isolated form neonatal rat pups, and the BV-2 celline. The BV-2 celline is derived from raf/myc-immortalized murine neonatal microglia and are now the most frequently used cell line model for the study of microglia. (Bocchini et al., 1992). Although, as for other cell lines, doubts have been raised whether these cells are good microglial models for primary cells. In some recent studies, it was shown that both BV-2 cells and PMG have very similar responses and the main differences that were observed were rather quantitative (*e.g.* BV-2 enhance NO production upon LPS stimulation but this enhancement is less pronounced compared to PMG) (Horvath et al., 2008; Henn et al., 2009). Compared to BV-2 cells, PMG have several disadvantages as well. PMG cultures necessitate the use of a lot animal pups (for each experiment about 10 pups are used), are costly, labour intensive, not 100% pure and probably not homogeneous either. Also, when considering its usage because they would be a better model for the true *in vivo* physiological situation, one has to keep in mind, that for the purification these cells are grown *in vitro* in mixed cultures (with astrocytes) for 2 weeks. During this period important non-physiological modifications can take place already.

In section 2 we provide molecular evidence for GlyR expression and show that that these receptors are not involved in the known immunomodulatory effects. Also a role of strychnine-independent NMDARs is ruled out. In section 3 we show that the immunomodulatory effects are correlated with a modulation of microglial calcium signaling and we suggest a role for

neutral amino acid transporters in this modulation. In section 4 we bring our findings together and hypothesize how they may be linked together.

2. GLYCINE MODULATES MICROGLIAL ACTIVITY BY A GLYCINE RECEPTOR-INDEPENDENT MECHANISM.

2.1 Introduction

In peripheral macrophages different studies provide molecular evidence for GlyR expression. GlyR α1, α4 and β-subunits were found in Kupffer cells while in lung and splenic macrophages α2, α4 and β-subunits were found (Froh et al., 2002). It was pharmacologically shown that these receptors are responsible for immunomodulatory properties in these cells. To our knowledge nothing is known about GlyR expression in microglial cells.
In microglia glycine was shown to enhance NO and superoxide production, metabolic activity and acid phosphatase activity (Tanaka et al., 1998; Yang et al., 1998), but the precise mechanism is not studied yet. NO is an important inflammatory mediator of macrophages and microglia. NO is synthesized from L-Arginine by the NO synthase (NOS) enzyme (Figure III.1).

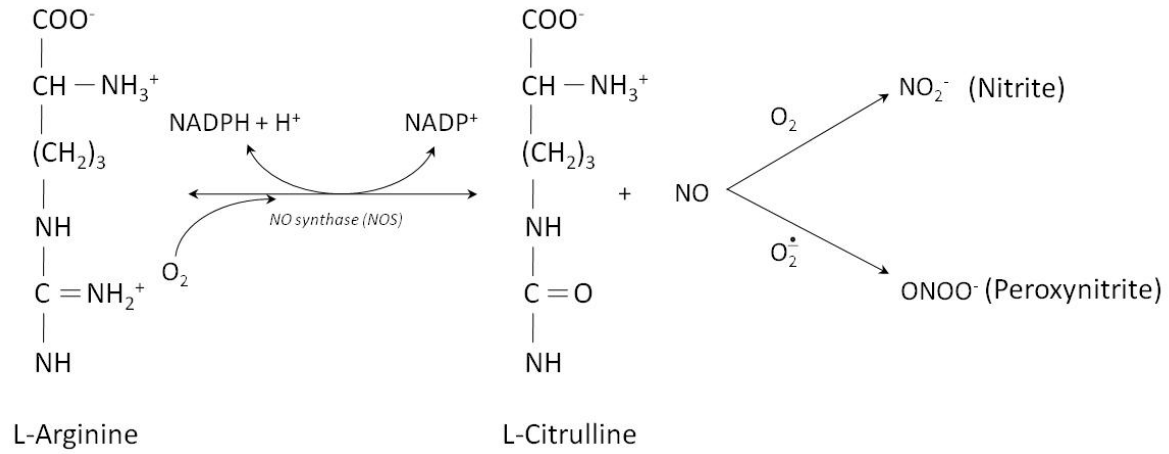

Figure III.1 Synthesis of nitric oxide (NO)

NO has a very short half life (less than one second) as it is quickly degraded to form nitrite or nitrate. In the presence of the superoxide anion it can form the highly reactive peroxynitrite, which causes lipid, protein and DNA oxidative damage and mitochondrial dysfunction, leading to cellular toxicity (Pacher et al., 2007). Three types of NOS are reported, encoded by different genes. Neuronal (nNOS or type I) and endothelial NOS (eNOS or type III) are constitutively active and are fast and directly activated by calcium. The inducible (iNOS or type II) isoform, which is expressed by immune cells and hence microglia, is activated more slowly at the transcriptional level (Minghetti and Levi, 1998). Different transcription factors are reported to activate iNOS in microglia: NF-κβ, ERK1/2 and NFAT. Although iNOS cannot be directly activated by calcium, it is important to realize that activation of the transcriptional factors mentioned is reported to be calcium dependent in immune cells, where calcium-calmoduline binding to calcineurine causes a dephosphorylation and translocation of these transcription factors to the nucleus (Ferrari et al., 1999; Martin et al., 2006). NO is reported to be both neuroprotective and neurotoxic (for review see (Calabrese et al., 2007)). If

NO is produced in excess, it can react with superoxide to form peroxynitrite, which is toxic to neurons. In many neurodegenerative (*e.g.* Alzheimer's Disease, Parkinson's Disease and Huntington's Disease) but also neuro-inflammatory disorders (*e.g.* MS) nitrosative damage has been suggested to be involved in the pathogenesis. However NO also has neuroprotective properties, as it is also reported to decrease the activity of NMDA-R and caspases (both by means of S-nitrosylation) and increase the activity of cAMP response element binding protein (CREB) and AKT-kinases (both cGMP dependent), which have been shown to be involved in neuroprotection.
In this section molecular evidence for GlyR expression in microglial cells is provided. Glycine modulation of microglial activity will be studied in cell culture experiments and the role of GlyRs will be determined and correlated to electrophysiological findings.

2.2 Materials and methods

2.2.1 Cell culture

Primary microglial cells were isolated from one to two day old neonatal Wistar rats (Harlan, Horst, The Netherlands). Briefly, cells were isolated from the forebrain of neonatal rats and the suspension was cultured for 10 - 12 days on PLL-coated (5 µg/ml) cell culture flasks in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Merelbeke, Belgium) containing 10% heat-inactivated fetal calf serum (FCS; Invitrogen), 2 mM Glutamine and 100 U/ml Penicillin and 100 µg/ml Streptomycin (1% PS; Invitrogen). Microglial cells were collected by shake off (180 rpm during 1h). Purity, as determined by immunocytochemistry after staining with mouse anti-rat CD11b/c (OX-42) 1/100 (Invitrogen) and 1.25µg/ml propidium iodide (Invitrogen) was 91 +/- 1%. For cell culture experiments cells were put on minimal essential medium (MEM, Gibco), supplemented with 10% FCS, 2 mM glutamine (Sigma) and 1% penicillin/streptomycine. Some experiments were carried out in serum-free medium, meaning that FCS was replaced by bovine serum albumin (BSA) 1 mg/ml. For nitrite and TNFα measurements and metabolic activity/viability assays, cells were pre-incubated with glycine (or other test compounds, *e.g.* L-serine) for 24 hours after which cells were stimulated with LPS 1 µg/ml (Sigma-Aldrich) and supernatants were collected at different time points after stimulation (Figure III.6). Control conditions were measured in the abscence of glycine (or other test compounds).
The immortalized *mouse microglial cell line BV-2* (Bocchini et al., 1992) was cultured in DMEM containing 10% FCS, 2 mM Glutamine and 1% PS. Cells were split twice a week and brought to a concentration of 100.000 cells per ml.

2.2.2 Chemicals

Glycine, L-serine, D-serine, taurine, β-alanine, MK-801, APV (DL-2-Amino-5-phosphonopentanoic acid), DCKA (5,7-Dichlorokynurenic acid monohydrate), picrotoxinine (PXN), BSA and lipopolysaccharide (LPS) were all purchased from Sigma Aldrich (Bornem, Belgium). KCl was purchased from Acros Organics (Geel, Belgium).

2.2.3 Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR experiments were performed as described in paragraph 2.2.3. Primers were designed using Primerdesign 3.0 software, except when mentioned otherwise, and synthesized by Eurogentec (Table III.1). PCR products were then separated on a 2% agarose gel, containing GelRed Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA) and visualized using a Gel doc system controlled by Quantity one software (Biorad, Hercules, CA, USA). Bands of interest were cut out and extracted from the gel using the Qiaquick gel extraction kit (Qiagen), purified and sequenced (Qiagen sequencing services, Hilden, Germany) to confirm cDNA identity.

Table III.1 Primers used for RT-PCR of microglia.

Species	Subunit	Accession number	Fw	Rev	Length	Ta (°C)
MOUSE	GlyR α1	NM_020492	GGAAGAGGCGACATCACAA	TGGACATCCTCTCTCCGGAC	301	55
MOUSE	GlyR α2	NM_183427	ATGGATGTCCAGACCTGTACAATG	GCAGTGACCCATCCCATAGCCACT	600	51
MOUSE	GlyR α3	NM_080438	GGCTGAAGGACTCACTTTGC	TACTCTAGCTGGGGCTGCAT	226	56
MOUSE	GlyR α4	NM_010297	CCACGTCTGGCCTACCGAGAA	GTAGCCCATCTGCCGCTCC	459	59
MOUSE	GlyR β	NM_010298	GGAGGTCATCTTCACCCTGA	CAGCGCCTTCACATAAGACA	220	54
MOUSE	geph	NM_172952	GTGGTCACCTGCAACCTC	ACGTACTGCTCTGTCTTTGGA	269	55
RAT	GlyR α1	NM_013133	CCGTCTCGCCTACAATGAAT	TCCATCGGGAAATTCTTCAG	231	53
RAT	GlyR α2	NM_012568	ATGGATGTCCAGACCTGTACAATG	GCAGTGACCCATCCCATAACCGCT	600	51
RAT	GlyR α3	NM_053724	GGCTGAAGGACTCACTTTGC	TACCCGAGCCGGAGCTGCAT	226	56
RAT	GlyR α4	XM_001054481	CCACGTCTGGCCTACCGAGAA	GTAGCCCATCTGCCGCTCC	457	59
RAT	GlyR β	NM_053296	GAAGAACACTGTGAACGGCA	GGCTTCTTGTTCTTTGCCTG	228	55
RAT	geph	NM_022865	CCATGGGGGAAAAGGACTAT	GGATTCCCTGGTAGTGCAAA	160	55

Depending on the cell type examined (primary cells have a rat origin, while BV-2 cells are of mice origin) different forward (Fw) or reverse (Rv) primers were used for GlyR subunit mRNA detection. All primers were designed using Primerdesign 3.0 software with the exception of the primer for GlyR α2, which were according to (Froh et al., 2002). Ta annealing temperature.

2.2.4 Western Blotting

Cell pellets containing 10 million cells were stored at -80°C until usage for western blotting experiments. The protein content of cell lysates was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA), following the instructions of the manufacturer. Thirty µg of cell extract was loaded on NuPAGE Novex Bis-Tris Mini Gels (Invitrogen) and subsequently electrophoresed at 200V for 35 minutes. The gel proteins were then transferred to a PVDF membrane, by blotting at 300mA for 90 minutes. After blocking with 2% Marvel Milk Powder in PBS, the membranes were incubated overnight at room temperature with primary antibodies. Primary antibodies used where the GlyR subunit aspecific mAb4a antibody 1/500 (recognizing all 48 kda α-subunits and with a lower affinity also 58 kda β-subunits) and the gephyrin specific 3B11 antibody 1/1000 (both from Synaptic Systems, Goettingen, Germany). After washing in PBS the membranes were incubated for 1 hour in a peroxidase-conjugated secondary antibody (Rabbit anti-mouse 1/1000; Dakocytamon, Glostrup, Denmark). Proteins were detected by adding the chromomeric substrate DAB (3,3' Diaminobenzidine) and 30% hydrogen peroxide.

2.2.5 Immunolabelling and confocal microscopy

A cell suspension of 50.000 microglial cells was put on glass coverslips and cells were allowed to incubate for 24 hours at 37°C. Fater incubation immunolabellings and confocal microscopy was performed as described in paragraph 2.2.5.

2.2.6 Nitric oxide measurements

Cells were cultured in 96-well plates as described in *cell culture.* Every culture condition was performed in triplicate wells. Microglial nitric oxide production was measured as nitrite accumulation in the supernatants of cells with the Griess reaction according to the instructions of the manufacturer. Briefly 100 µl of supernatant was mixed with 100 µl Griess reagens (Sigma-Aldrich) and after incubation for 15 minutes at room temperature the absorbance at 540 nm, with reference absorbance at 690 nm, was measured in a Fluostar Optima microplate reader (Isogen Life Science, De Meern, The Netherlands). A calibration was performed with known nitrite concentrations in culture medium and final nitrite concentrations in each experimental condition was calculated. To evaluate glycine effects on nitrite production, we normalized every experimental condition to control (glycine free) conditions, and expressed them as "normalized nitrite production".

2.2.7 TNFα-ELISA

Cells were cultured in 96-well plates as described in *cell culture.* Every experiment was performed in triplicate wells. Microglia were stimulated with LPS (1 µg/ml) and supernatants were collected after 6 and 10 hours of incubation. TNFα was measured using a sandwich ELISA based on mouse- and factor-specific antibody pairs (R & D Systems, Wiesbaden, Germany) following the procedure of the manufacturer. The color reaction was analyzed in a Fluostar Optima microplate reader.

2.2.8 Metabolic activity/Cell viability assay

Cells were cultured in 96-well plates as described in *cell culture.* Every experiment was performed in triplicate wells. After incubation (37°C, 5% CO_2) cell viability was assayed by measuring the activity of mitochondrial dehydrogenases in cell cultures as an indicator of total viable cell number. The activity was measured by means of the "DHL cell viability and proliferation assay kit" (Tebu-Bio, Boechout, Belgium), following the instructions of the manufacturer. Briefly 20 µl of reagent and 100 µl culture medium was added to each well and the change in fluorescence was measured during a one hour time interval as an indicator of total dehydrogenase activity, using a Fluostar Optima microplate reader with excitation filters set at 540 nm and emission filters set at 590 nm. Caution is necessary because changes in metabolic activity without changes in cell number will also lead to changes in fluorescence. This means that every positive result using this test should be correlated with a true viability assay. For simplicity we will only use the term "metabolic activity" for this assay.

2.2.9 Electrophysiology*

For patch clamp experiments BV-2 cells were resuspended in culture medium and seeded in Nunclon Petri dishes (35 mm, Nunc, Roskilde, Denmark) at 25.000 cells/ml and incubated in a humidified incubator at 37°C with 5% CO_2 for 1 to 5 days before the experiments. Whole cell patch clamp recordings were made at room temperature with an EPC9 or EPC10 amplifier (HEKA electronik, Lambrecht, Germany). Gigaseals were obtained and the membrane potential was clamped at –60 mV after rupturing the membrane patch. Fast solution changes were done by means of a Warner SF-77B fast step superfusion system (Warner Instruments LLC, Hamden, CT, USA). Currents were sampled at 1 kHz and filtered at 0.2 kHz. Data acquisition was done with Pulse or Patchmaster (HEKA electronik, Lambrecht, Germany). The extracellular solution contained (in mM) 130 NaCl, 5 KCl, 10 glucose, 10 HEPES, 1 $MgCl_2$ and 2 $CaCl_2$ and the pH was adjusted to 7.4 with NaOH. The intracellular solution contained (in mM) 120 kCl, 11 EGTA, 10 HEPES, 2 MgCl and 1 $CaCl_2$ and the pH was adjusted to 7.3 with KOH. For every experiment a new Petri dish with cells was taken.

2.2.10 Statistics

Data are expressed as means ± S.E.M. Statistical significance was evaluated using ANOVA followed by Dunnett's comparison to control test, or student t-testing when comparing only 2 sets of data. P-values of less than 0.05 were considered significant.

2.3 Results

2.3.1 Molecular evidence for glycine receptor expression in microglial cells

We started by screening microglial cells for GlyR expression by western blotting. The mAb4a mainly recognizes 48 kDa α-subunits and to a lesser extent also 58 kDa β-subunits as can be seen in the positive spinal cord control (Figure III.3). In both primary and BV-2 microglial cells the 48 kDa GlyR α-subunit could be detected, although the band was located slightly higher compared to the spinal cord control. It was not possible to detect a band corresponding to the 58 kDa β-subunit, although it could be weakly detected in spinal cord positive control. Also when the amount of protein was increased (from 30 µg/ml to 70 µg/ml) no β-subunit could be detected (data not shown). Using the 3B11 anti-gephyrin antibody

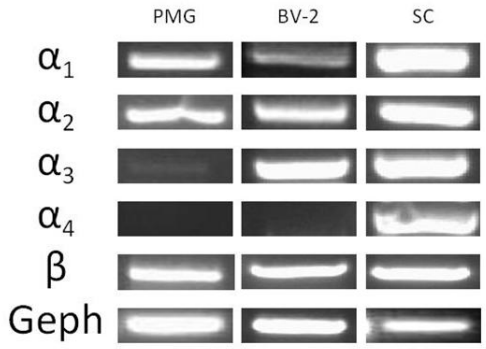

Figure III.2 Reverse Transcriptase PCR detection of microglial GlyR subunits
Using RT-PCR we amplified cDNA of GlyR α1, α2 and β-subunits as well as gephyrine in both BV-2 as primary microglial cells (PMG), while GlyR α3-subunit cDNA was only detected in BV-2 cells. Spinal cord (SC) was used as a positive control.

* Electrophysiological experiments were performed by Dr.B.Brône but are mentioned here for their crucial relevance in this research project

we could also detect the 93 kDa GlyR clustering protein gephyrin (Figure III.3). To further characterize the GlyR subunits that were expressed by microglial cells we performed Reverse Transcriptase PCR with specific primers for cDNA of GlyR α1, α2, α3, α4 and β subunits and gephyrin (Table III.1). mRNA was detected for α1, α2 and β subunits and gephyrin, for both BV-2 and primary cells (Figure III.2). Sequencing of the PCR products and subsequent blasting of the sequenced products confirmed the identity of the mRNA with matches that were minimal 97%. In BV-2 cells we could also detect α3 subunits (although the match after sequencing was only 90%), but this mRNA was absent in primary cells where only a weak band was detected that was identified as α2- subunit cDNA after sequencing.

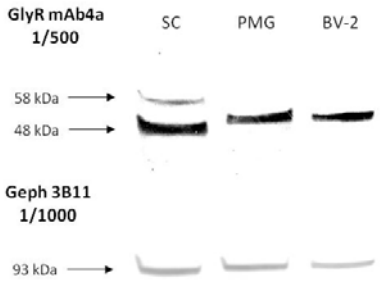

Figure III.3 Western blot detection of microglial GlyR proteins
Cell protein extracts (30 µg/ml) from primary microglial cells (PMG) or BV-2 microglial cells were loaded and blotted as indicated in materials and methods. Using the anti-glycine receptor monoclonal mAb4a (upper part) a clear 48 kDa band, corresponding to glycine receptor α-subunits could be detected. This band was located slightly higher than the spinal cord control. The anti-gephyrin monoclonal 3B11 antibody detected a 93 kDa band, corresponding to gephyrin (lower part).

To confirm and extent these results and analyze the location of these GlyR subunits, we performed confocal microscopy. After fixation and immunolabelling with anti-GlyR mAb4a of microglial cells, we observed a punctuate expression of GlyR subunit proteins. These proteins seemed to be predominantly located intracellularly, both in BV-2 and PMG, although partial localization at the plasma membrane could not be excluded (Figure III.4).

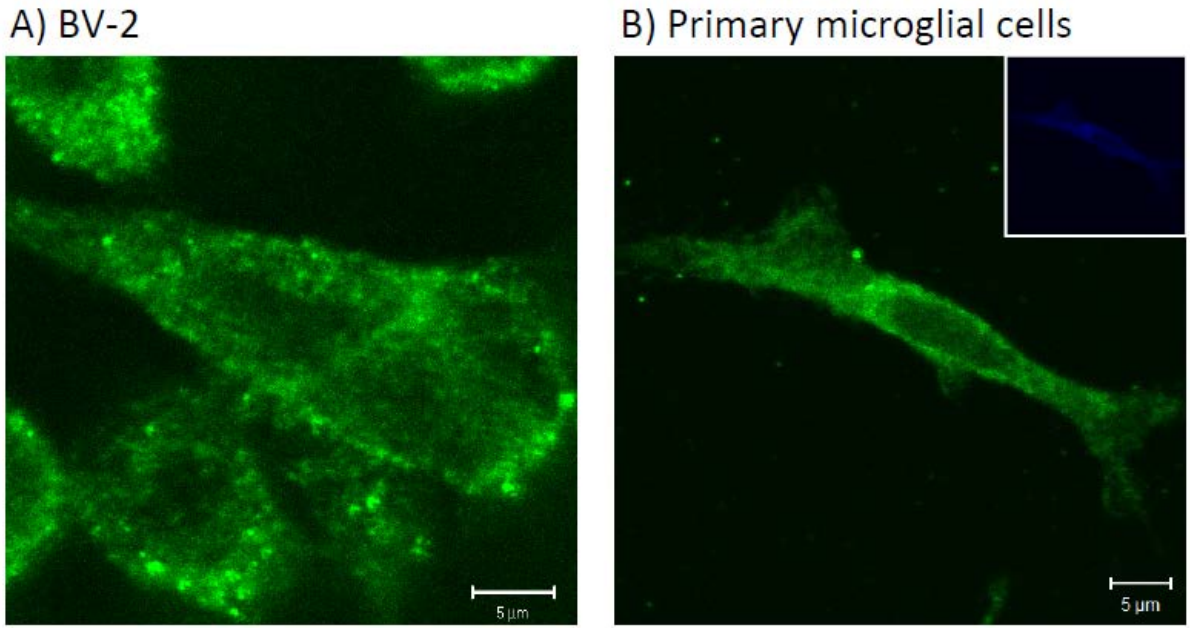

Figure III.4 Confocal imaging of GlyR expression in microglial cells
Images were taken at 63x magnification with a digital zoom of 4 (A) or 3 (B). Using the anti-glycine receptor monoclonal mAb4a (1/100) we clearly detected GlyR expression in both BV-2 (A) and primary microglial cells (B). Primary cells were dual labeled with OX42 for microglial identification as shown in the insert of panel B. In both microglial cell types GlyR proteins seemed to be mainly intracellularly, rather than in the plasma membrane. Scale bars = 5 um.

2.3.2 Glycine and L-serine induce strychnine-independent currents in BV-2 microglial cells.*

To examine the functionality of these receptors we performed whole cell patch clamp recordings of BV-2 microglial cells. When cells were perfused with glycine 1 mM, small inward currents could be observed (2.4 ± 0.5 pA) (Figure III.5A and B). These currents could not be blocked 1 µM strychnine, a concentration of which it was shown that GlyR are maximally blocked (see chapter I, p20). These data indicate that teh currents they where not mediated by GlyRs. The currents were sodium dependent as they were completely abolished in a sodium free extracellular solution (Figure III.5E). These currents could also be evoked by perfusing cells with L-serine 1 mM (7.4 ± 1.4 pA). L-serine currents were significantly larger than glycine currents and were also abolished in sodium free extracellular solution. Although these currents were small in amplitude the effect on membrane potential was substantial (+3.5 mV for glycine and + 8.7 mV for L-serine as measured in current clamp conditions, Figure III.5C and D). Figure III.5F shows concentration-response relationships of glycine and L-serine. EC_{50} were found to be 3.62 and 0.43 mM for glycine and L-serine respectively.

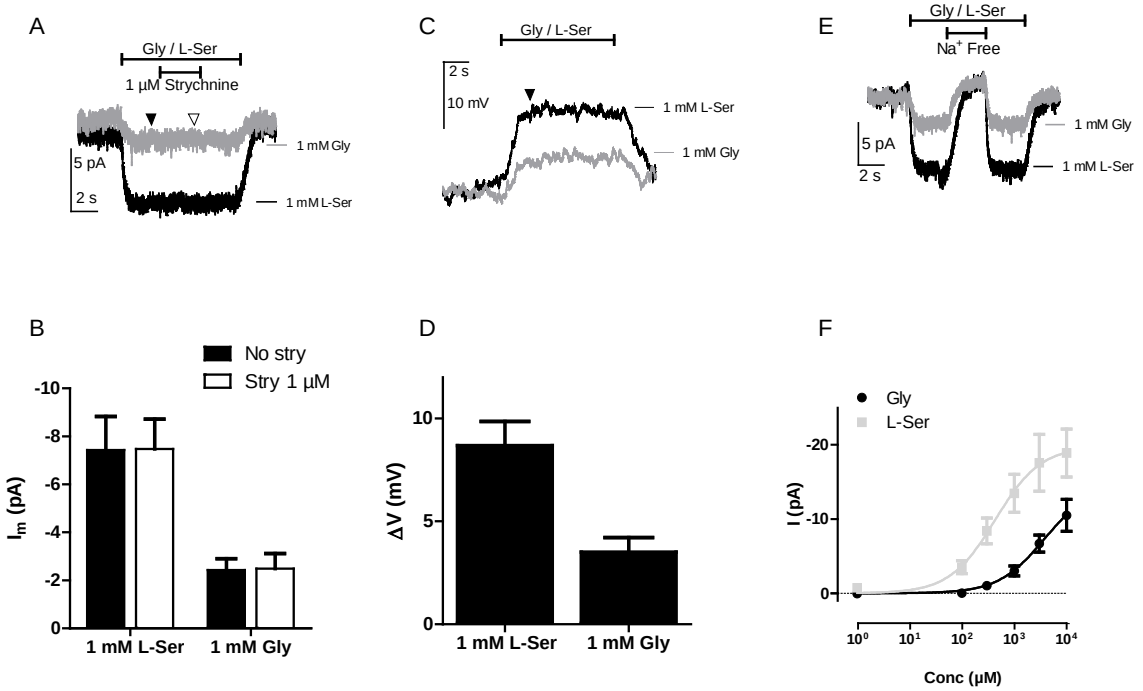

Figure III.5 The effect of glycine and L-serine on the electrophysiological properties of BV-2 cells.
A and B. Perfusion of BV-2 cells with a solution containing 1 mM Gly or L-Ser induced a maintained inward current that was insensitive to strychnine (1µM). Representative trace shown in A and calculated mean currents in the absence (closed bars and arrowheads) or presence (open bars and arrowheads) of strychnine, calculated from 6 independent experiments, shown in B. C and D. In current clamp conditions the resting membrane potential of BV-2 cells was measured. Glycine and L-serine application depolarized cells (representative trace shown in C and mean currents, calculated from 6 independent experiments shown in D). E. representative trace showing that the removal of Na^+ abolished the glycine- or L-serine-induced currents. F. Glycine and L-serine dose-response relationship on inward currents.

* Electrophysiological experiments were performed by Dr.B.Brône but are mentioned here for their crucial relevance in this research project.

2.3.3 Glycine modulates microglial nitrite and TNFα production

When primary microglial cells were stimulated with LPS 1 µg/ml and nitrite production was measured in the supernatant at different time points after stimulation, a clear rise in nitrite production could be detected starting from 24 hours after stimulation (Figure III.6A). When first pre-incubating cells with glycine 1 mM for 24 hours, an enhancement of microglial nitrite production could be observed (Figure III.6A and Figure III.7A). This enhancement could already be observed 24 hours after stimulation (p=0.047) and became obvious 48h after stimulation (p=0.003).

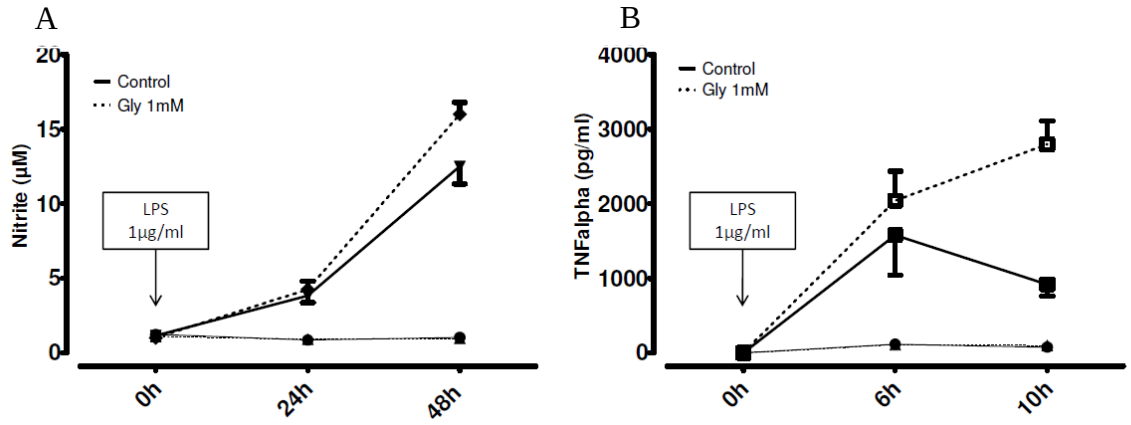

Figure III.6 Glycine time-dependently enhances microglial nitrite and TNFα production in primary microglia.
Primary microglial cells were pre-incubated for 24h under control (full line) or glycine (1 mM, dashed line) conditions as indicated in materials and methods. Then they were stimulated with LPS (1 µg/ml). Nitrite (A) or TNFα (B) was determined in the supernatant after different incubation times as indicated in the abcis. A) Glycine significantly enhanced nitrite production after 24h (p=0.047), and this became highly significant after 48h (p=0.003). B) Glycine significantly enhanced TNFα production 10h after LPS stimulation (p=0.01). Results are expressed as means ± S.E.M. from four (NO) or three (TNFα) independent experiments, each analysed in triplets. The lower curves show LPS unstimulated controls with or without glycine pre-incubation (overlapping curves). For clarity error bars are only indicated for the stimulated conditions and are shown above for glycine and below for control conditions.

When normalizing LPS-induced nitrite production to the control (glycine free) conditions to analyze the extent of the glycine effects, a rise of 12% and 35% could be detected after 24 and 48 hours respectively (Table III.2). This change in nitrite production was not caused by a change in metabolic activity (Figure III.7B).
To investigate the concentration range of the glycine effects, a dose response was measured on microglial cells. Figure III.8A shows that the main part of the response takes place between 10 and 1000 µM with a half maximal concentration (EC_{50}) of 99 µM.
We also measured TNFα-production in the supernatant after LPS stimulation. TNFα peaked already 6 hours after stimulation and decreased again after 10 hours in control conditions. Glycine also enhanced TNFα production, which became significant after 10 hours (p=0.01) (Figure III.6B).

Table III.2 Summary of glycine effects on microglial nitrite production after LPS (1 µg/ml) stimulation in primary microglia.

		Mean	95% CI	p-value	n
24h	Glycine 1mM	112%	102-122%	0.033	4
	Strychnine 1µM	99%	77-121%	ns	4
	Glycine 1mM + Strychnine 1µM	130%	109-150%	0.019	4
	L-serine	166%	136-195%	0.0056	4
48h	Glycine 1mM	135%	125-146%	<0.001	12
	Strychnine 1µM	104%	99-109%	ns	4
	Glycine 1mM + Strychnine 1µM	135%	101-168%	0.047	4
	L-serine	146%	131-160%	<0.001	12
	Picrotoxinine 30µM	108%	77-138%	ns	4
	Glycine 1mM + Picrotoxinine 30µM	137%	102-172%	0.043	4
	β-alanine 1mM	104%	97-112%	ns	8
	Taurine 1mM	89%	82-95%	0.004	8
	MK-801 30µM	109%	92-127%	ns	4
	Glycine 1mM + MK-801 30µM	131%	110-152%	0.018	4
	APV 30µM	107%	80-133%	ns	4
	Glycine 1mM + APV 30µM	144%	120-167%	0.01	4

CI Confidence Interval, n number of independent experiments, each analyzed in triplicate wells. 24h and 48h indicate 24 or 48 hours after LPS stimulation respectively.

2.3.4 Glycine modulates microglial viability in serum free conditions

Because a small amount of glycine in (10%) FCS cannot be excluded, we also examined glycine effects under serum-free culture conditions to evaluate the active glycine concentrations and the extent of the effect. Although the results were similar to the one discussed in the previous paragraph (Figure III.7C), contrary to these results significant changes in metabolic activity were found (Figure III.7D), making the effects on nitrite production difficult to interpret. (Note that 48h after LPS stimulation, the metabolic activity under serum free control conditions was only 46% of the activity under 10% FCS control conditions, suggesting a lower number of viable cells in serum free conditions (data not shown).) Figure III.9 shows that the effect on metabolic activity was taking place at lower glycine concentrations, compared to the effects on nitrite production, as an EC_{50} of 8 µM could be found.

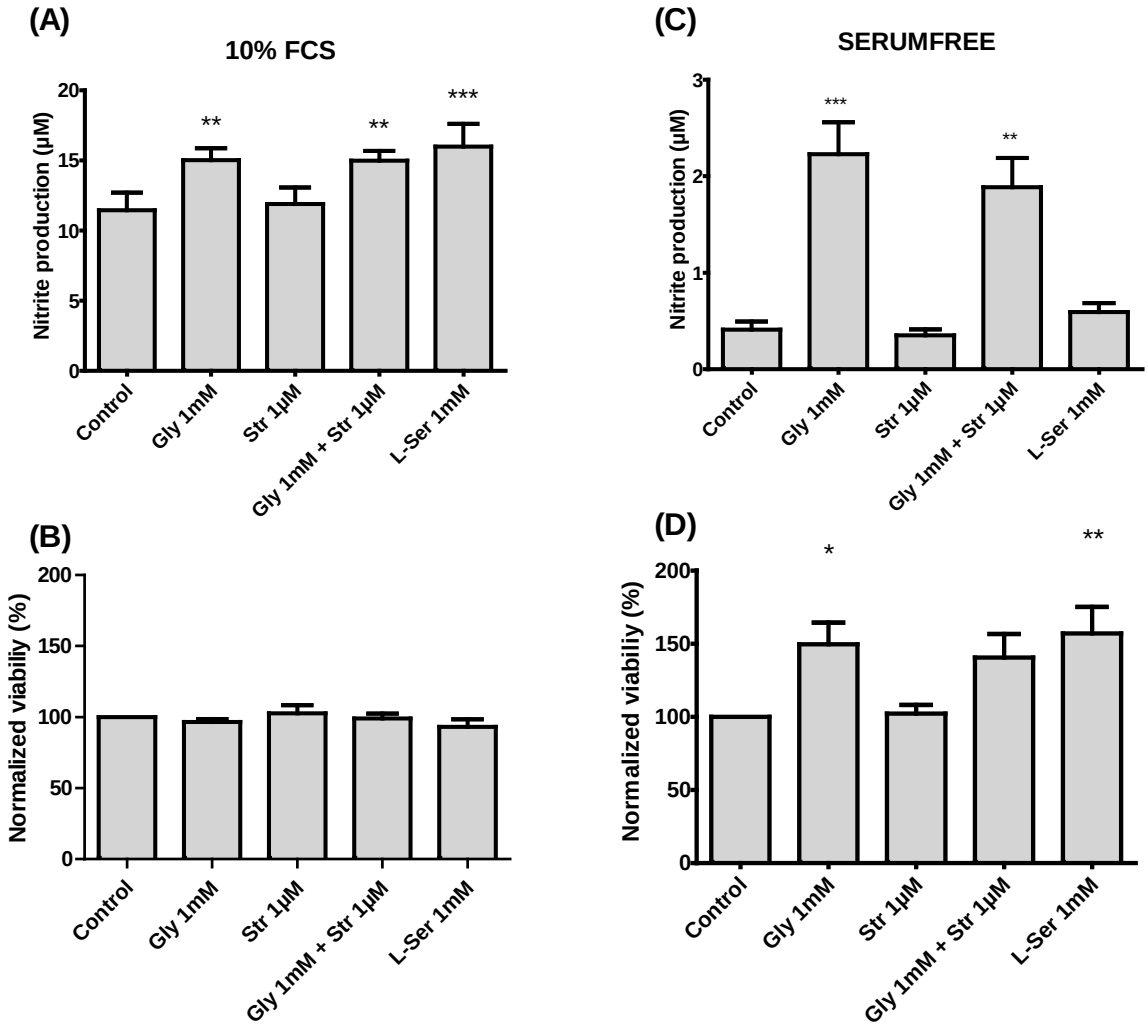

Figure III.7 Glycine stimulates microglial nitrite production 48h after LPS stimulation in primary microglia.
Glycine significantly enhances nitrite production 48h after LPS (1 µg/ml) stimulation, both in serum (A) as in serum-free (C) culture conditions. The specific glycine receptor antagonist strychnine (1 µM) could not block the effects of glycine suggesting a glycine receptor independent mechanism. L-serine (1 mM) mimicked the effects of glycine, but only in serum conditions. B) Under serum conditions metabolic activity was unaltered after LPS stimulation and when adding strychnine to cell cultures. D) Under serum-free culture conditions glycine (and L-serine) significantly stimulated metabolic activity as well. Results are expressed as means ± S.E. from four independent triplicate experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$

2.3.5 Immunomodulatory properties of glycine are not mediated by strychnine sensitive glycine receptors

To test whether these glycine immunomodulatory properties on PMG were mediated by GlyRs we tried to block the effects with the specific GlyR antagonist strychnine and a more aspecific homomeric GlyR antagonist picrotoxinine. We used concentrations of both antagonists of which it has been clearly shown that GlyRs are maximally blocked. Neither strychnine (1 µM), nor picrotoxinine (30 µM) could reverse the effects of glycine on microglial cells (Figure III.7, Table III.2). Also the glycine dose-response relationship on LPS-induced nitrite production could not be shifted by strychnine (Figure III.8A), nor could increasing concentrations of strychnine change glycine effects (Figure III.8B).

Next it was checked whether the physiological agonists taurine or β-alanine could mimic the effects of glycine. Table III.2 shows that no agonist effect on microglial nitrite production could be observed. Note that taurine did however significantly decrease nitrite production, which may have been due to a decrease in cell viability (metabolic activity) that was found when adding taurine to cell cultures (data not shown). Also glycine effects on metabolic activity are not altered by strychnine (Figure III.9).

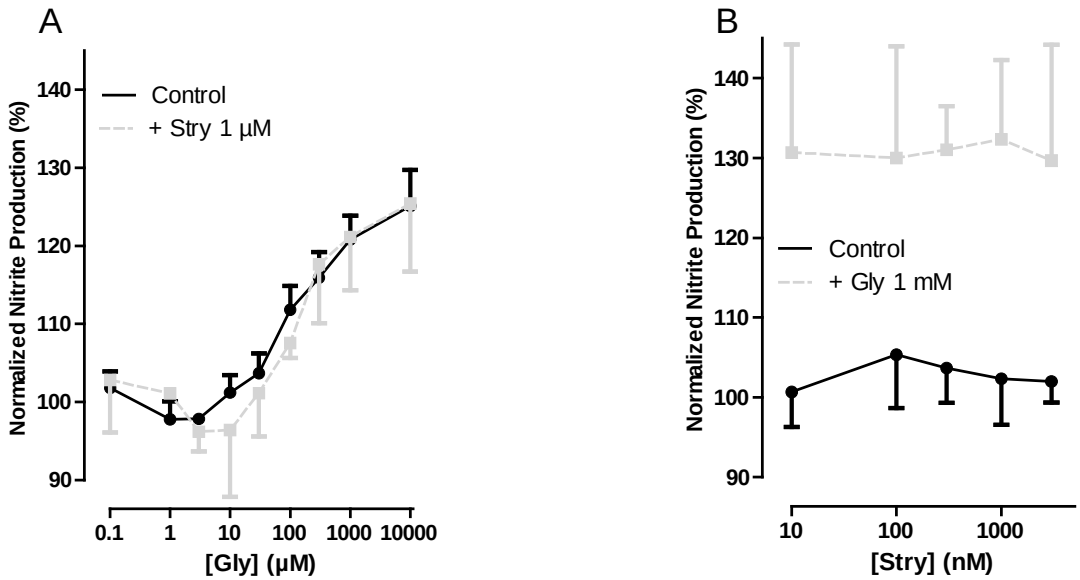

Figure III.8 Glycine and strychnine concentration-response relationships on LPS-induced nitrite production in primary microglia.
Nitrite was measured 48h after LPS (1 µg/ml) stimulation. A. Glycine dose-response relationship. The main part of the glycine effect is taking place between 10 and 1000 µM. This dose response curve could not be altered by strychnine (1 µM). For clarity data without strychnine are shown in solid black with error bars drawn above curve, while data with strychnine are shown in dashed grey with error bars drawn below curve. Results are expressed as means ± S.E.M. from four independent triplicate experiments. B. Strychnine dose-response relationship. Strychnine does not modulate LPS-induced nitrite production, nor does it block the glycine enhancement of LPS-induced nitrite production. For clarity data without glycine are shown in solid black with error bars drawn below curve, while data with glycine are shown in dashed grey with error bars drawn above curve. Results are expressed as means ± S.E.M. from three independent triplicate experiments.

2.3.6 Immunomodulatory properties of glycine are not mediated by strychnine insensitive NMDA receptors

As is obvious form Figure III.7 and Table III.2, L-serine could mimic the effects of glycine on LPS-induced nitrite production in primary microglial cells. Although L-serine is known as a very weak agonist of glycine at the GlyR, L-serine effects could not be blocked by either strychnine or picrotoxinine (data not shown). Remarkably L-serine effects were taking place earlier compared to glycine with significant enhancement already found after 12 hours of LPS stimulation ($[NO_2^-]$ = 1.1 µM in control conditions compared to 1.8 µM in L-serine (1 mM) conditions, p = 0.02). Also L-serine was more potent in enhancing nitrite production compared to glycine and the EC_{50} was lower (23 µM). As both glycine and the D-serine, the enantiomer of L-serine, are co-agonists at the NMDA receptor we examined the possible

involvement of NMDA receptors. Our first approach was to investigate whether D-serine by itself could mimic L-serine (or glycine) effects on nitrite production. As is obvious from Figure III.10A D-serine did not mimic L-serine effects. It should be noted that a significant drop in cell viability (and hence nitrite concentration) was noticed when adding 300 µM of D-serine or more (Figure III.10B). D-serine cytotoxicity is further discussed in chapter V, paragraphs 3.3.5-3.3.7 (p.117-118). Furthermore the dose response relationship of glycine on nitrite production was not altered in the presence of 100 µM D-serine or of 30 µM of the glycine site antagonist DCKA (data not shown). The next approach was to try and antagonize NMDA receptor activation by means of the NMDA receptor antagonists MK-801 and APV. These substances changed the control response (absence of glycine) nor the glycine response either (Table III.2).

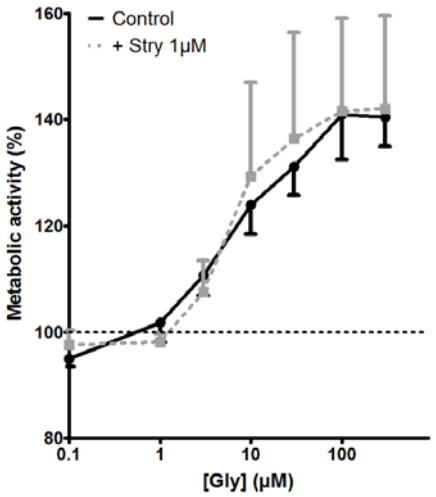

Figure III.9 Dose response relationship of glycine effects on metabolic activity of primary microglia under serum-free culture conditions.
Glycine dose dependently stimulated metabolic activity 48h after LPS (1 µg/ml) stimulation. This effect was significant at 10 µM and higher. The dose response was not influenced by strychnine (1 µM) addition to cell cultures.

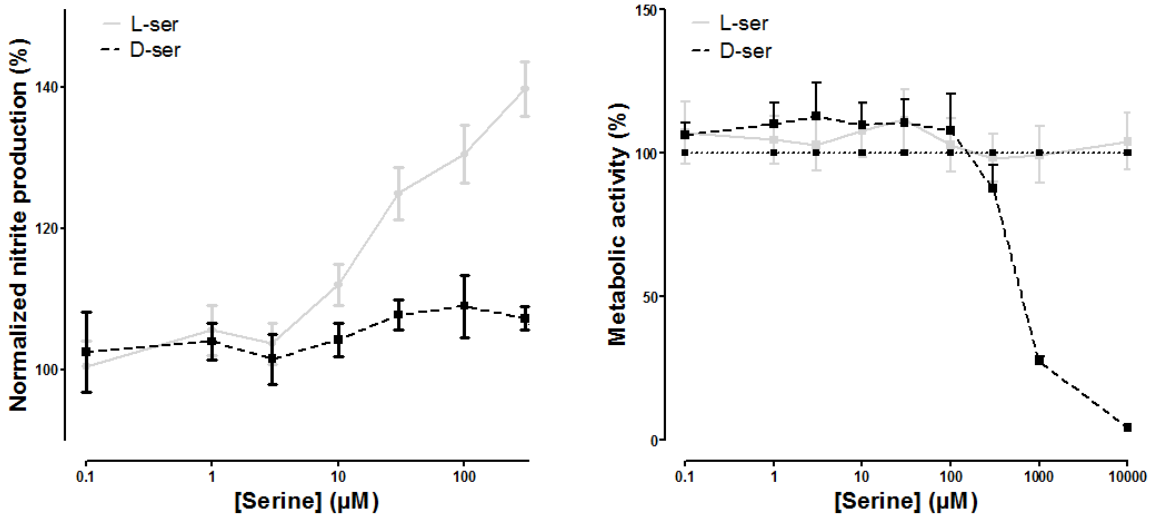

Figure III.10 L-Serine but not D-serine mimics glycine effects on primary microglial nitrite production.
A) L-serine (solid grey line) but not D-serine (dashed black line) enhance microglial nitrite production 48 hours after LPS stimulation. While the dose response relationship of L-Serine is comparable to glycine, D-serine does not modulate NO production. B) Viability control experiments 48 hours after LPS stimulation. L-serine did not change viability in concentrations up to 10 mM, as measured by changes in metabolic activity (see materials and methods). D-serine significantly decreased metabolic activity at concentrations of 1 mM and higher ($p<0.001$). Data are expressed as means ± S.E.M. of 4 to 6 independent experiments, each performed in triplicate.

2.3.7 Hyperkalemic induced-depolarization mimics glycine immunomodulation of microglial cells

As we found electrophysiological evidence that glycine and L-serine induce inward currents, mediated by sodium-coupled amino acid transporters, leading to a membrane depolarization of microglial cells, we tested the hypothesis that a depolarization could mimic the observed glycine effects on primary microglial cells in our cell culture conditions. We mimicked membrane depolarization by adding increasing concentrations of potassium chloride up to 30 mM to the cell culture medium. As shown in Figure III.11 a significant rise in nitrite production could be observed starting from the addition of 10 mM of KCl. Viability was unaltered under hyperkalemic culture conditions (data not shown).

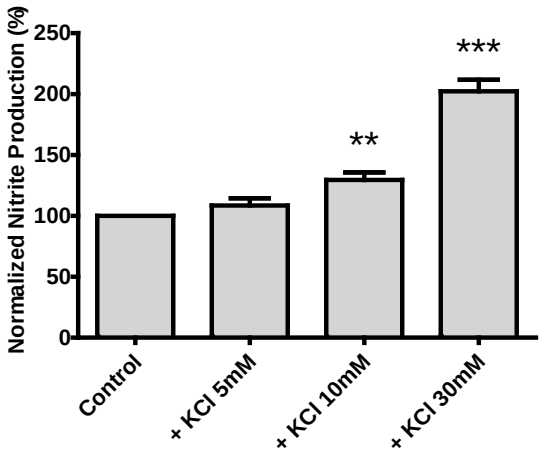

Figure III.11 Hyperkalemic cell culture conditions enhance microglial nitrite production.
Increasing concentrations of potassium chloride were added to the culture medium, leading to an enhancement of nitrite production. Results are expressed as means ± S.E.M. from four independent experiments. ** $p<0.01$; *** $p<0.001$

2.4 Discussion

As nothing is known about GlyR expression in microglial cells, we first looked for **molecular evidence** of GlyRs. Our western blot data clearly showed 48 kDa α-subunit expression, which was confirmed with RT-PCR showing mRNA presence for GlyR α1 and α2 subunits. The 48 kDa band was again located a little higher compared to the spinal cord control, similar to the results discussed in chapter II for T lymphocytes and described for macrophages. We could also demonstrate mRNA presence for β-subunits, which could not be demonstrated at the protein level using mAb4a. Although this seems to point to the absence of GlyR β-subunit protein expression, it can also be caused by the rather low affinity of the mAb4a antibody to these subunits. Increasing the amount of protein did not change the results. Glycine receptors have also been demonstrated in other macrophages although their subunit composition differs. To our knowledge β-subunits have been demonstrated in every macrophage studied so far and α1 subunits could be demonstrated in Kuppfer cells, but not in other macrophages where there was mainly α2 expression. Alpha3 expression was not found in macrophages so far. Our GlyR immunofluorescence images showed a somewhat heterogeneous staining of GlyR. This may point toward GlyR clustering, which has been demonstrated to be mediated by gephyrine, which we could also demonstrate by western blotting and RT-PCR. The location or GlyRs seemed to be predominantly cytoplasmic, making the punctuate appearance to be more likely linked to intracellular location in organelles. As these receptors were not located in the plasma membrane, at least in our cell culture conditions, they may not be functional in these conditions, which was confirmed by our electrophysiological findings which excluded GlyR-mediated currents in BV-2 microglial cells. Also Schilling and Eder could not detect

any GlyR-mediated currents in BV-2 cells (Schilling and Eder, 2004). Remarkably, also in macrophages (or other immune cells), to our knowledge no electrophysiological patch clamp studies ever demonstrated GlyR-mediated currents, although pharmacological evidence for these receptors in cell culture experiments or calcium response measurements is obvious (see also chapter I)!

In all macrophages studied so far an inhibition of immune function was found in micro- to low millimolar glycine concentrations. It was suggested that glycine causes a GlyR-mediated hyperpolarization, which inhibits calcium influx in macrophages by means of voltage gated calcium channels (VGCC). As calcium is a very important second messenger, necessary for activation of these cells, blunting these fluxes might decrease activation explaining the decreased production of nitric oxide, superoxide and cytokines, such as TNFα. This inhibition is the opposite of the enhancing role on microglial cells that was found by the group of Tanaka (Tanaka et al., 1998; Yang et al., 1998). As we found molecular evidence for GlyRs, we studied the potential role of these receptors in the modulating properties of glycine on microglial activity. Since in most papers studying glycine effects on immune cells so far, effects were maximal at glycine concentrations of 1 mM, we used this concentration in our experiments. We confirmed the enhancing effects of glycine on NO production and extended them to TNFα. The production of both substances is a consequence of a transcriptional activation and it has been suggested that two separate pathways exist, one for early release of mediators (*e.g.* TNFα and IL-1β) and one for late release (*e.g.* NO and IL-6) (Nakamura et al., 1999). Our results indicate that both pathways are influenced and the effect should be somewhere upstream of these pathways. We demonstrated that TNFα release peaked at 6 hours and in control conditions declined again after 10 hours. This was also found by others, both in microglial cells as peripheral immune cells (Nakamura et al., 1999; Spittler et al., 1999). As iNOS is the enzyme that is responsible for NO production in microglial cells, and this enzyme is mainly regulated at the transcriptional level, NO production is a process with a certain delay, which could also be seen in our longitudinal experiments, in which NO started increasing obviously only after 24 to 48 hours of stimulation. Glycine caused a 35% enhancement of NO production at the latter timepoint. Although this effect seems to be a slow one it is important to emphasize that the signal for an increase in transcription of iNOS most likely starts at the initial activation of these cells. It has been demonstrated indeed that astrocytic derived glycine and L-serine increase mRNA and protein expression of iNOS, pointing to a regulation at the transcriptional level (Yang et al., 1998). We also found a glycine dependent increase in metabolic activity/viability under serum-free culture conditions. This was also found by Tanaka and co-authors who, based upon trypan blue staining showing no changes in viability, suggested this was a change in metabolic activity and not viability (Tanaka et al., 1998). The same group however also reported similar glycine and L-serine effects on the viability (and not metabolic activity) of neurons, with active concentrations between 1 and 30 µM (Yang et al., 2000). This issue is explored and discussed further in the next chapter.

We used microglial NO production as the primary parameter to examine **the mechanism behind the glycine effects**. To determine whether these glycine effects where mediated by GlyRs we used a double approach. First we tried to block the effects by adding GlyR antagonists and second we tried to mimic them by adding GlyR (partial) agonists to cell

cultures. The most important antagonist that is used for in vitro experiments is strychnine. Strychnine is a competitive antagonist that blocks GlyRs with a K_i of 2-15 nM (Legendre, 2001). We used 1 µM in our experiments to have a maximal block of GlyRs on the one hand and still be GlyR specific on the other hand, as it has been shown that strychnine becomes a blocker of other receptors (*e.g.* $GABA_A$) at higher concentrations (Shirasaki et al., 1991). The use of strychnine for demonstrating GlyRs in cell culture experiments has been successfully done by other authors as well (Spittler et al., 1999). When adding strychnine to our microglial cultures no differences in glycine-induced effects could be observed. This was further confirmed by measuring concentration-responses of glycine, which were not shifted by strychnine. The same experiments were conducted with picrotoxinine, a more aspecific homomeric GlyR antagonist (Pribilla et al., 1992), which also failed to reduce glycine effects. These data clearly demonstrate that the mechanism of glycine modulation is GlyR independent. Further support for the GlyR independency comes from experiments with the GlyR partial agonists β-alanine and taurine. Both agonists are in fact physiological agonists and it has even been suggested that instead of glycine they may act as the primary endogenous agonists under certain circumstances (Mori et al., 2002). These agonists failed to mimic glycine effects in our experiments. Although L-serine has been shown to be a weak agonist of GlyRs as well and we observed NO stimulating properties of L-serine, it is very unlikely that this is GlyR mediated as taurine and β-alanine are far more potent agonists and did not have any effect. Also we tested the effect of strychnine on the L-serine dose response relationship and did not observe any block (data not shown). As one *in vivo* paper in chicken suggested that L-serine effects in the CNS could be mediated by $GABA_A$-R (Shigemi et al., 2008), we also tried blocking the L-serine effects with the $GABA_A$-R blocker picrotoxinine, but again this did not change responses (data not shown).

Another hypothesis was that NMDA receptors might mediate these effects. Although functional NMDA receptors have not been demonstrated on microglial cells yet, there is some molecular evidence for their presence under certain conditions (Gottlieb and Matute, 1997). NMDA receptors are primarily gated by glutamate, but glycine and D-serine function as (strychnine insensitive) co-agonists at these receptors, increasing the potency of glutamate. Since it has been demonstrated that microglial cells can synthesize glutamate in concentrations high enough to activate neuronal NMDA receptors (Piani et al., 1991) we tested the hypothesis that glycine could modulate (autocrine) glutamate-gated NMDA activity on microglial cells. Along with this hypothesis L-serine effects could be explained by the conversion of L-serine to D-serine by serine racemase, the converting enzyme that is known to be present in microglial cells (Wu and Barger, 2004). Applying D-serine directly however failed to mimic L-serine effects on microglial cells. Confirming these results, both the non-competitive NMDA-R antagonists MK-801 and the competitive antagonist APV did not have any effect on NO production. These data hence exclude the role of NMDA receptors in glycine immunomodulation of microglial cells. As there is limited evidence for primary glycine-gated NMDA receptors (Chatterton et al., 2002), unresponsive to MK-801 and APV, we also examined the glycine-site antagonist DCKA. No inhibition of glycine effects could be found arguing once more against the involvement of these receptors. This was further supported by the fact that D-serine did not shift the glycine dose-response (data not shown).

As already indicated our electrophysiological results are in agreement with Schilling and Eder, who provided electrophysiological evidence that no GlyRs are active under basal conditions in microglia cells (Schilling and Eder, 2004). They did also find small inward currents that were activated by glycine. These currents were sodium dependent but chloride and strychnine independent, suggesting they were transporter mediated. They further suggested that the immunomodulatory properties of glycine might be mediated by sodium coupled system A neutral amino acid transporters (SNATs). Although the currents they measured were quite small (a few pA), their effects on the microglial membrane potential was quite big (more than 10 mV), as can be explained by the very high membrane resistance of these cells, which has been shown to be several gigaohms (Newell and Schlichter, 2005). The lack of leaky currents makes these cells very sensitive to small depolarizing currents. We extended these results to L-serine. To test the hypothesis that a membrane depolarization might be responsible for the observed glycine effects on microglial activation, we tried mimicking a membrane depolarization in microglial cell cultures by adding potassium. We could find a significant enhancement of microglial NO production when adding 10 mM potassium or more, suggesting that depolarizing the cell membrane may indeed be responsible for the observed effects. The hypothesis of amino acid transporters being responsible for glycine effects is further examined in section 3.

As already mentioned, in many immune cells it has been suggested that a membrane hyperpolarization caused an inhibition of immune activation. As we found an enhancement of activation, correlated with a membrane depolarization, a plausible hypothesis is that depolarizing immunological cell membranes enhances their function, while hyperpolarizing could have the opposite effects. Although this is obviously an oversimplification of truth, many studies reported modulating effects of different substances on microglial activity, which correlate with changes in potassium conductance and hence membrane potential (Kuhn et al., 2004; Farber et al., 2005; Farber and Kettenmann, 2006a; Pannasch et al., 2006). Changes in membrane potential might alter calcium fluxes which are key signals in immunological cell activation. These fluxes are mediated by voltage-gated calcium channels and store-operated calcium channels, both of which are sensitive to membrane potential changes in microglial cells (Franchini et al., 2004). It has been suggested that these calcium levels also regulate NO production (Hoffmann et al., 2003; Farber and Kettenmann, 2006b).

Besides this membrane depolarization it is also possible that glycine effects are mediated by some metabolic pathway. This is supported by the fact that glycine and L-serine can easily be converted by the enzyme serine hydroxymethyltransferase (SHMT). It has been suggested that glycine and L-serine are in fact essential amino acids in microglial cells because of the low expression of 3-phosphoglycerate dehydrogenase, a key enzyme in L-serine biosynthesis (Sugishita et al., 2001). This is however contradicted by the high glycine and L-serine synthesis capacities by microglial cells (Hayashi et al., 2006).

Although molecular evidence for GlyR expression in microglial cells is obvious, this study demonstrated that glycine enhances NO production by a GlyR-independent mechanism. We suggest a transporter-mediated membrane depolarization to be responsible for the observed effects, although a metabolic effect cannot be completely ruled out, but seems less likely considering the very low concentrations at which glycine exerts its effects. The role of microglial GlyRs remains to be elucidated. The following section investigates whether this

modulation correlates with changes in calcium signals, as has been already reported in peripheral immune cells.

3. GLYCINE ENHANCES MICROGLIAL INTRACELLULAR CALCIUM SIGNALING. A ROLE FOR NEUTRAL AMINO ACID TRANSPORTERS.

3.1 Introduction

Microglia are considered to be the macrophages of the central nervous system (CNS), which after activation can become phagocytic, present antigens and secrete cytokines. Besides this important immune function, there is increasing evidence for a broader role of these cells. In development they have an important function in determining neuronal fate, differentiation and synaptogenesis (Polazzi and Contestabile, 2002; Bessis et al., 2007). Also their role in different pathologies seems to be more important than originally thought. These cells have been linked to neuro-inflammatory disorders (Muzio et al., 2007), neuropathic pain (Scholz and Woolf, 2007), neurodegenerative diseases (Gao and Hong, 2008), epilepsy (Aronica et al., 2007) and even psychiatric disorders (Bayer et al., 1999).

Glycine is one of the main inhibitory neurotransmitters in the CNS, acting through strychnine-sensitive glycine receptors (GlyRs). Increasing evidence suggests that glycine exerts a more general signaling role than the pure transmission of neuronal inhibition. In non-neuronal cells glycine was shown to protect different cell types against ischemic cell death (*e.g.* renal cells, hepatocytes and endothelial cells). Furthermore, glycine was shown to have modulatory properties in macroglial cells, immune cells and sperm cells (reviewed recently in (Van den Eynden et al., 2009)). The immunomodulatory properties were best studied in Kupffer cells, the resident macrophages of the liver, where an inhibition of cytokine production (*e.g.* tumour necrosis factor α) was found, explaining beneficial glycine effects in animal models of alcohol hepatitis and endotoxic shock (Ikejima et al., 1996; Ikejima et al., 1997; Yamashina et al., 2005). In these cells, glycine activates GlyRs, leading to chloride influx and membrane hyperpolarization. As these cells can be activated by lipopolysaccharide (LPS), leading to membrane depolarization, it was hypothesized that glycine inhibition can be explained by counteracting this depolarization and hence blunting of calcium fluxes. A role for voltage gated calcium channels (VGCC) was hypothesized (Ikejima et al., 1997). Similar findings have been obtained with other macrophages (*e.g.* splenic and pulmonary macrophages) (Wheeler and Thurman, 1999; Li et al., 2001).

In brain macrophages or microglial cells much less is known about glycine-induced immunomodulation. It has been suggested that astrocytes can modulate microglial activity by secreting glycine and L-serine (Tanaka et al., 1998; Yang et al., 1998). This would lead to an increase in microglial activity, contrary to the inhibitory role that is given to glycine in the peripheral immune system. However, the mechanism of this modulation is not known. It was suggested that glycine can activate Na^+-coupled neutral amino acid transporters, leading to a cellular depolarization (Schilling and Eder, 2004). Given the role of microglial cells in different pathologies, the exact mechanism may be of special interest in the perspective of potential new therapeutic targets.

As glycine was shown to inhibit Kupffer cell activation by GlyR-dependent blunting of calcium influx whereas glycine enhances activation of microglial cells, we hypothesized that the latter might correlate with an enhancement of microglial calcium fluxes, which may be mediated by neutral amino acid transporters, as suggested by Schilling and Eder. In

macrophages and microglial cells calcium signaling is crucial for several downstream effects. Calcium signals are linked to the activation of several transcription factors (*e.g.* nuclear factor of activated T cells (NFAT), nuclear factor κβ (NFκβ)), responsible for immune cell effector functions (*e.g.* cytokine production, proliferation) (Ferrari et al., 1999; Kim et al., 2004; Martin et al., 2006). Calcium signaling can be induced by different mediators in microglial cells (Moller, 2002; Farber and Kettenmann, 2006b). Both metabotropic (P2Y) and ionotropic (P2X) purinergic receptors were described in microglial cells (Farber and Kettenmann, 2006a). The main purinergic receptors responsible for calcium signaling are P2Y and $P2X_7$ receptors. While $P2X_7$ receptor activation by higher concentrations of ATP (≥1mM), leads to a sustained calcium influx from the extracellular environment, P2Y receptors, activated by lower concentrations of ATP (<1mM), leads to a transient calcium release from intracellular stores (Moller et al., 2000; McLarnon, 2005). LPS is a frequently used *in vitro* activator of macrophages and microglial cells. The effects of LPS on calcium signaling are complex. LPS was reported to induce both fast transient and slow chronic changes in intracellular calcium concentrations, from both intra- and extracellular sources (Bader et al., 1994; Ikejima et al., 1997; Choi et al., 2002; Hoffmann et al., 2003; Kim et al., 2004; Yi et al., 2005; Martin et al., 2006; Beck et al., 2008). Thapsigargin (TG) is an artificial agent, used to study the calcium machinery in many cells. TG is a blocker of the endoplasmic reticulum calcium-ATPase, leading to a net gradual release of calcium from these stores (Thastrup et al., 1990). In this section we will use the three inducers ATP, LPS and TG to study potential glycine modulation of calcium responses.

3.2 Materials and Methods

3.2.1 Cell cultures

BV-2 microglial cells were cultures as described in paragraph 2.2.1. Twenty four hours before experiments cells were transferred to 96-well plates (for calcium measurements) or glass cover slips (for immunolabelling) and put on (glycine-free) Minimum Essential Medium (MEM; Invitrogen) supplemented with 10% FCS, 2 mM glutamine and 1% P/S (simply called MEM-medium in the remainder of the article).

3.2.2 Chemicals

Glycine, L-serine, L-leucine, L-valine, α-aminoisobutyric acid (AIB), strychnine, probenecid, pluronic acid, ATP, lipopolysaccharide (LPS) and thapsigargin (TG) were all purchased from Sigma Aldrich (Bornem, Belgium). Mannitol was supplied by Acros Organics (Geel, Belgium). Fluo-3 AM (Invitrogen) was dissolved in a 20% solution of pluronic acid in dimethylsulfoxide (DMSO) to avoid spontaneous de-esterification.

3.2.3 Calcium measurements

Cells were plated in MEM-medium at a density of 50.000 cells/well into 96-well plates. After plating, cells were allowed to incubate for 24 hours (37°C, 5% CO_2). After 24 hours, medium was removed and cells were rinsed in a physiological buffer containing 130 mM NaCl, 5 mM

KCl, 2 mM CaCl (or without when using a calcium free buffer for some experiments), l mM $MgCl_2$, 10 mM HEPES and 10 mM glucose, supplemented with 2.5 mM Probenecid to avoid dye leaking, and brought to a final pH of 7.4 by HCl addition. Measurements were carried out at 37°C using the Fluostar optima plate reader (Isogen Life Science, De Meern, The Netherlands), with filters set to detect excitation at 490 nm and emission at 520 nm. After background measurements, cells were loaded with 5 µM Fluo-3-AM in buffer for 30 minutes at 37 °C in a 5% CO_2 incubator. The relatively high K_d of Fluo-3 (864 nM at 37°C) makes this tracer very sensitive to detect higher calcium concentrations (Merritt et al., 1990). Once dye-loaded, the cells were washed thoroughly with buffer to remove any unincorporated dye. The different test compounds (*e.g.* glycine) were added in their final concentration at this stage. Before measurements, cells were incubated in the plate reader for 15 minutes at 37°C, which allowed modulation of the cells by the test compounds, ensured full de-esterification of the Fluo-3 dye inside the cells and allowed temperature stabilization inside the reader. After establishing baseline fluorescence, LPS (dissolved in buffer containing 5% rat serum), ATP or TG was injected and the response was measured. Measurements were performed ever 1 second for ATP and LPS and every 15 seconds for TG.
The calcium response was expressed as $\Delta F/F_0$, *i.e.* the ratio of changes in fluorescence intensity ($\Delta F=F-F_0$) relative to basal fluorescence (F_0). The maximal calcium response after stimulation was defined as the peak calcium response. Calcium response modulation of glycine (or any other compound tested), was defined as the percentage of the peak calcium response in glycine conditions relative to the peak calcium response in control conditions (no test compound added). In this way, values higher than 100% would indicate enhancement of calcium (peak) responses, while values lower than 100% would indicate inhibition of calcium responses.

3.2.4 Immunolabelling and confocal microscopy

BV-2 cells were transferred onto glass cover slips in MEM-medium at a density of 25.000 cells/glass and allowed to incubate for 24 hours (37°C, 5% CO_2). After washing with the same physiological buffer as used for calcium measurements, cells were exposed to different test compounds. Following 30’ incubation, cells were fixed for 5 minutes in 4% paraformaldehyde. Triton X-100 (Sigma Aldrich) 0.2% was then applied for 10 minutes to ensure permeabilization, followed by blocking for 30 minutes with 3% filtered goat serum (Millipore, Brussels, Belgium). For glycine detection a 1/1000 dilution of rat anti-glycine antibodies (ImmunoSolution, Queenslands, Australia) was added to the cells for 1 hour. Detection of the primary antibody was done by adding a 1/500 dilution of Alexa Fluor 555 goat anti-rat IgG (Invitrogen) for 1 hour. Phosphate buffered saline (PBS) was used for dilution of PFA, Triton X-100, goat serum, primary and secondary antibody and for thorough washing of the cells between each step after fixation and all labellings were performed at room temperature. After this procedure, cover slips were mounted on glass slides using ProLong Gold antifade reagent (Invitrogen).
Microscopical imaging was carried out on a Zeiss LSM 510 META one-photon confocal laser-scanning microscope (Zeiss, Jena, Germany). We used a helium neon laser, with excitation at 543 nm and filters set to detect emission above 560 nm to detect alexa 555

fluorescence. Images were made at 63x magnification and quantification was done using ImageJ software (National Institute of Health, Bethesda, USA). For quantification of glycine uptake, the background signal was subtracted from each image, fluorescence intensity of each individual cell was measured and normalized to the mean cellular fluorescence of control (glycine-free) conditions.

3.2.5 Statistics

Data are expressed as means ± S.E.M. Statistical significance was evaluated using ANOVA followed by post-hoc Tukey-Kramer testing when comparing different conditions, or student-t testing relative to a fixed value (100%) when comparing the different conditions to control responses. P-values of less than 0.05 were considered significant. P values of less than 0.05; 0.01 and 0.001 are indicated by one, two or three asterisks or number signs (* or #) respectively in the figures.

3.3 Results

3.3.1 Characterization of calcium responses in microglial cells

3.3.1.1 Serum factors induce intracellular calcium transients in microglial cells.

Most studies investigating glycine effects on macrophages use 10 µg/ml LPS in the presence of 5% rat serum to induce calcium reponses. The addition of rat serum was shown to be necessary for calcium responses to be induced. We did indeed confirm that LPS 10 µg/ml by itself did not induce any calcium response on its own (Figure III.12A). When LPS was injected in the presence of rat serum, a fast and transient calcium response was measured (Figure III.12B). Surprisingly this response was also measured in the presence of rat serum alone (Figure III.12C), indicating that serum factors and not LPS are responsible for calcium transients in microglial cells.

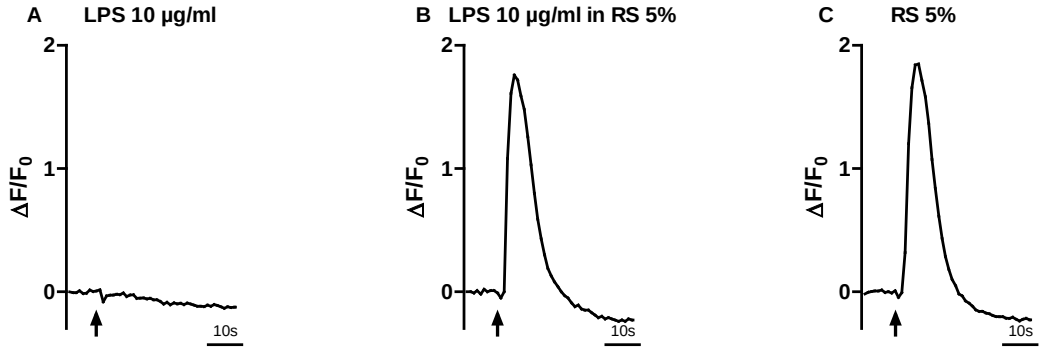

Figure III.12 Rat serum induces calcium transients in BV-2 cells, independently of LPS.
Representative examples of individual traces are shown. $\Delta F/F_0$ corresponds to changes in intracellular calcium concentrations. The arrow below each graph indicates the time point of application of different compounds. A. Application of LPS 10 µg/ml by itself did not induce any calcium response. B. When LPS 10 µg/ml was applied in the presence of 5% rat serum (RS), a fast transient calcium response was evoked. C. When RS was applied without LPS the same response as shown in B. could be evoked.

These findings were extended to FCS. When 0.5% FCS was applied to cells a similar fast transient response was measured (Figure III.13A). This response was completely blocked when intracellular calcium stores were depleted by pre-incubating cells for 15 minutes with TG 500 nM first. The reponse was still present in the absence of extracellular calcium, indicating that it was primarily caused by intracellular calcium release, rather than by extracellular calcium influx. Next the dose response of FCS on peak calcium responses was determined. The response started at 0.1% FCS and higher and was maximal at 3% (Figure III.13B).

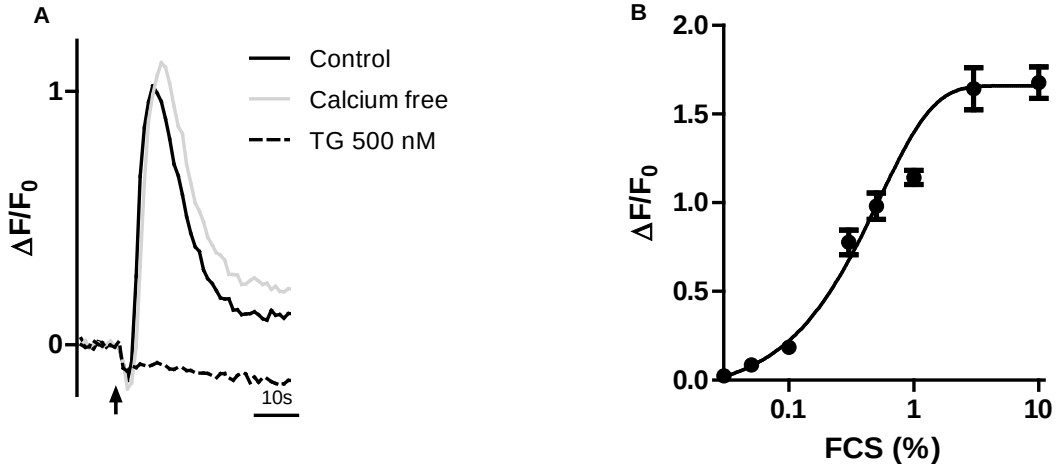

Figure III.13 Fetal calf serum induces intracellular calcium transients in BV-2 cells.
A. Representative traces are shown. The arrow indicates the time point of FCS 0.5% application. FCS induced a fast and transient calcium response, which could be completely blocked by thapsigargin (500nM) pre-incubation of cells, but was independent of extracellular calcium. B. Dose response relationship between the concentration of FCS and the mean peak calcium response. Results are expressed as means ± S.E.M. of minimal 6 experiments.

3.3.1.2 Low and high concentrations of ATP induce different calcium responses in microglial cells.

ATP is one of the best studied calcium response inducers in microglial cells. We first applied a low concentration of ATP (100 µM) to microglial cells. As shown in Figure III.14A a fast and transient response could be evoked. As this response was completely blocked when intracellular calcium stores were depleted by pre-incubating cells for 15 minutes with TG 500 nM, but not in a calcium-free extracellular environment, the primary sources are likely intracellular stores. When a high concentration of ATP (1 mM) was applied to microglial cells, a completely different, more pronounced sustained response was measured (Figure III.14B). This response was partially blocked when cells were pre-incubated with TG or when they were measured in the absence of extracellular calcium. Moreover the pattern changed between these two conditions. While TG seemed to abolish the initial peak, leading to a somewhat slower exponential response, the calcium-free extracellular environment rather caused a more pronounced initial peak with a pattern that was more similar to the reponses measured with 100 µM ATP. These results point to a mixed source of calcium, both from the extracellular environment and from intracellular stores, when 1 mM ATP is applied.

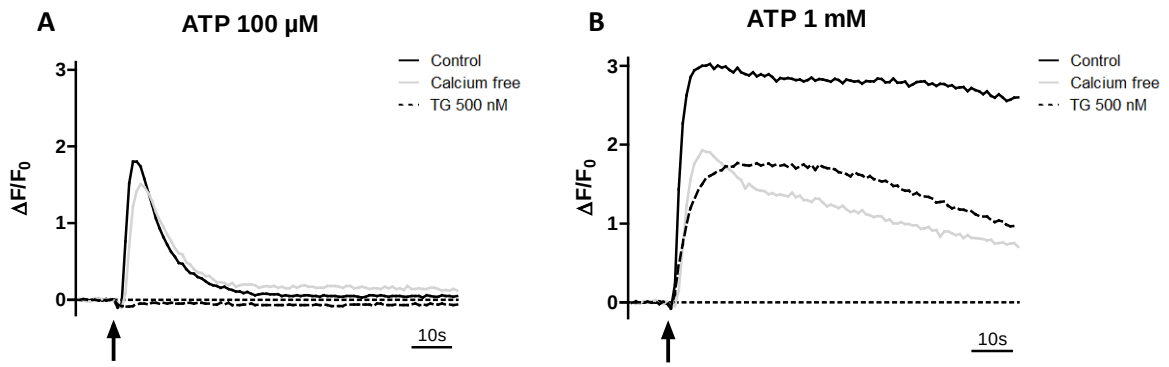

Figure III.14 ATP 100 µM and 1 mM induce different calcium responses in BV-2 cells.
Representative examples of individual traces are shown. $\Delta F/F_0$ corresponds to changes in intracellular calcium concentrations. The arrow below each graph indicates the time point of application of different inducers. A. Application of 100 µM ATP induced a transient calcium response, which could be almost completely blocked by pre-incubating cells with thapsigargin (500 nM), but was not altered when the responses where measured in a calcium free extracellular environment. B. Application of 1 mM ATP induced a sustained calcium response. In the presence of thapsigargin (500 nM) the initial peak disappeared and the amplitude of the response decreased. A calcium-free extracellular environment decreased the amplitude while the initial peak became more apparent.

3.3.2 Glycine enhances microglial intracellular calcium transients

Glycine, or any of the other test compounds used in this study, did not elicit a calcium response on its own (data not shown). Previous reports studying glycine effects on calcium transients mostly used LPS, dissolved in 5% rat serum to induce calcium responses. Although our experiments demonstrated that 10 mM glycine caused a significant enhancement of calcium responses, induced by LPS/rat serum (26 ± 2%; $p<0.001$; n=20), we showed in the previous paragraph that this response is not caused by LPS, but secondarily to an unknown factor in serum (Figure III.12), making the relevance of these findings unclear.
Therefore we decided to use ATP (100 µM), which was shown to induce similar calcium responses, mainly attributed to intracellular store release (Figure III.14). When cells were incubated for 15 minutes in 10 millimolar glycine conditions and calcium responses where induced by ATP (100 µM), mean peak calcium responses increased from 1.85 ± 0.08 to 2.16 ± 0.11, corresponding to an enhancement of 17 ± 3% ($p<0.001$; n=27) (Figure III.15 A and B). To further study the modulating role of glycine on intracellular calcium transients, we used thapsigargin (TG) 500nM, which is known to cause intracellar calcium release by blocking SERCAs. Pre-incubation of cells with 10 mM glycine for 15 minutes increased the peak calcium response from 0.82 ± 0.02 to 1.10 ± 0.02, corresponding to an enhancement of 37 ± 3% ($p<0.001$; n=86) (Figure III.15C and D). Also at a concentration of 1 mM glycine induced a significant enhancement of calcium responses (11 ± 3%; $p=0.009$; n=8) (Figure III.15D).

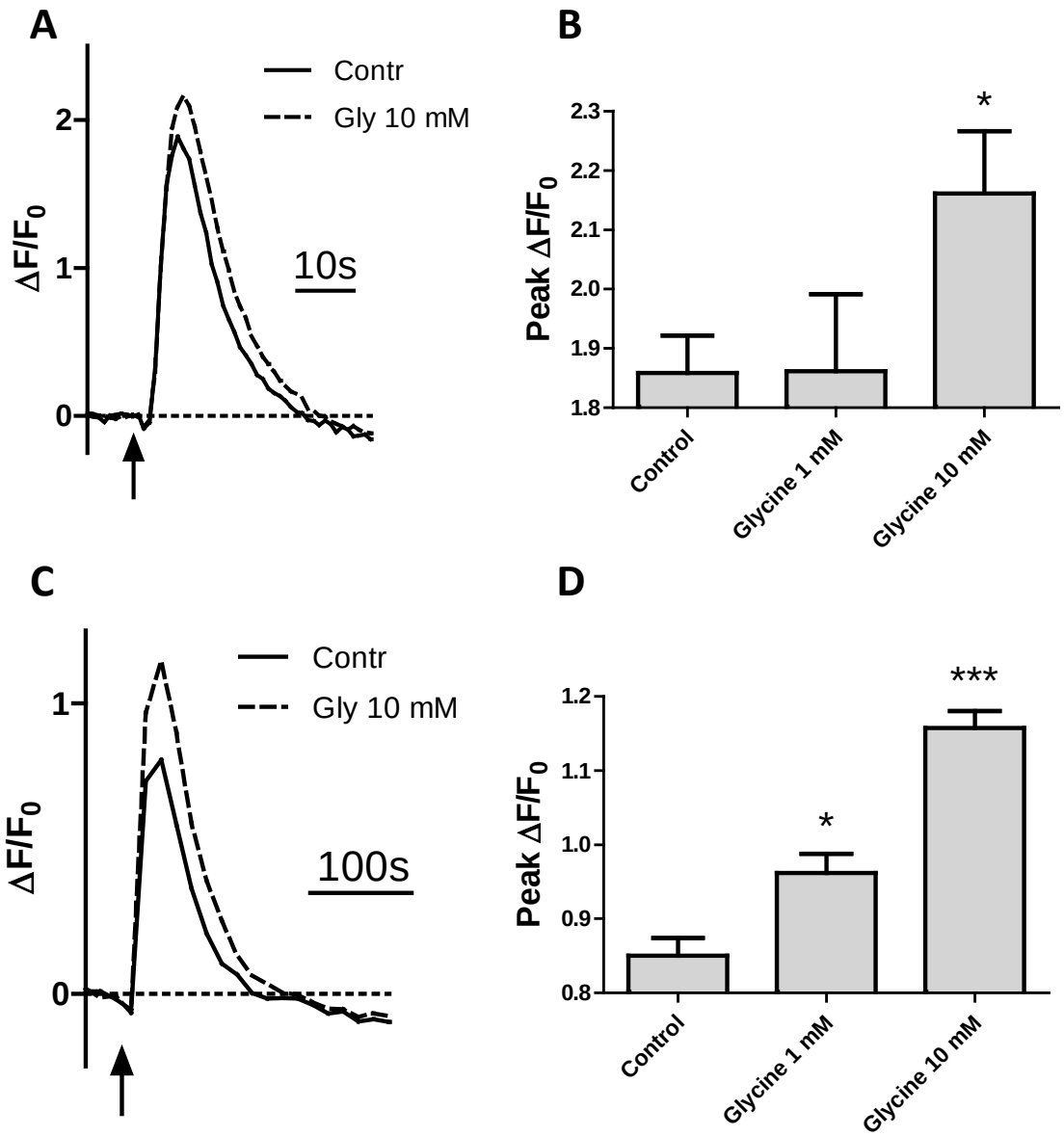

Figure III.15 Glycine enhances BV-2 intracellular calcium transients in millimolar concentrations.
Cells were incubated for 15 minutes in glycine or control conditions. A and C. Examples of individual traces showing the calcium response ($\Delta F/F_0$) after ATP 100 µM (A) or TG 500 nM (C) application (arrow) in control (solid line) or 10 mM glycine (dashed line) conditions. B and D. Mean peak calcium response (Peak $\Delta F/F_0$) in control and glycine conditions. Results are expressed as means ± S.E.M. of minimal 8 experiments.

3.3.3 Glycine receptors are not responsible for glycine effects

To investigate the potential involvement of GlyRs in the glycine modulating properties on calcium transients, we used a double approach in which we tried to block the glycine effects with strychnine, a GlyR antagonist, on the one hand, and to mimick the effects with the GlyR partial agonists β-alanine and taurine on the other hand. Strychnine was co-applied with glycine, in a concentration known to specifically block GlyRs (1 µM). Figure III.16 shows that when strychnine was simultaneously added with glycine to the cells, the calcium response modulation of glycine did not change. Taurine did not modulate peak calcium responses, while a small but not-significant enhancement could be found when β-alanine was applied.

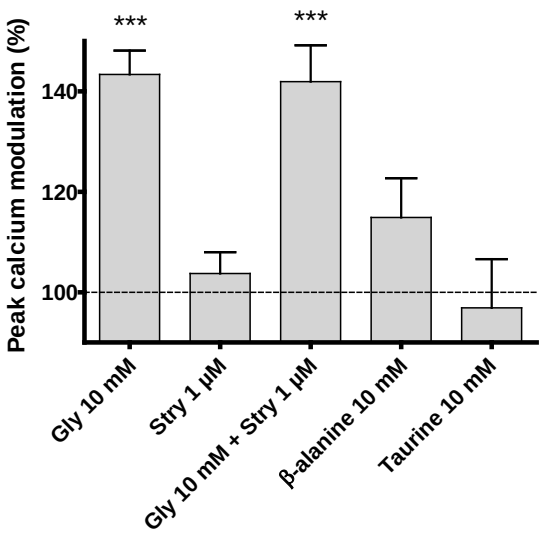

Figure III.16 Influence of GlyR agonists and antagonists on BV-2 cell calcium response modulation.
Calcium transients were induced with 500 nM TG. Glycine induced a significant enhancement of peak calcium responses. When the GlyR-antagonist strychnine (1 µM) was co-applied with glycine the glycine effect was not significantly altered. β-alanine (10 mM) caused a small, but not significant enhancement of calcium responses, while the response was unaltered when taurine (10 mM) was applied. Results are expressed as means ± S.E.M. of minimal 9 experiments.

3.3.4 Glycine modulatory effects are dependent on neutral amino acid transporters

Next, we examined the potential involvement of neutral amino acid transporters in glycine modulation of calcium transients. We used the competitive antagonist α-aminoisobutyric acid (AIB), which blocks both system L and system A neutral amino acid transporters. Figure III.17 shows that equimolar concentrations of AIB (10 mM) almost completely blocked glycine (10 mM) effects, compared to AIB control conditions, indicating that neutral amino acid transporters are essential for glycine modulation of calcium transients. Note that AIB by itself also caused a small enhancement of peak calcium responses (113 and 114% for 1 and 10 mM respectively). The involvement of glycine transporters type 1 (GlyT1) was also tested by applying the GlyT1-blocker ALX-5407, which failed to block the glycine effects (Table III.3). Because neutral amino acid transporters are known to co-transport sodium, leading to membrane depolarization, peak calcium responses were also determined under hyperkaliemic and hence depolarizing conditions. When KCl 30 mM was applied and TG-induced calcium responses were measured, a mean modulation of 113 ± 4% was found (Table III.3).

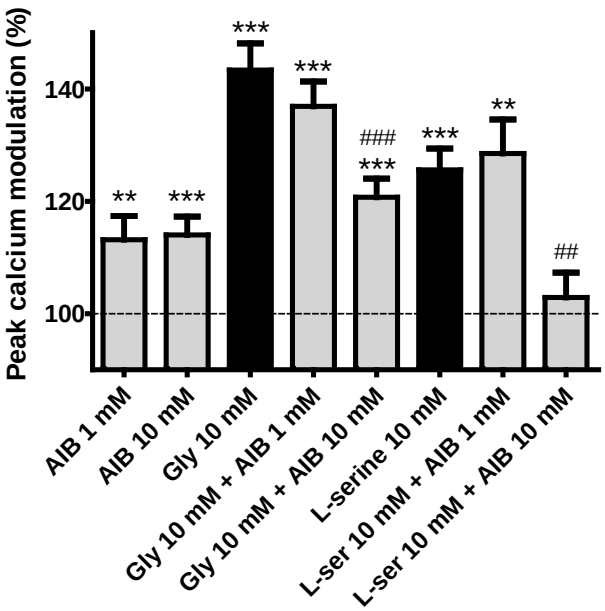

Figure III.17 α-Amino isobutyric acid (AIB) blocks glycine modulation of calcium responses in BV-2 cells. Calcium transients were induced with 500 nM TG. Glycine (10 mM) induced a significant enhancement of peak calcium response, which could be significantly blocked by co-application of 10 mM AIB. Also L-serine (10 mM) enhanced peak calcium response, which was blocked by 10 mM AIB as well. AIB by itself also caused a small enhancement of calcium response. Significant differences from control (100%) are indicated with asterisks (*), while relevant significant differences from glycine/L-serine effects are indicated with number signs (#). Results are expressed as mean ± S.E.M. of minimal 9 experiments.

3.3.5 Neutral amino acid transporter substrates mimic glycine effects

Next the amino acid specificity of the effects was examined. Besides glycine also millimolar concentrations of (L-)serine, (L-)alanine, (L-)glutamine and (L-)valine significantly enhanced calcium transients induced by TG (Table III.3). As shown for glycine, serine effects on TG-induced calcium responses could also be blocked by equimolar concentrations of AIB (Figure III.17). (L-)Leucine (10 mM) however did not cause any changes in calcium responses. As an amino acid concentration of 10 mM can alter osmolarity, possibly leading to biologic effects, we used mannitol to examine whether changes in osmolarity might have effects on calcium responses. Table III.3 shows that mannitol did not modulate calcium responses and summarizes the modulatory properties on peak calcium transients of the different compounds tested in this study.

Table III.3 Summary of BV-2 cell TG-induced peak calcium modulation by different compounds tested in this study

	Peak calcium modulation				
	Mean	***95% CI***	***n***	***p (contr)***	***p (gly/ser)***
Gly 1 mM	111%	104-118%	8	0.009	
Gly 10 mM	137%	132-142%	86	<0.001	
+ AIB 1 mM	137%	127-147%	9	<0.001	ns
+ AIB 10 mM	121%	114-128%	24	<0.001	<0.001
+ Stry 1 µM	142%	125-159%	9		ns
+ ALX 10 nM	139%	111-167%	5	0.018	ns
Tau	97%	75-119%	9	ns	
β-ala	115%	97-133%	9	ns	
Ser 1 mM	122%	112-131%	9	<0.001	
Ser 10 mM	128%	125-132%	50	<0.001	
+ AIB 1 mM	129%	115-143%	9	0.002	ns
+ AIB 10 mM	103%	93-113%	9	ns	0.002
Ala 10 mM	138%	120-155%	9	0.001	
Gln 10 mM	137%	111-163%	9	0.012	
Val 10 mM	124%	110-138%	12	0.003	
Leu 10 mM	104%	95-112%	9	ns	
AIB 10 mM	114%	107-120%	33	<0.001	
Man 10 mM	102%	96-109%	9	ns	
KCl 30 mM	113%	104-123%	12	0.011	

BV-2 cells were pre-incubated for 15 minutes with the test compound, after which calcium transients were induced by application of 500 nM TG. The peak calcium was normalized to the control conditions as indicated in *materials and methods*. 95% CI: 95% confidence interval; p(contr): p-value compared to control (100%) conditions; p(gly/ser): p-value of blockers compared to glycine or serine conditions

3.3.6 Neutral amino acid transporters mediate glycine uptake in microglial cells

As we found that there may be a role for neutral amino acid transporters for glycine modulation of calcium responses, we examined glycine uptake under our experimental conditions. When microglial cells were incubated for 30 minutes in 10 mM glycine and/or 10 mM AIB conditions and immediately fixed and immunostained for glycine, an extensive intracellular cytoplasmic labelling could be found which could be largely blocked by 10 mM AIB (Figure III.18). Figure III.19 shows the results of the cellular quantification for glycine uptake. Compared to control conditions (Figure III.18A), glycine uptake was substantial (474 ± 15%) after incubation in 10 mM glycine (Figure III.18C). AIB (10 mM) had no effect on basal glycine uptake (95 ± 4%, Figure III.18B), but when AIB (10 mM) was co-applied with 10 mM glycine, glycine uptake was reduced to 222 ± 10% (Figure III.18D). These experiments confirm extensive glycine uptake under glycine conditions which could be blocked for 67% if equimolar concentrations of AIB were co-applied.

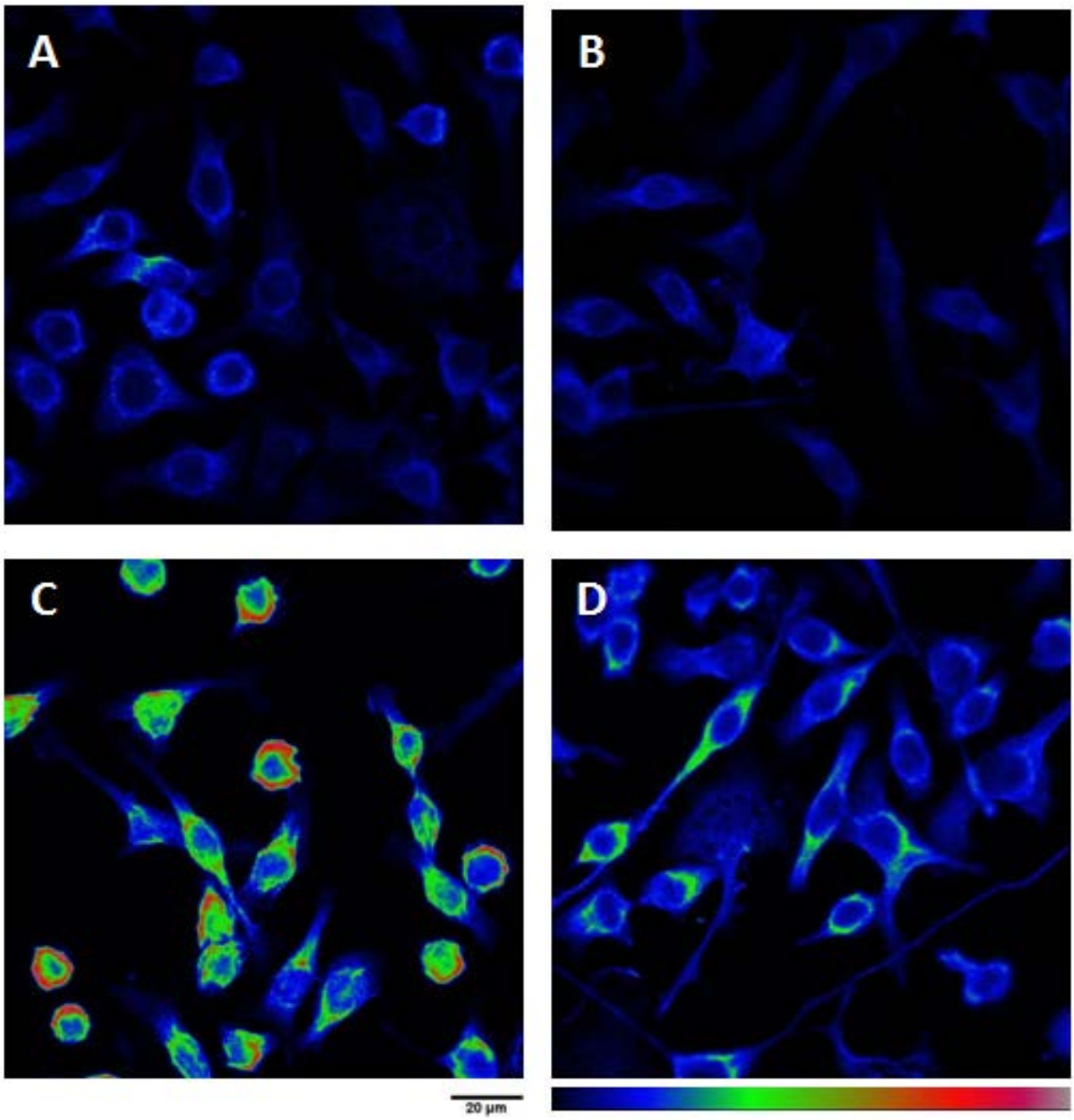

Figure III.18 Glycine immunofluorescence images showing glycine uptake by BV-2 microglial cells. Microglial cells were incubated for 30 minutes in glycine (10 mM) and/or AIB (10 mM) conditions and labelled for glycine. Under (glycine-free) control (A) or AIB conditions (B) glycine immunofluorescence is relatively low. After incubation in 10 mM glycine an extensive cytoplasmic glycine fluorescence can be seen (C), which can be largely blocked by 10 mM AIB (D). Scale bar and colour scale are indicated below the figures.

3.4 Discussion

In this study we showed for the first time that glycine was able to enhance microglial calcium transients, induced by different agents known to induce calcium signaling. This enhancement was shown to be GlyR-independent, contrary to findings in other macrophages. We showed that this positive modulation could be mimicked by some structurally related neutral amino acids (*e.g.* serine and alanine) and that sodium-coupled neutral amino acid transporters (SNATs) are essential for the effects to take place.

In macrophages and other immune cells (*e.g.* neutrophils or monocytes) outside the CNS glycine was shown to inhibit immune cell activation (Ikejima et al., 1996; Spittler et al., 1999;

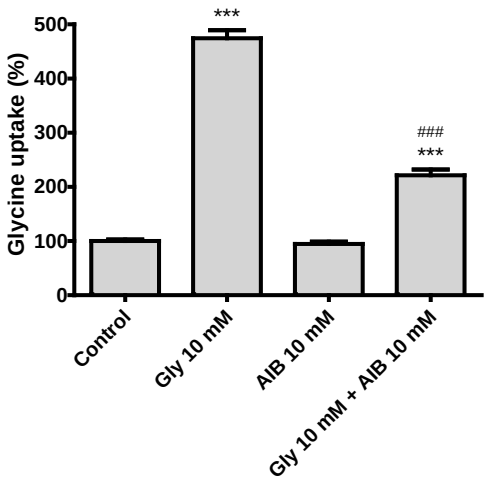

Figure III.19 Immunofluorescence quantification of BV-2 cell glycine uptake. Under glycine (10 mM) conditions microglial glycine uptake increased to 474 ± 15%. AIB (10 mM) could reduce this uptake back to 222 ± 10%, indicating a 67% reduction in glycine uptake. A total of 43-123 cells of 3-5 independent experiments were quantified. Significant differences from (glycine-free) control are indicated with asterisks (*), while relevant significant differences from glycine effects are indicated with number signs (#).

Wheeler and Thurman, 1999; Wheeler et al., 2000a; Li et al., 2001). Glycine was found to blunt calcium signaling. It was suggested that glycine binds to GlyRs and hyperpolarizes cells. As LPS was found to depolarize cells and suggested to activate VGCCs, resulting in calcium influx, it was hypothesized that the glycine-induced hyperpolarization counteracts the LPS-induced depolarization and hence blunts calcium fluxes via VGCCs (Ikejima et al., 1997; Van den Eynden et al., 2009). In microglial cells, the macrophages of the brain, opposite findings were made in cell culture experiments. It was suggested that astrocytes secrete both glycine and L-serine and that these amino acids somehow enhance LPS-induced microglial activation and can even induce morphological changes, but the precise mechanism was not studied (Tanaka et al., 1998; Yang et al., 1998). It was suggested that the effect could have a non-specific metabolic origin, since microglial cells were shown to have a low expression of 3-phosphoglycerate dehydrogenase, a key enzyme in L-serine and glycine biosynthesis. This could mean that glycine and L-serine are in fact essential amino acids for microglial cells (Sugishita et al., 2001). This conclusion is however contradicted by the high glycine and L-serine synthesis capacities of microglial cells (Hayashi et al., 2006). Another study by Schilling and Eder, together with the findings described in the previous section of this work, demonstrated that glycine can induce GlyR-independent depolarizing currents in BV-2 microglial cells. These authors conclude that system A neutral amino acid transporters (SNATs) were responsible for these currents. Moreover, they hypothesized that these currents, although small, could have a signaling role via membrane depolarization (Schilling and Eder, 2004). Indeed, glycine-induced depolarization was substantial (up to 30 mV increase in membrane potential was measured) despite the low amplitude of the currents (around 10 pA), and could be explained by the high membrane resistance (up to 8 gigaOhm) of microglial cells (Newell and Schlichter, 2005).

We further explored this hypothesis by studying microglial calcium signals. Although glycine modulating effects on calcium signaling in peripheral immune cells have been extensively studied, nothing is known about its modulation of calcium signals in microglial cells. **LPS** acts on Toll-like 4 receptors, although the mechanism behind induction of calcium responses with LPS is not entirely clear. Both acute transient and sustained chronic responses were described and both intracellular and extracellular sources were suggested to explain calcium rises in microglia (Bader et al., 1994; Choi et al., 2002; Hoffmann et al., 2003; Yi et al., 2005). Remarkably the acute transient calcium responses could only be induced when LPS was dissolved in 5% rat serum and not by LPS on its own. Indeed, most reports studying glycine modulation of calcium fluxes so far used LPS (10 µg/ml), dissolved in 5% rat serum,

to induce calcium transients (Ikejima et al., 1996; Ikejima et al., 1997; Seabra et al., 1998; Wheeler and Thurman, 1999; Wheeler et al., 2000a). This was suggested to be caused by the obligatory presence of an LPS binding protein in rat serum. We confirmed the induction of calcium transients after LPS/rat serum application in BV-2 microglial cells (and found an enhancing role of micromolar glycine concentrations), and not after LPS application by itself, as has been suggested by previous studies. To our surprise however we found the same responses when 5% rat serum was applied by its own. This could not be due to complement factors as the serum was heat-inactivated (30' at 56°C). These data shade a new light on LPS-induced calcium signalling. Although we cannot exclude that other studies reporting LPS-induced calcium transients are truely due to a LPS effects (most authors mention, but do not show that 5% rat serum alone was inactive), caution is necessary when interpreting these findings and correlating them to cell culture experiments. These findings also question the existence of LPS-induced acute transient calcium responses. We extended these data to (heat-inactivated) FCS, as this serum is used in almost every cell culture protocol and is even part of loading procedures of calcium microfluorimetric experiments (Buntinx et al., 2002). Again we found an acute transient calcium response, which was induced by 0.1% FCS and maximal at 3% FCS, all concentrations below the generally used percentage of FCS in cell culture experiments (10%). We showed that this calcium response was entirely caused by intracellular calcium release as TG pre-incubation could completely block the response while the removal of extracellular calcium did not change the response. Although the serum factor responsible for these transients needs to be present 100x above its saturating concentration in serum (as 1% presence is already enough to evoke almost maximal responses), is relatively heat-stable (56°C) and evokes only intracellular calcium responses, its precise identity remains unclear at present. Indeed, different serum components were previously reported to be active on microglial calcium signalling (*e.g.* Albumin, PGE_2, Thrombin, Platelet-activating factor) and may be potential candidates for the induction of the signals we measured (Hooper et al., 2005; Farber and Kettenmann, 2006b). We decided to use **ATP**, another inducer of calcium responses in microglial cells. Purinergic signaling is extensively studied in microglial cells (for review see (Farber and Kettenmann, 2006a)). ATP is known to act on ionotropic P2X and metabotropic P2Y receptors, the latter causing inositol 1,4,5-triphosphate (IP_3) release and hence intracellular calcium release from the endoplasmic reticulum (ER). When low concentrations of ATP (100 µM) were applied, we measured a thapsigargin-sensitive acute transient calcium response, which was only slightly reduced in a calcium-free extracellular environment. This is in agreement with other studies, showing that 100 µM of ATP mainly activated P2Y receptors, leading to IP_3 release and calcium release from the ER (Moller et al., 2000; McLarnon, 2005). Millimolar concentrations on the other hand activated a more complex, acute and sustained response, with a higher amplitude. The amplitude and shape of this response could be altered by both thapsigargin and removal of extracellular calcium, indicating a combination of extracellular calcium influx and intracellular store release. These results are in agreement with activation of ionotropic $P2X_7$ receptors (besides P2Y receptors), causing direct calcium influx from the extracellular environment, which was shown to have a substantial effect on total intracellular calcium concentration changes (McLarnon, 2005). We used **thapsigargin (TG)** to characterize intracellular calcium response modulation. TG is an artificial calcium inducer which blocks calcium reuptake by SERCAs

(sarcoplasmic endoplasmic reticulum ATPase), causing a net intracellular calcium release from the ER (Thastrup et al., 1990). This substance was previously shown to induce the activation of different transcription factors (*e.g.* NFAT and NFκβ) (Pahl and Baeuerle, 1995; Ferrari et al., 1999; Chen and Lin, 2001). We found again a transient, although slower and smaller calcium response when applying TG, which is in agreement with other studies (Thastrup et al., 1990; Nagano et al., 2005).

Glycine by itself did not cause any calcium response in our experimental set-up. This was never noticed in other immune cells either. Glycine did however enhance the calcium transients that were induced by both ATP (100 µM) and TG (500 nM). This enhancement was higher for TG (+37%), compared to ATP (+17%). This modulation was found to be GlyR-independent, based on the findings that the GlyR antagonist strychnine (1 µM) could not block the effects on the one hand, and that the GlyR partial agonists β-alanine or taurine could not significantly mimic the effects on the other hand. The involvement of GlyT1 was excluded as well because of the insensitivity of the effect to the GlyT1 antagonist ALX 5407. We found that this calcium modulation was rather dependent on SNATs, as it could be blocked by AIB. Our immunofluorescence experiments confirmed the activity of amino acid transporters. We showed that in our experimental setup there was substantial glycine uptake, which could be blocked by AIB, suggesting the involvement of SNATs. AIB blocks both sodium-dependent system A and sodium-independent system L transporters (Hyde et al., 2003). Leucine, which is a substrate for system L but not system A transporters did not change calcium responses. These findings point towards a role for sodium-dependent system A type SNATs. These transporters all transport glycine, glutamine, alanine and serine, all compounds that were shown to have similar effects on calcium transients. As we could also find agonistic effects with valine, which is only transported by SNAT1 (Hyde et al., 2003), it seems plausible that this SNAT1 transporter is responsible for the modulating effects we found in this study. These findings are similar to our electrophysiological findings described earlier, where we found an EC_{50} of 3.6 mM for glycine on these transporters, correlating with the millimolar concentration-response relation that we found for the glycine modulation of calcium signaling.

Although this study clearly shows a role for system A SNATs, the precise **mechanism** still remains unclear. If sodium-dependent SNATs were to be responsible for the effects, then are two main pathways which could explain the effects, a direct one via sodium transport and an indirect one via glycine uptake by itself. Sodium, which is co-transported, causes a depolarization, which may directly alter calcium fluxes. It was suggested by Schilling and Eder that the transporter-induced depolarization might have a signaling role (Schilling and Eder, 2004). This hypothesis is further supported by the fact that the modulation that we found seemed to be transporter specific (SNAT) but not amino acid specific (glycine, serine, alanine, glutamine as well as valine have similar effects). Also the correlation with (the opposite) findings on Kupffer cells is striking. In Kupffer cells a hyperpolarization, blunting of calcium signals and cytokine production was found while our findings in microglial cells, together with the previously mentioned studies of Yang and Schilling, now rather point to a depolarization and an enhancement of calcium signals and cytokine production. Previous studies in microglia already showed that high concentrations of potassium (see also paragraph 2.3.7), which are expected to depolarize cells, can enhance microglial cytokine production

(Chang et al., 2000). These findings point to a role of membrane potential changes in modulation of immune cell signaling. A role of a depolarization in calcium response modulation could be partially confirmed by our findings that a depolarizing hyperkalemic extracellular environment caused a small (+13%), but significant enhancement of intracellular calcium reponses. The next question then becomes: how does a change in membrane potential alters calcium fluxes? The role of VGCCs that was suggested for Kupffer cells does not seem to apply for microglial cells as the functional role of these channels in microglia is unclear (Moller, 2002; Farber and Kettenmann, 2006b) and the different inducers we used all seemed to have an effect mainly on intracellular calcium release. Furthermore acute application of KCl (30 mM) did not induce any calcium response in our setup (data not shown). Calcium release activated channels (CRAC) are shown to be important for calcium signaling in immune cells. The activation of these channels would cause a peak-plateau response instead of the transients responses we measured, and would change the responses in the absence of extracellular calcium, which was not the case. Also a depolarization would rather decrease the flow of calcium through these channels, both because of a decrease in the calcium electrochemical gradient and of a decrease in channel conductance (McLarnon, 2005). Sodium calcium exchangers (NCX) are important regulators for calcium homeostasis and one of the main systems responsible for calcium extrusion. A transporter-induced increase in intracellular sodium (and membrane potential) would decrease the activity or even reverse these exchangers, leading to decreased extrusion capacity and hence increased calcium concentrations upon stimulation, explaining our findings. This mechanism would also mean that not only the depolarization by itself, but also the change in intracellular sodium could contribute to the effect. This might explain the rather limited effect of hyperkalemic experimental conditions (+13%), compared to glycine. Interestingly Nagano and co-workers found a decrease in TG-induced calcium response in microglia when NCX was stimulated by nitric oxide, making this hypothesis plausible. This could also explain our findings that the effects of glycine were more pronounced when calcium transients were evoked by TG (137%) compared to ATP (117%), as in the latter case the SERCA can still compensate for a potential decreased activity of NCX and hence calcium extrusion. Another possible explanation for the lower glycine modulation of ATP-, compared to TG-induced calcium responses is the activation of P2X-activated sodium currents by 100 µM of ATP. This could partially mask the glycine effect, assuming that increases in intracellular sodium or membrane depolarizations are the primary mechanisms.

Another mechanism that could explain our findings are the indirect effects of glycine (or related amino acids) by itself, although this seems to be less likely as discussed earlier. An aspecific metabolic mechanism (*e.g.* protein synthesis) because of glycine being an essential amino acid may explain findings in long term cell culture experiments, but is unlikely in our set-up since we could detect changes in calcium responses after 15 minutes already. Another possibility is that glycine and structurally related amino acids bind to an unknown intracellular receptor leading to downstream effects on calcium signals, as has been demonstrated for leucine (Hundal and Taylor, 2009).

In conclusion this study shows for the first time that glycine enhances intracellular calcium transients, induced by different compounds, and suggests a role for neutral amino acid transporters, most likely of the system A type. We suggest a role for transporter-induced

membrane depolarization, which somehow enhances calcium responses and hence cytokine production, contrary to the findings in other macrophages or immune cells. Further research is necessary to further characterize these transporters, both functionally and at the molecular level, and to examine the precise role of the membrane potential in calcium flux regulation.

4. CONCLUSION

In this chapter we examined glycine effects on microglial cells, the macrophages of the brain. We provided molecular evidence for the expression of α- and β-subunit containing GlyRs, although our confocal images seemed to point to a predominant cytoplasmic location. These data are in agreement with electrophysiological studies of our group and others showing that microglial cells do not show GlyR-mediated currents upon glycine perfusion. We confirmed the reported enhancing role of glycine on LPS-induced NO production and extended this to TNFα. We next examined whether the GlyRs we found might be involved in the glycine modulating properties. Based on our pharmacological study using NO production as the primary parameter, this does not seem to be the case. We also excluded a possible role for NMDA receptors. As there is electrophysiological evidence for transporter-mediated depolarizing currents upon glycine perfusion and it was suggested that glycine might influence microglial activity via membrane depolarizations, we mimicked this depolarization by increasing potassium concentrations in our culture medium. This resulted in similar effects on NO production, making a role of depolarizations possible. Our next approach was to check whether glycine also modulated microglial calcium signaling, as has been reported in other macrophages. We showed that millimolar concentrations of glycine indeed enhanced calcium transients, and again this was independent on GlyRs. To further test the hypothesis that transporters were involved, we tested other amino acids and tried blocking the effects by a transporter substrate antagonist. These results clearly pointed to a role of neutral amino acid transporters. The activity of these transporters was confirmed by glycine immunostaining of microglial cells.

Figure III.20 brings our current research findings together and hypothesizes how they may be linked together. The microglial activator ATP or an unknown serum factor (but not LPS, see previous discussion in paragraph 3.4) stimulate calcium (indicated as blue dots) release from the endoplasmatic reticulum by means of metabotropic IP_3-release (the question mark indicates that there is some uncertainty concerning the “serum pathway”). The resulting increase in cytoplasmic calcium concentrations might result in the activation of transcription factors (*e.g.* NF-κβ, ERK1/2 and NFAT). These activated transcription factors subsequently increase the transcription of iNOS (leading to NO release) and cytokines such as TNFα. Glycine enhances this calcium release and hence downstream effects. Glycine receptors (indicated in green) are present and are mainly located in the cytoplasm and hence not active under our culture conditions. We hypothesize that they might traffic to the membrane in a certain, but currently unknown, physiological environment as discussed extensively in chapter I. In this context studies on brain slices may be interesting to perform. Glycine does however activate (system A) neutral amino acid transporters (SNATs), which seem to be crucial in calcium signal modulation. The most likely mechanism seems to be a transporter-mediated sodium influx and depolarization in which we hypothesize a role for sodium calcium exchangers (NCX). Future research is necessary to confirm this hypothesis. Note however that another possible role of SNATs may be the direct transport of glycine inside the cells. Although an aspecific metabolic origin for our findings does not seem to be very likely as discussed earlier, we cannot exclude that glycine and related amino acids such as L-serine

activate some yet unknown intracellular signaling pathway explaining our findings. One major criticism of such an hypothesis is that we do not mention a role for store operated channels (SOCs) in this figure. SOCs were shown to be the major source of calcium in immune cells, but do not seem to play a role in the BV-2 microglial model we used as discussed above. These data certainly need confirmation in primary cells were SOCs were reported to have an important contribution to calcium fluxes, leading to peak-plateau instead of transient responses. As the mechanism seems to be aspecific and downstream we hypothesize that also plateau responses may be increased.

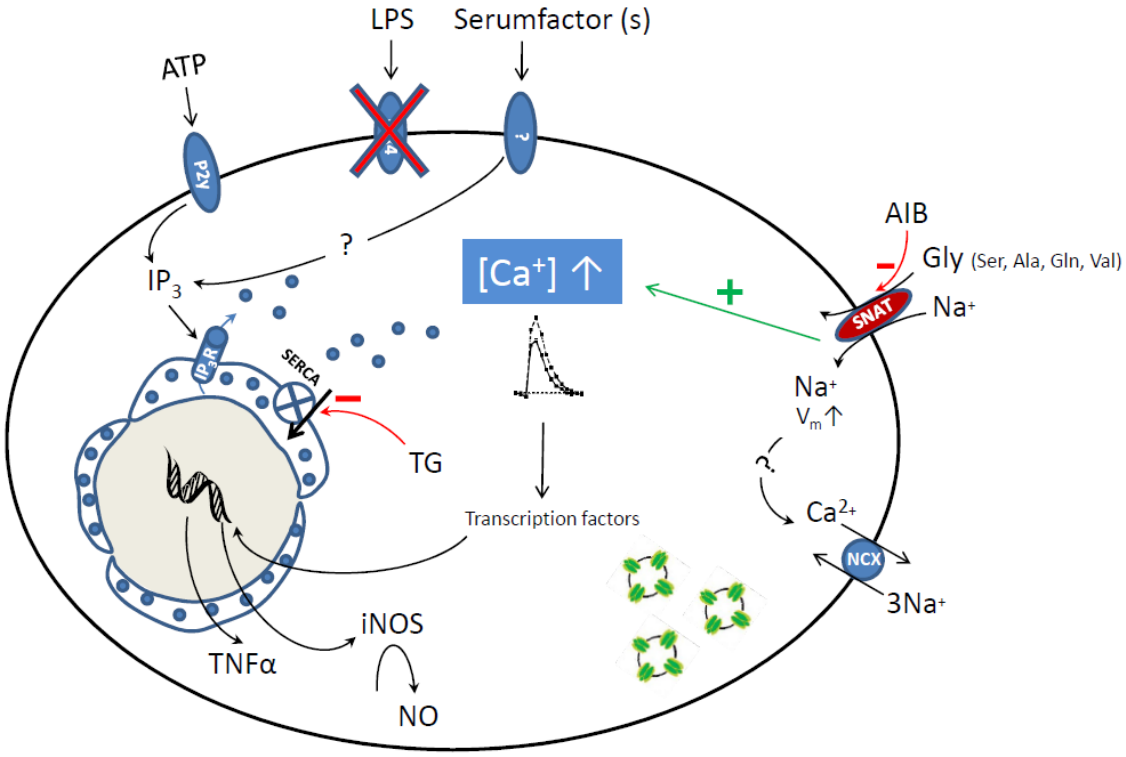

Figure III.20 Summary and hypothesis of current research findings on microglial cells
See text for explanation.

Table III.4 compares our current research findings of glycine modulation of microglial cells with the findings reported on peripheral macrophages (with Kupffer cells being the most studied cell type). In macrophages, glycine (or GlyR agonists such as β-alanine or taurine) activate GlyRs, leading to chloride influx and a membrane hyperpolarization. This hyperpolarization is suggested to counteract an LPS-induced depolarization and hence activation of VGCCs, leading to blunting of calcium fluxes and a decrease in the production of inflammatory mediators (*e.g.* NO, superoxide, TNFα). In microglia, glycine (and L-serine, but not GlyR agonists such as taurine) activates SNATs, leading to sodium influx and a membrane depolarization. This depolarization enhances calcium responses and increases the production of inflammatory mediators. Although it seems that the findings are cell type specific, one has to be cautious in this conclusion. Indeed, both cell types have the same mesodermal origin and the differences we find may result from the physiological environment (or cell culture conditions), rather than intrinsic cell specific properties. In this context it

seems plausible that GlyRs are expressed in macrophages making an hyperpolarized membrane predominate under glycine conditions. If however GlyRs are down-regulated (as is apparently the case in microglia), the smaller transporter-mediated depolarization (or other effect?) may predominate leading, to opposite effects. This is further investigated in the next chapter where we will repeat our experiments in peripheral macrophages to determine the specificity of our findings.

	Macrophages	Microglia
Stimulus	Glycine, taurine, β-alanine	Glycine, L-serine
Interacting protein	GlyR	SNAT
Membrane potential	Hyperpolarized	Depolarized
Calcium signals	Decreased	Increased
Inflammatory mediators	Decreased	Increased

Table III.4 Comparison of the reported glycine findings on peripheral macrophages with the glycine findings on microglia we report in this chapter.

Although the hypothesis postulated above is attractive and this sequence of events was suggested by all studies studying glycine effects in immune cells before, the truth is probably more complex than this. Indeed when correlating the modulating effects of glycine on calcium signaling with the effects on the production of inflammatory mediators, some remarks have to be made. In cell culture experiments NO (or TNFα) production was measured after microglial activation with LPS, while the modulatory effects on calcium transients were observed after (micromolar) ATP stimulation. Although previously authors suggested calcium transients to be evoked by LPS in the presence of rat serum, our data questionized these findings. This means that, although LPS clearly activates microglial cells, it does not induce calcium transients. A possible explanation may be that the positive modulation of calcium transients is not directly responsible for immune cell downstream effects on immune cell function, but rather represent a general effect on calcium signaling. In this way other calcium signals that may be more important for transcriptional activation, and that were not measured in our experiments, may be modulated as well. Indeed, it has been shown that LPS evokes a slow chronic elevation in microglial cells as well (Hoffmann et al., 2003). Although the techniques used in this work were not suitable to measure chronic changes in calcium levels, this point certainly deserves more future attention. Another striking discrepancy between cell culture experiments are the concentration ranges where the effects take place. Effects on NO production have EC_{50}-values lower than 100 µM, while the effects on calcium modulation take place in millimolar concentrations. Although these differences are hard to explain, one has to keep in mind that the setting of both types of experiments is completely different. For cell culture experiments, cells incubate for 2 or 3 days in an environment containing different amino acids and serum factors, which changes during the course of the experiments because of cellular production or breakdown of different mediators. In the calcium experiments glycine is incubated in a physiological buffer, for no longer than 15 minutes before measurement. One can speculate that the EC_{50} discrepancies may result from the different settings. Another way to explain the different discrepancies mentioned here is the fact that the modulation of inflammatory mediators on the one hand and calcium modulation on the other hand are based on an entirely different mechanism. The question than becomes again what the mechanism is behind the cell culture findings, as we excluded the most plausible pathways

(GlyR and NMDA receptors). One hypothesis that was not ruled out and certainly deserves further attention is the possibility of an unknown receptor. As no currents could be measured in micromolar concentrations this should be a metabotropic receptor (GPCR). The main agonists should be glycine and L-serine, with lower Km-values for the latter. It is remarkable in this context that for most neurotransmitters known (*e.g.* glutamate, GABA, acetylcholine, serotonine, ATP) both ionotropic as metabotropic receptors have been described, while this is so far not the case for glycine. This issue certainly deserves further attention in the future.

CHAPTER IV.GLYCINE SIGNALING IN MACROPHAGES

1. INTRODUCTION

In the previous chapter, we showed that glycine enhanced LPS-induced cytokine production and microglial calcium signaling by a GlyR-independent mechanism. We suggested a role for neutral amino acid transporters. As these findings are in fact exactly the opposite of the findings described in peripheral macrophages so far, the main aim of this chapter is to investigate whether these properties are microglia-specific or rather more general macrophage-specific and, hence, more dependent on the extracellular environment.
We used a murine macrophage cell line (RAW 264.7) to answer these questions and cultured them in exactly the same conditions as we did for microglial cells. This cell line is established from the ascites of a tumour, induced by intraperitoneal injection of Abelson Leukaemia Virus (Raschke et al., 1978). It is now widely used as a model to study signal transduction pathways in macrophages.
In the next section we show that under the same culture conditions as we performed our microglial experiments, glycine was again able to enhance calcium signaling, although less pronounced than in microglia. Remarkably these findings correlated to a smaller contribution of neutral amino acid transporters in total glycine uptake, compared to microglial cells. In section 3 we show that, similar to microglial cells, glycine enhanced metabolic activity/viability. We show that viability rather than metabolic activity by itself is altered. Moreover we suggest an NMDA-independent cytotoxic role for the gliotransmitter D-serine. An unexpected finding was the property of glycine to induce a bipolar morphology in RAW 264.7 cells. In section 4 we summarize our findings in RAW 264.7 macrophages.

2. GLYCINE ENHANCES CALCIUM SIGNALING IN RAW 264.7 MACROPHAGES.

2.1 Introduction

In macrophages ATP has been known for a longtime to be an inducer of intracellular calcium release (Greenberg et al., 1988). In blood and alveolar macrophages, different subtypes of metabotropic P2Y and ionotropic P2X were demonstrated and ATP (micromolar concentrations) was shown to induce calcium transients, mainly attributed to P2Y activation and hence intracellular release (Hanley et al., 2004; Myrtek et al., 2008). Furthermore it has been shown that gene expression of iNOS and TNFα, induced by transcription factors such as NF-κβ and ERK1/2, is critically dependent upon intracellular calcium release in macrophages (Kim et al., 2004; Martin et al., 2006). This means that any substance that alters this calcium release may result in altered transcription and release of inflammatory mediators.
One of the striking differences between our findings on microglial cells and findings on peripheral macrophages are the modulating effects of glycine on calcium signals. While in peripheral macrophages a clear GlyR-dependent inhibition or even blunting of calcium signals was found, in microglia we found a GlyR-independent enhancement. This section investigates whether the modulation of calcium signaling that was reported for microglial cells in the previous chapter also applies for peripheral macrophages.

2.2 Materials and Methods

2.2.1 Cell cultures

The mouse macrophage cell line RAW 264.7 was used in all experiments. Cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) containing 2 mM L-glutamine, 10% heat-inactivated Fetal Calf Serum (FCS; Invitrogen) and 100 U/ml Penicillin and 100 µg/ml Streptomycin (1% P/S; Invitrogen). Twenty four hours before experiments cells were transferred to 96-well plates or glass cover slips and put on (glycine-free) Minimum Essential Medium (MEM; Invitrogen) supplemented with 10% FCS, 2mM glutamine and 1% P/S (simply called MEM-medium in the remainder of the article).

2.2.2 Chemicals

See paragraph 3.2.2.

2.2.3 Calcium measurements

See paragraph 3.2.3

2.2.4 Immunolabelling and confocal microscopy

See paragraph 3.2.4

2.2.5 Statistics

See paragraph 3.2.5

2.3 Results

2.3.1 Glycine enhances ATP- and TG-induced calcium responses at millimolar concentrations

Glycine by itself did not induce any calcium response (data not shown). Compared to the calcium responses we obtained in BV-2 microglial cells, the application of 100 µM ATP induced a more sustained but still transient increase in intracellular calcium concentrations (Figure IV.1A). Similar to our findings in BV-2 cells, this response was completely blocked by pre-incubation of cells with TG (500 nM) and not altered in a calcium-free extracellular environment (data not shown).

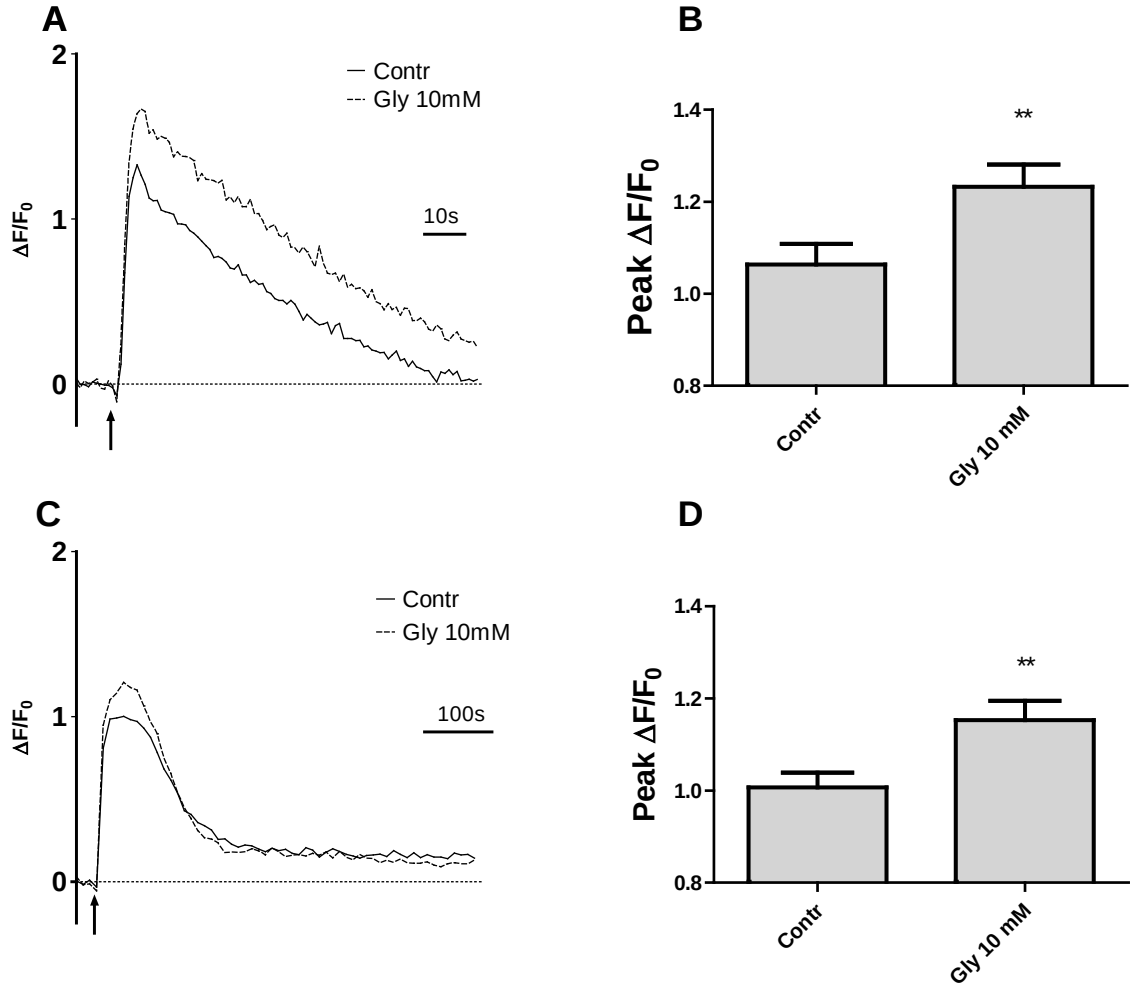

Figure IV.1 Glycine effects on ATP- and TG-induced calcium responses in RAW 264.7 macrophages
Cells were pre-incubated for 15 minutes in glycine (10 mM) or (glycine-free) control conditions. A and C. Examples of individual traces showing the calcium response ($\Delta F/F_0$) upon application of ATP 100 µM (A) or TG 500 nM (C) (arrow) in control (solid line) or 10 mM glycine (dashed line) conditions. B and D. Mean modulation of peak calcium response (Peak $\Delta F/F_0$), induced by 100 µM ATP (B) or 500 nM TG (D). 10 mM glycine significantly enhanced peak calcium responses after ATP-induction or after TG-induction. Results are expressed as means ± S.E.M. of 21 (TG) or 48 (ATP) experiments.

When cells were incubated for 15 minutes in millimolar glycine conditions, an enhancement of ATP-induced calcium transients could be found (+18%), with mean peak calcium responses shifting from 1.06 ± 0.05 in control conditions to 1.23 ± 0.05 in 10 mM glycine conditions (p=0.01; n=48) (Figure IV.1B). This enhancement was not altered by addition of 1 µM strychnine (data not shown). Also TG (500 nM) induced calcium responses that were significantly enhanced by 10 mM glycine (Figure IV.1C). Mean peak calcium responses were 1.01 ± 0.03 in control conditions and shifted to 1.15 ± 0.04 in 10 mM glycine conditions (p=0.007; n=21) (Figure IV.1D), correlating to a mean enhancement of 15%. AIB (10 mM) application decreased this enhancement from 15% to 8%, although this was not found to be significantly different from glycine conditions (p=0.23; n=12).
Similar to our findings in BV-2 cells we also found an induction of a transient calcium response when LPS (10 µg/ml), together with 5% rat serum, was applied. Although this response could be enhanced by glycine 10 mM (+22 ± 6.3 %; data not shown), it was entirely secondary to the application of 5% rat serum, as can be seen in Figure IV.2.

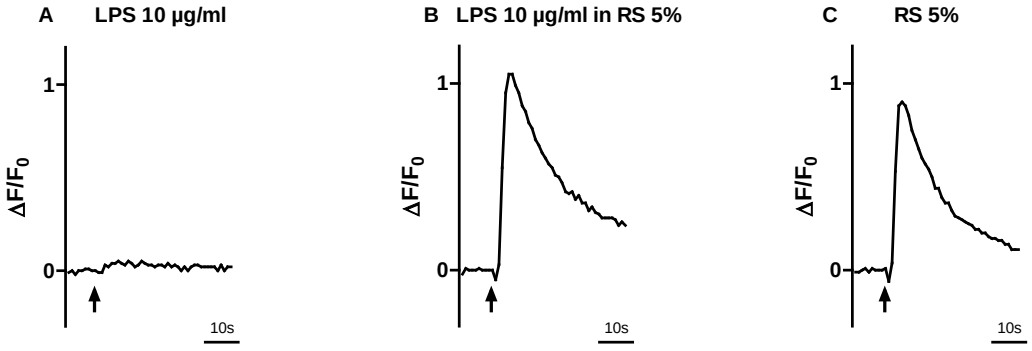

Figure IV.2 Rat serum induces calcium transients in RAW 264.7 macrophages, independently of LPS. Representative examples of individual traces are shown. $\Delta F/F_0$ corresponds to changes in intracellular calcium concentrations. The arrow below each graph indicates the time point of application of different inducers. A. Application of LPS 10 µg/ml by itself did not induce any calcium response. B. When LPS 10 µg/ml was applied in the presence of 5% rat serum (RS), a fast transient calcium response was evoked. C. When rat serum was applied without LPS the same response as shown in B could be evoked.

2.3.2 Calcium response modulation correlates with extensive glycine uptake in RAW 264.7 cells.

We next examined whether we could correlate these findings again with glycine uptake as was demonstrated in microglial cells. When RAW 264.7 cells were incubated for 30 minutes in 10 mM glycine conditions and immediately fixed and immunostained for glycine, an extensive intracellular cytoplasmic labelling could indeed again be found (957±46%, Figure IV.3A). This effect was partially, but not significantly blocked by AIB (842±38%, indicating a 13% block), as can be seen in the quantification of glycine uptake in Figure IV.3B.

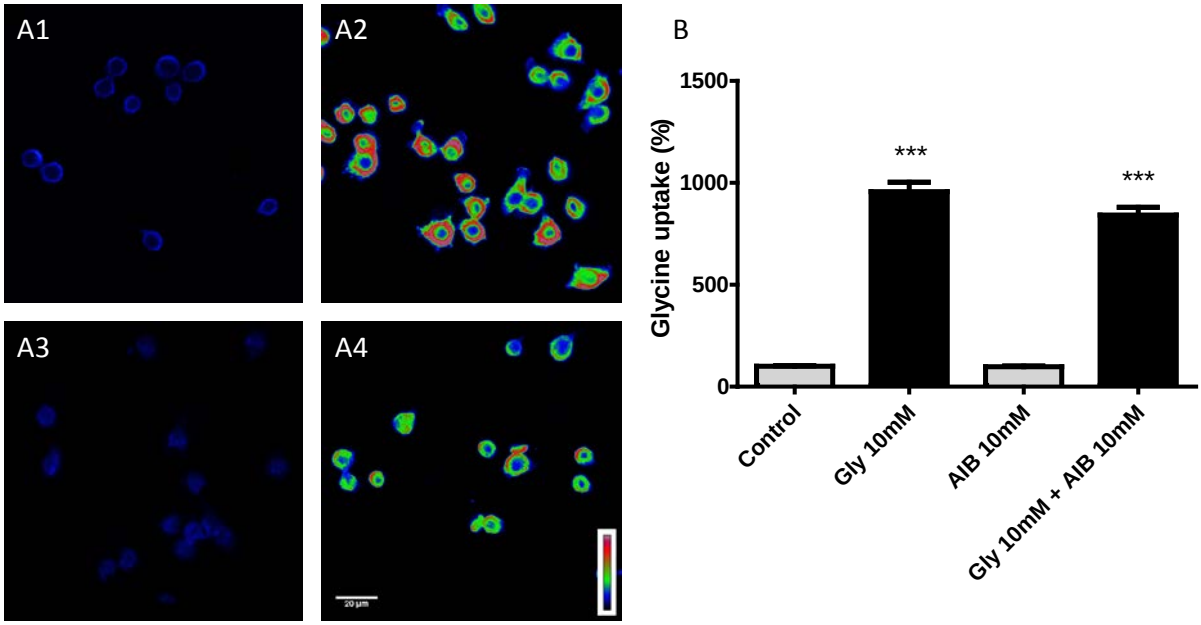

Figure IV.3 Glycine immunofluorescence images showing glycine uptake by RAW 264.7 cells.
RAW 264.7 cells were incubated for 30 minutes in control (A1), 10 mM glycine (A2), 10 mM AIB (A3) or 10 mM glycine and 10 mM AIB (A4) conditions and labelled for glycine. Scale bar and colour scale are indicated on the lower right. Figure B shows the results of the glycine uptake quantification (42-56 cells analyzed from 3 independent experiments). After incubation in 10 mM glycine an extensive cytoplasmic glycine fluorescence can be observed (957±46% of control conditions). This uptake was partially but not significantly blocked by AIB (842 ±38%; p=0.06 when compared to gly conditions).

2.4 Discussion

The main question that was asked in this section was whether glycine enhancing properties on calcium responses were microglia-specific or could be a more general property of macrophages. We showed that under the same experimental conditions, glycine again enhances, rather than inhibits calcium responses, indicating that the property is not microglia-specific. Although the extent of the enhancement of the ATP-induced response was similar to the findings in BV-2 microglial cells, the enhancement of TG-induced responses was only +15%, compared to +38% in microglial cells.

When the patterns of the calcium responses of RAW 264.7 cells were compared with the patterns in BV-2 microglial cells, more sustained (though still transient) responses were noticed, indicating that the different players in the cellular calcium machinery may somewhat differ. We however demonstrate that glycine enhanced the calcium response, so this seems to be independent on the precise pattern of the calcium response. The fact that glycine modulates the calcium response independently on the precise machinery involved (a conclusion that could also be made in microglial cells because of the relative independency of the effect on the precise calcium inducer) clearly points to some general mechanism that is modulated by macrophages. Two differences in glycine modulation of calcium responses between microglial cells and macrophages were noticed: the extent of the glycine effect on TG-induced calcium responses and the AIB-insensitivity. Our uptake experiments demonstrated extensive glycine uptake in our experimental conditions, although there was only a limited block by AIB (13% block, compared to 67% in microglial cells), indicating that other

transporters, next to AIB-sensitive system A neutral amino acid transporters (SNATs), are responsible for glycine uptake in macrophages compared to microglia.
Taken together these results partially confirm our hypothesis that glycine enhancing properties are not microglia specific, but rather represent a more general macrophage property. Under the hypothesis that AIB-sensitive SNATs are necessary for glycine-induced calcium modulation in macrophages, the differences we found between mciroglia and peripheral macrophages might be caused by differences in transporter expression, although molecular studies need to confirm this hypothesis.

3. GLYCINE AND D-SERINE DIFFERENTLY ALTER VIABILITY AND MORHOLOGY IN RAW 264.7 MACROPHAGES

3.1 Introduction

In chapter III we found an enhancement of metabolic activity/viability when culturing microglial cells in micromolar glycine or L-serine concentrations in serum-free conditions. In this section we will examine whether these effects can also be observed in RAW 264.7 macrophages, or are rather microglia-specific. Furthermore we will determine whether the effects we measured by our metabolic activity assay (which measures the activity of mitochondrial dehydrogeneases as dicussed earlier) are due to an enhancement of cell viability or a change in cellular mitochondrial dehydrogenase activity by itself. Since the effects were only present in serum-free culture conditions, we will also focus on the influence of serum and changes in the amino acid concentrations.

3.2 Materials and methods

3.2.1 Cell culture

RAW 264.7 were cultured as described in paragraph 2.2.1. For cell culture experiments, cells were put on (glycine-free) Minimum Essential Medium (MEM; Invitrogen), supplemented with 1 mg/ml BSA, 2 mM glutamine and 1% P/S (simply called “serum-free MEM-medium” in the remainder of the section).

3.2.2 Chemicals

Glycine, L-serine, D-serine, α-amino isobutyric acid (AIB), L-arginine, staurosporine (STS), APV, MK-801, Picrotoxinine (PXN), reduced glutathione (GSH), digitonine, propidium iodide (PI) and strychnine were all purchased from Sigma Aldrich (Bornem, Belgium). L-alanine was purchased from Acros Organics (Geel, Belgium).

3.2.3 Metabolic activity/Cell viability assay

Cells were cultured in 96-well plates as described in *cell culture.* After 24 hours of incubation (37 °C, 5% CO_2) metabolic activity/cell viability was assayed as described in paragraph 2.2.8.

3.2.4 Propidium Iodide viability assay

To differentiate between changes in metabolic activity and changes in cell viability we used a fluorescence plate reader-based viability assay after propidium iodide (PI) staining of cells, as described by (Sarafian et al., 2002). This assay is based on the fact that PI becomes fluorescent after binding to DNA, which can only occur when (death) cells have lost their membrane integriy and allow PI to penetrate the cell membrane. The assay first determines the number of death cells by measuring the fluorescence (F) of the PI-uptake. Then the fluorescence of the total cell population is determined (F_{max}), by permeabilizing cells using

digitonine, leading to PI uptake of all cells in the population. The ratio F/F_{max} then correlates to the fraction of death cells in the population. Cells were cultured in 96-well plates as described in *cell culture.* Every experiment was performed in triplicate wells. After 24 hours of incubation (37 °C, 5% CO_2), 5 µl of PI (final PI concentration = 1.25 µg/ml) was added to cells bathing in 200 µl of culture medium or cell-free culture medium for background signal (F_{bgr}) determination. After 15 minutes of incubation in the dark fluorescence was determined (=F, a measurement for the total number of necrotic cells) using a Fluostar optima plate reader with excitation set at 540 nm and emission set at 612 nm. Then 5 µl of digitonine (final concentration = 250 µM) was added to permeabilize cells and after 20 minutes of incubation in the dark fluorescence was determined again (=F_{max}, a measurement for the total number of cells). The percentage of necrotic cells was then determined by the following formula:

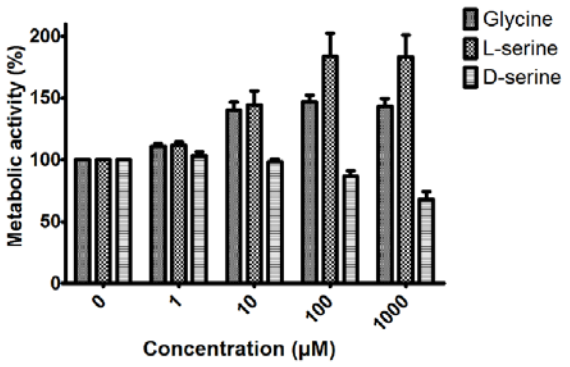

Figure IV.4 Opposing effects of Glycine and L-serine versus D-serine on RAW 264.7 macrophage metabolic activity.
After 24 h incubation, glycine and L-serine enhance metabolic activity in low micromolar concentrations. Maximal enhancement of L-serine (184±19%) was higher than glycine (147±6%). D-serine did not mimic glycine or L-serine effects and caused a decrease in activity in high micromolar concentrations, with significant decreases found at 100 µM and higher. Data are expressed as mean ± SEM from 10 – 21 independent experiments, each performed in triplicate.

$$Necrosis\ (\%) = \frac{F - F_{bgr}}{F_{\max} - F_{bgr}}$$

3.2.5 Statistics

Data are expressed as means ± S.E.M. Statistical significance was evaluated using ANOVA or student t-testing when comparing only 2 sets of data. P-values of less than 0.05 were considered significant.

3.3 Results

3.3.1 Glycine and L-serine have opposing effects on metabolic activity compared to D-serine.

RAW 264.7 macrophages were cultured for 24 hours in the presence of different concentrations of glycine as indicated in materials and methods. Glycine caused a significant increase in metabolic activity as can be seen in Figure IV.4 This enhancement, with maximal values at 147±6%, was significant at concentrations of 1 µM and higher. When the same experiments were performed in the presence of different concentrations of L-serine, the same effects could be observed, but with maximal values increasing to 184±19% (Figure IV.4).
D-serine could not mimic the effect of glycine and L-serine, and rather caused a dose-dependent decrease in metabolic activity at concentrations of 100 µM and higher (Figure

IV.4), with metabolic activity decreasing to 87(±4)%, 68(±6)% and 50(±3)% at 100, 1000 and 10000 µM respectively.

3.3.2 Glycine increases viability in RAW 264.7 macrophages.

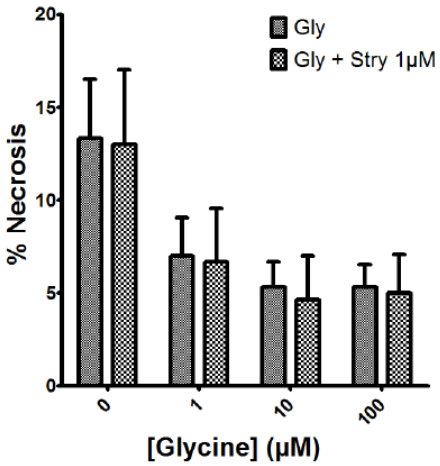

Figure IV.5 Glycine reduces necrosis in low micromolar concentrations in RAW 264.7 macrophages.
After 24 hours of incubation in different glycine concentrations, a significant decrease of necrosis could be found at concentrations of 1 µM and higher. Addition of strychnine (1 µM) did not block the effect of glycine. Data are means ± S.E.M. of three different experiments , each performed in triplicate

To differentiate whether the previous findings were due to true changes in viability or just to changes in metabolic activity, we performed a PI viability assay as described in materials and methods. As can be seen in Figure IV.5 the effects of glycine can at least partially be explained by an effect on macrophage viability. Indeed we found a significant decrease of total cellular necrosis when glycine was added in concentrations of 1 µM and higher. To test for involvement of GlyRs we tried to block the effects with strychnine (1 µM). This did not change the effects of glycine at all.

3.3.3 Glycine and L-serine induce a bipolar morphology in RAW 264.7 macrophages.

While we were actually studying viability changes in RAW 264.7 cells, we observed changes in the morphological phenotype, depending on the amino acid tested, a case of serendipity. When cells were cultured under serum-free conditions in the presence of glycine and to a lesser extent also of L-serine, a small percentage of cells lost their normal round appearance and showed a more bipolar morphology (Figure IV.6 A & B). These changes were only seen with L-serine or glycine and not with any other amino acids. We quantified the number of bipolar cells by a binary approach in which cells were determined to be either round or not round. We tried pairing the experiments as much as possible when comparing different conditions (*e.g.* glycine vs. AIB, serum dose responses). Figure IV.6 shows that the effect is mainly taking place at millimolar concentrations and glycine is more potent at 1 mM or higher than L-serine (being significant only at 10 mM). D-serine on the other hand did not cause any morphological effect (apart from the fall in viability in higher concentrations that is discussed earlier in this chapter). We then tested whether these changes were also (neutral amino acid) transporter-dependent by adding AIB to cell cultures. This caused a small decrease in the number of bipolar cells at millimolar concentrations, although this was only significant at 1 mM ($p=0.04$) and not at 10 mM ($p=0.12$). This bipolar phenotype was not a necrotic or apoptotic phenotype (as examined by staining of apoptotic cells with PI and Annexine V, data not shown) and no differences could be observed with MHCII immunostaining between round and bipolar cells (data not shown).

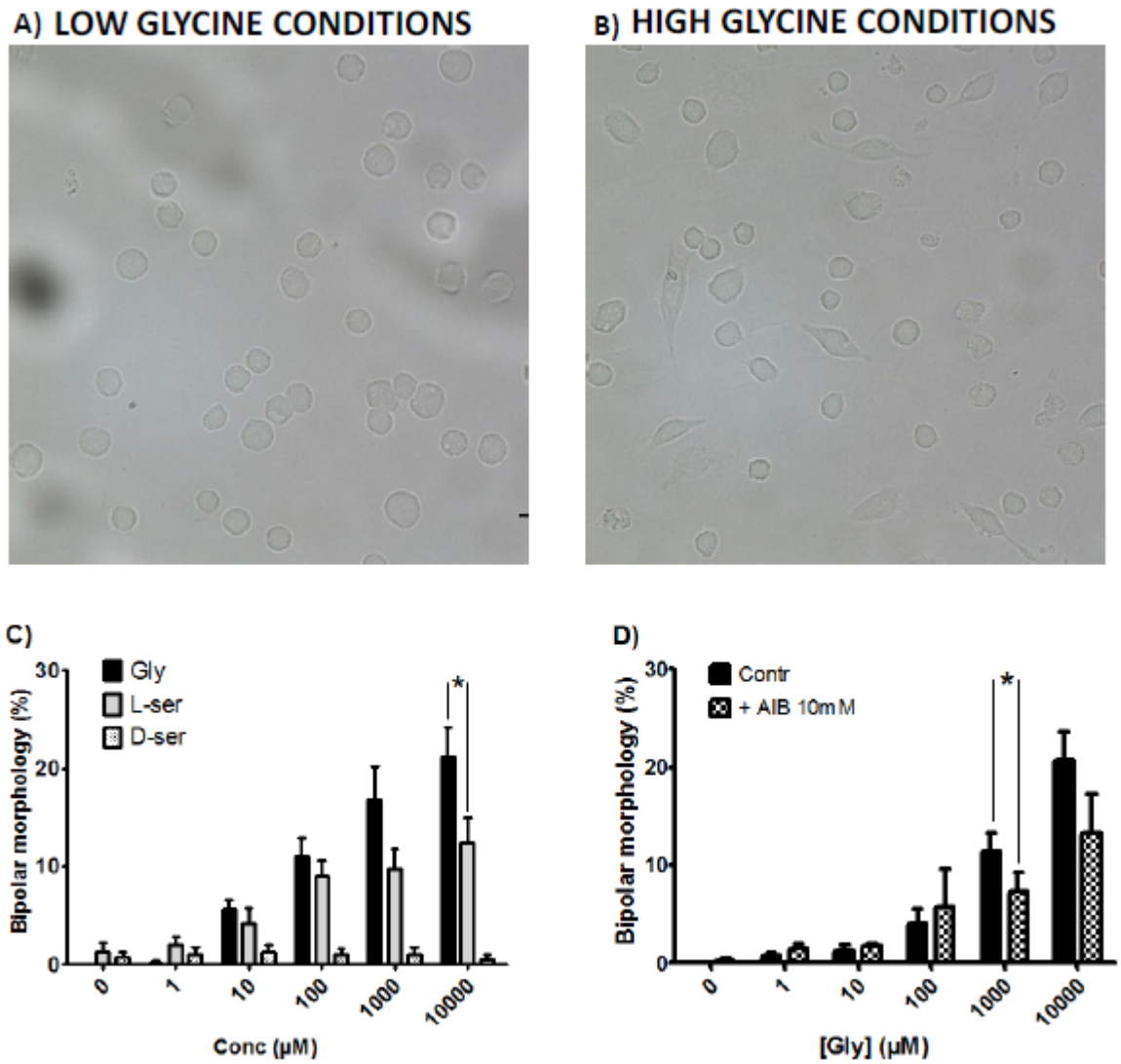

Figure IV.6 Glycine induces a bipolar morphology in RAW 264.7 macrophages.
Cells were cultured for 24 hours in serum-free cell culture condtions. A and B. Light microscopical images of cells incubated in low glycine (10 µM) or high glycine concentrations (10 mM). When cells were incubated at low glycine conditions (A) all cells showed a round morphological phenotype. When cells were incubated at high glycine conditions part of the cells displayed a more bipolar phenotype. The number of bipolar cells was quantified (C and D). C. Glycine and to lesser extent also L-serine induced a dose-dependent bipolar morphology at concentrations of 100 µM and higher ($p<0.001$). Glycine effects were significantly higher than L-serine effects at 10 mM ($p<0.05$). D. AIB reduced the effects of glycine on the number of bipolar cells in millimolar glycine concentrations, although this was only significant at 1 mM ($p<0.05$). Results are expressed as means ± S.E.M. of 4 to 6 independent experiments.

3.3.4 Glycine and L-serine effects are serum dependent and can be influenced by other amino acids.

In chapter III we mentioned similar effects on microglial metabolic activity, which could only be found in the absence of FCS. This was the underlying reason to perform the experiments in this chapter in serum-free cell culture conditions. To confirm this serum-dependency in RAW 264.7 macrophages and further examine this effect, we performed dose response measurements of different serum concentrations on the macrophage metabolic activity and studied the influences of both glycine and L-serine on this relationship. A clear and significant rise in metabolic activity can be observed in control conditions at 1% serum and

higher (Figure IV.7A). After adding glycine and L-serine to the culture medium the previously described enhancement on metabolic activity in serum-free conditions could be observed. This effect tends to decrease with higher serum concentrations and completely disappears at 3% FCS. At higher serum concentrations there even seems to be a tendency for the effect to reverse (Figure IV.7A).

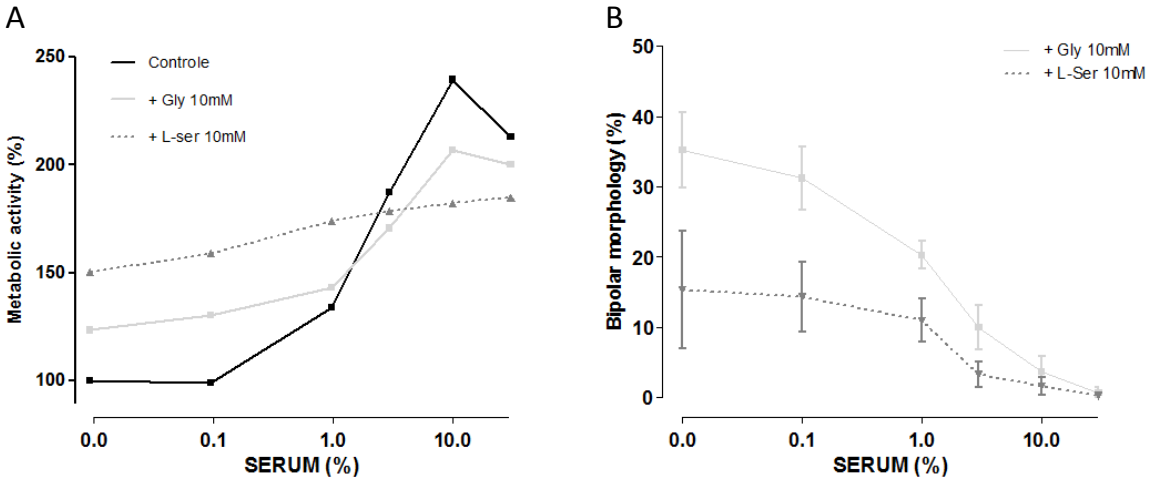

Figure IV.7 Serum dependency of glycine and L-serine effects on RAW 264.7 macrophages.
Cells were cultured for 24 hours in different concentrations of FCS and metabolic activity (A) or the number of cells with bipolar morphology (B) were measured. A. At 1% FCS and higher a significant rise in metabolic activity can be observed under control conditions. Glycine and L-serine enhanced metabolic activity in serum-free conditions. This effect decreased at increasing serum concentrations, disappeared at 3% FCS and had a tendency to reverse at 10% FCS. Data are expressed as means of 3–5 independent experiments, each performed in triplicate. For clarity error bars are not indicated on the figure. B. In serum-free culture conditions and at 0.1% FCS both glycine (solid black line) and L-serine (dashed gray line) induced a bipolar morphology. At 1% FCS and higher, glycine effects were significantly reduced compared to 0% FCS control conditions ($p<0.01$ at 1% and $p<0.001$ at higher serum concentrations). L-serine effects were significantly reduced compared to (FCS-free) control conditions at 10% FCS and higher ($p<0.05$). Data are expressed as means ± S.E.M. of 3 or 4 different experiments (for glycine and L-serine respectively).

As glycine (and L-serine) effects seemed to depend on serum concentrations, we next examined whether other amino acids might influence these effects (Figure IV.8). L-alanine (10 mM) or AIB (10 mM) had no effect on glycine nor L-serine dose responses. Note that AIB caused a small decrease in metabolic activity (suggesting a small decrease in viability) under control conditions, but the pattern of the dose response of glycine/L-serine did not change. In the presence of L-serine (10 mM) glycine had no additive effect on metabolic activity anymore. In the presence of cationic amino acids arginine (10 mM) and to a smaller extent also lysine (10 mM) both glycine and L-serine effects were completely blocked. D-serine effects are discussed in paragraphs 3.3.5-3.3.7.

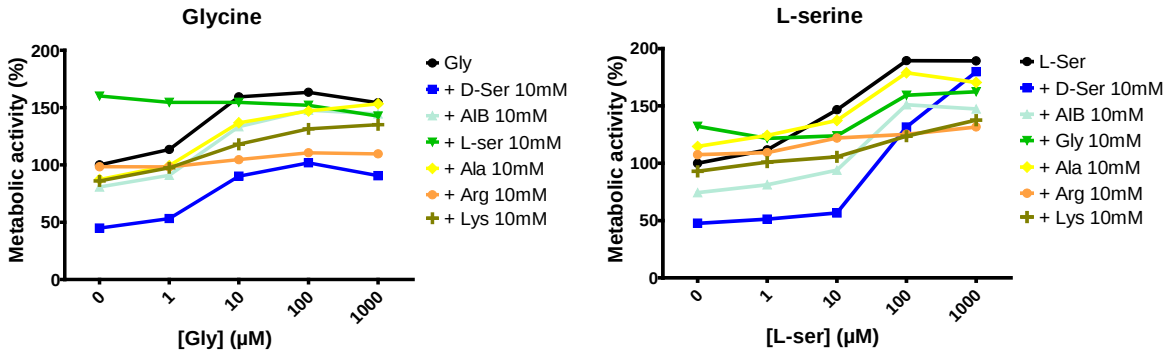

Figure IV.8 Interaction of different amino acids with glycine and L-serine effects on RAW 264.7 macrophage metabolic activity.
Cells were cultured for 24 hours in different concentrations of glycine (left figure) or L-serine (right figure) in the presence of different amino acids. See text for explanation. Data are expressed as means of 3-19 independent experiments, each performed in triplicate. For clarity error bars are not indicated on the figure.

The dependency of the effects of glycine and L-serine on serumconcentrations was also shown for the previously described morphological changes. As shown in Figure IV.7B glycine and to a lesser extent also L-serine again induced a bipolar phenotype in serum-free conditions. However with increasing concentrations of FCS the effects tend to decrease, with almost no changes seen anymore at 10% FCS. For glycine the decrease was significant at 1% and higher compared to 0% FCS conditions ($p<0.01$). For L-serine significant changes (compared to FCS-free conditions) could only be found at 10% FCS, although variability was quite high.

3.3.5 D-serine reduces viability in RAW 264.7 macrophages.

When analyzing stereospecificity of L-serine effects we observed a reducing effect of D-serine on macrophage metabolic activity in high micromolar to millimolar concentrations (Figure IV.9). We correlated these findings again with a PI viability assay. As can be seen in Figure IV.9 the drop in metabolic activity correlates with an increase in necrosis, which becomes significant at 1 mM D-serine, suggesting that the D-serine effects were explained by changes in cell viability.

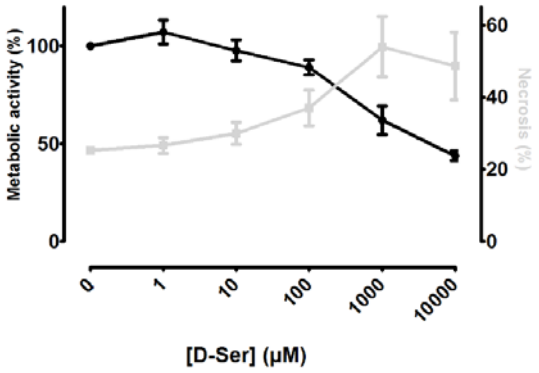

Figure IV.9 D-serine is cytotoxic to RAW 264.7 macrophages.
After 24 hours of incubation in different D-serine concentrations, a significant decrease of metabolic activity could be found at concentrations of 100 µM and higher (left ordinate). This correlated to an increase in total necrosis (right ordinate), which became significantly different from control at 1 mM. Data are expressed as mean ± S.E.M. from 3-6 independent experiments, each performed in triplicate.

3.3.6 D-serine effects are independent on NMDA receptors

Because D-serine is known as a gliotransmitter, acting on NMDA-receptors, leading to neurotoxicity in different neuropathological conditions (see discussion), we next examined the potential

involvement of NMDA receptors. The fact that glycine does not mimic these D-serine effects already argued against NMDA-R involvement. NMDA-Rs can only be involved if one assumes some glutamate secretion in the culture medium, which already acts at NMDA-Rs in control conditions. As can be seen in Figure IV.10A, the NMDA-R blockers MK-801 and APV did not change control responses, strongly arguing against the activity of these receptors. Also the D-serine effects were not altered in the presence of either blockers.

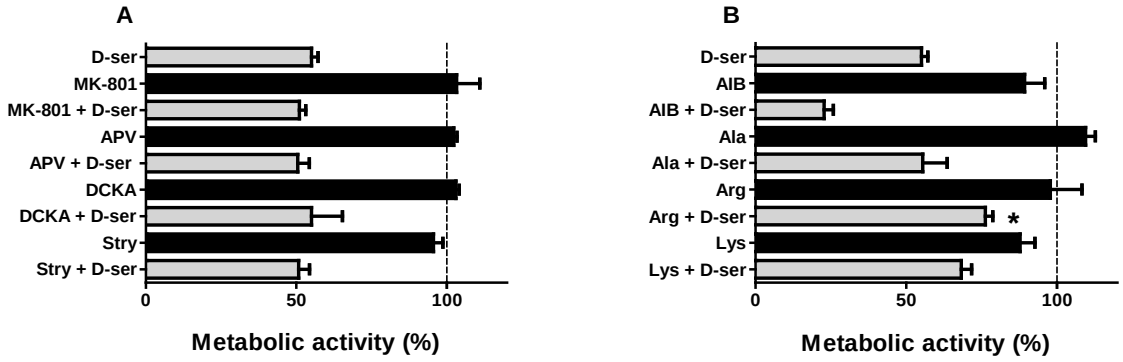

Figure IV.10 D-serine cytotoxic effects on RAW 264.7 macrophages are independent of NMDA receptors but dependent on cationic amino acids.
Cells were incubated for 24 hours in the presence of D-ser (10 mM) together with different NMDAR-blockers (A) or amino acids (B). A. None of the NMDAR blockers tested was able to block D-serine effects, nor change control responses. The following concentrations were used: 10 µM MK-801, 10 µM APV, 3 µM Stry (the latter is not a NMDAR-blocker). B. The basic amino acids arginine and lysine showed a trend towards a reduction of the D-serine effects, although this was only significant for arginine (indicated with the asteriks), as analysed by ANOVA. Alanine or AIB did not reverse D-serine effects. All amino acids were added in equimolar concentrations (10 mM). The (D-serine free) control is indicated with the dashed line. Data are expressed as means ± S.E.M. of at least 4 independent experiments, each performed in triplicate.

3.3.7 D-serine effects are completely blocked by L-serine and partially by glutathione.

As can be seen in Figure IV.8 the reduction of metabolic activity in the presence of 10 mM of D-serine can be completely blocked by 1 mM of L-serine, but not by glycine. This is confirmed by Figure IV.11 were the dose response relationship of D-serine on metabolic activity was measured in the presence of glycine and L-serine. While glycine did not cause any change in the relationship, addition of L-serine (10 mM) to the culture medium completely inhibited the dose-response relationship. Remarkably, in the presence of L-serine there even seemed to be a tendency towards a reversal of the D-serine dose response relationship with D-serine becoming stimulating on metabolic activity.We tested some other amino acids as well (Figure IV.10B). Neither the transporter blocker AIB, nor the SNAT-substrate alanine blocked D-serine effects, but in the case of AIB rather seemed to potentiate it ($p<0.001$), although some toxicity could be observed in control conditions as well (not significant). The cationic amino acids arginine and lysine were found to partially inhibit the serine cytotoxic effect, although this was only significant for arginine ($p<0.05$). Because D-serine metabolism leads to hydrogen peroxide formation and possibly oxidative stress, we next investigated the effects of the anti-oxidant glutathione on D-serine effects. Glutathione

could significantly ($p<0.01$) block D-serine effects though the effect seemed to decrease at 10 mM D-serine.

3.4 Discussion

In this part, we studied the effects of glycine, L-serine and D-serine on macrophage viability. We screened for changes in viability by means of a metabolic activity assay and correlated the findings with a PI viability assay. The main findings were that glycine and L-serine both enhanced viability at low micromolar concentrations, while D-serine decreased viability at high micromolar to low millimolar concentrations. Apart from these viability changes we also showed that glycine, and to a smaller extent also L-serine, induced a bipolar differentiation in cultured cells.

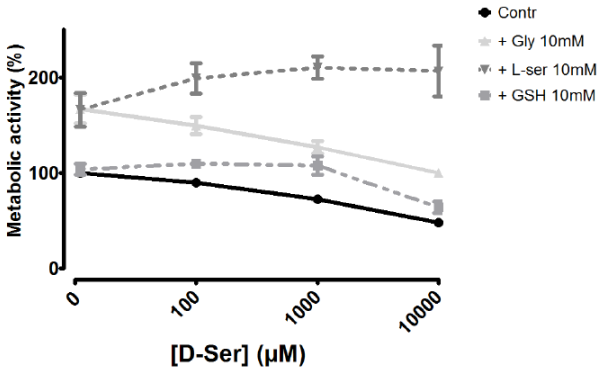

Figure IV.11 L-serine and glutathione block D-serine cytotoxicity on RAW 264.7 macrophages.
Cells were incubated for 24 hours in the presence of increasing concentrations of D-serine, leading to a reduction of metabolic activity. Glycine increased metabolic activity in control conditions (as reported earlier), but did not change the D-serine response. L-serine also increased the activity in control conditions but also blocked the dose response relationship of D-serine completely and this relationship even showed a tendency towards an inversement. Also glutathion (GSH) caused a signifcant block of D-serine effects in all concentrations tested, although this effect tended to decrease at 10mM. Data are expressed as means ± S.E.M. of 3-10 independent experiments, each performed in triplicate.

In chapter III we already described changes in metabolic activity/viability when cells were cultured in serum-free MEM-medium, without exploring this issue any further. In this part, we extended these findings to peripheral macrophages by using RAW 264.7 cells as a macrophage model, further suggesting that these effects are dependent on the extracellular environment and are not intrinsic cellular properties. Similar effects under serum-free culture conditions and micromolar concentrations were previously reported for both neurons and microglia (Tanaka et al., 1998; Yang et al., 2000). Although for neurons it was shown that these changes related to viability, for microglia it was believed these changes were due to an increase in general metabolic activity rather than viability. Here we used a fluorescent platereader-based assay after labeling necrotic cells with propidium iodide (PI). This allows a reliable and fast measurement of large cell populations and has been successfully used by others as well (Trost and Lemasters, 1994; Rudolph et al., 1997; Sarafian et al., 2002). Using this assay, we could clearly demonstrate that the increase in metabolic activity correlated to a decrease in total necrosis, suggesting that at least part of the effects could be explained by changes in viability and not just in metabolic activity.

One remarkable finding about the PI assays is the rather high percentage of necrotic cells we found in control conditions (15-20%). Such values (up to 35%) were also reported by the group of Tanaka when culturing microglial cells in similar culture conditions (Tanaka et al., 1998). These high values can be explained by the culturing of cells in serum-free cell culture conditions in MEM medium, which is already scarse in nutrients. We assume this leads to a form of starvation stress ultimately leading to cell death. Apparently, this stress is necessary for glycine (and L-serine) effects to be unmasked. Indeed the effects were strongly dependent

on the amount of serum added to the cultures and were only present in concentrations lower than 3%. This serum-dependency was also noted in microglial cells where changes in metabolic activity could only be detected in serum-free and not serum containing MEM medium. Staurosporine-induced apoptosis of RAW 264.7 cells was also investigated in glycine conditions, but the presence of glycine did not provide any protection (data not shown).

The effects of L-serine were more pronounced than those of glycine and both amino acids did not cumulatively influence each other. Indeed, under saturating L-serine conditions, no glycine effect could be observed anymore, indicating a common mechanism explaining the effect. Another remarkable finding was the complete block of both glycine and L-serine effects when the experiments were performed in the presence of arginine. The reason for this arginine effect is unclear as we do not know the mechanism behind the glycine/L-serine effects. A simple interaction with glycine uptake is unlikely as glycine uptake was not found to be altered in equimolar arginine concentrations (data not shown). These findings show that these potentially important biological effects can be influenced by simple amino acids and are critically dependent on cell culture conditions. Further insights in these interactions and mechanisms may provide new potential tools, possibly as simple as nutritional, to modulate immune function.

When examining stereoselectivity of L-serine effects we noticed that D-serine decreased metabolic activity in high micro- to millimolar concentrations. This effect is not limited to peripheral macrophages since we found the same D-serine responses in microglial cells Figure III.10B). We correlated these findings in RAW 264.7 macrophages again to our PI viability assay, which clearly showed a decrease in cellular viability when 1 mM or more D-serine was added to the culture medium. Because D-serine is known as a gliotransmitter, secreted by astrocytes, but also by microglial cells, which causes neuronal cell death via NMDA-R activation (Wu et al., 2004; Katsuki et al., 2007; Wolosker et al., 2008), we next examined the potential involvement of these receptors, under the assumption that in our cell culture experiments glutamate is secreted in amounts high enough to activate NMDA-R. In this case the hypothesis would be that D-serine potentiates the activity of these NMDA-Rs. As for microglia, it has also been shown for macrophages that soluble factors can be secreted that activate NMDA-Rs (Xiong et al., 2004). However we rejected this hypothesis based on the following findings: 1) glycine did not agonize D-serine effects, while being also a co-agonist at NMDA-receptors. 2) NMDA-R blockers did not change control responses, arguing against our assumption of glutamate acting on NMDA-Rs in our culture conditions. 3) NMDA-R antagonists also did not block D-serine responses. This indicates that other mechanisms are likely to exist and to explain D-serine-induced cytotoxicity. As D-serine is degraded by the enzyme D-amino acid oxidase (DAO) yielding the toxic hydrogen peroxide, a possible way to explain the cytotoxic effects of D-serine is through oxidative stress. This mechanism was previously suggested to explain D-serine-induced nephrotoxicity (Krug et al., 2007). A recent report also showed the potential of D-serine to cause lipid and protein oxidative damage by a NMDAR-independent mechanisms (da Silva Lde et al., 2009). To investigate the potential involvement of oxidative stress in D-serine-induced cytotoxicity, we added glutathione to our cell culture, which could indeed shift the cytotoxicity to higher D-serine concentrations, arguing in favor of this metabolic hypothesis. As this mechanism

implies D-serine uptake, there may be an important regulating role for D-serine transporters. Different transporters are suggested to transport D-serine, both sodium-dependent (*e.g.* alanine-serine-cystein-like transporter type 2 (ASCT2)) and sodium-independent transporters (*e.g.* alanine-serine-cystein transporter type 1 (asc1)) (Utsunomiya-Tate et al., 1996; Nakauchi et al., 2000; Helboe et al., 2003). As their name implies these transporters (which are in fact amino acid exchangers, see Figure IV.13) are known to be aspecific and to transport other neutral amino acids (*e.g.* alanine, glycine, threonine, serine). This means that substrate competition exists between different amino acids and addition of high concentrations of competing substrates to the cell culture medium might block D-serine uptake and hence cytotoxicity. Our results indicate that L-serine but not glycine inhibits D-serine induced cytotoxicity, arguing against the involvement of such aspecific transporters. This conclusion is further supported by the insensitivity to AIB, which blocks transporters like asc1. Although specific D-serine transporters have not been identified yet, an unknown alanine-insensitive transporter was described in neurons, transporting both L-serine and D-serine (Javitt et al., 2002). Interestingly, this transporter had a Km of 1.1 mM for D-serine and L-serine prevented D-serine uptake in concentrations between 0.1 and 1 mM. Both findings correlate perfectly with the findings reported here. Transporter studies, using tritium-labelled D-serine seem to be necessary to further elucidate the precise regulation of D-serine uptake and identify new specific D-serine transporters. The current hypothesis explaining D-serine findings is summarized in Figure IV.12.

Although the aim of this section was the study of glycine (and related amino acids) effects on macrophage viability, we observed a bipolar differentiation in a minority of cells under millimolar glycine conditions. This effect, although less pronounced, was also present when cells were incubated in millimolar concentrations of L-serine, but not after D-serine addition. None of the other amino acids tested in this section mimicked these effects. Similar to glycine and L-serine effects on viability, the changes in morphology were also serum-dependent. RAW 264.7 cells were previously reported to differentiate in multinuclear osteoclast-like (giant) cells upon activation of the NFκβ pathway by the RANK ligand, or by LPS under the appropriate culture conditions (Islam et al., 2007). Interestingly, L-serine (0.1 mM), leading to the activation of NFAT, was shown to be required for this differentiation to take place. Out of different non-essential amino acids tested, the authors only found glycine to be able to weakly mimic L-serine-induced activation of NFAT (Ogawa et al., 2006). Also hypoxic conditions, leading to

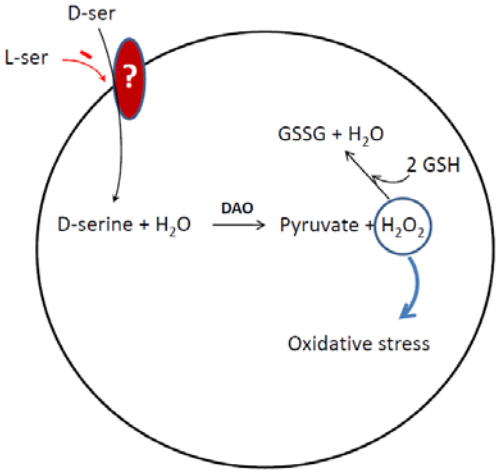

Figure IV.12 Current hypothesis explaining D-serine cytotoxicity in macrophages.
D-serine is taken up by an unknown alanine-insensitive, but L-serine sensitive transporter. In the cell D-serine is metabolized by means of the D-amino acid oxidase (DAO), leading to hydrogen peroxide production and hence oxidative stress, ultimately leading to cell death. Reduced glutathione (GSH) can detoxify hydrogen peroxide and convert it back to water.

mitochondrial stress, increases in cytosolic calcium and activation of calcineurin were shown to promote osteoclast differentiation (Srinivasan and Avadhani, 2007). Although these reports were all on RAW 264.7 differentiation in osteoclast-like cells, our differentiated cells did not resemble multinucleated giant cells. In fact, we do not have a convincing hypothesis about the functional meaning of this bipolar differentiation. However, the role that was suggested for L-serine and glycine and for calcium-sensitive intracellular mediators (*e.g.* calcineurin, NFAT and NFκβ) shows striking resemblance with our results. Also in microglial cells, low concentrations of glycine (5 µM) and L-serine (25 µM) were shown to induce a more branched phenotype (Tanaka et al., 1998). Also thapsigargin (15 nM) was shown to induce an increased ramification in microglial cells, an effect that was potentiated by culturing cells in a serum-free culture medium (Yagi et al., 1999). Furthermore, human blood monocytes were shown to be able to differentiate into dendritic cells, a process that is inhibited by serum and involves calcium signaling and activation of NFκβ (Lyakh et al., 2000).

In conclusion this section shows that glycine and L-serine both enhance RAW 264.7 macrophages viability at low concentrations (<100 µM), whereas, at higher concentrations (>100 µM), both amino acids induce a bipolar morphological differentiation. Remarkably, for the first effect, L-serine was more potent, while for the latter effect glycine was more potent. Moreover, millimolar concentrations of D-serine were shown to be toxic for cells, an effect independent of NMDA-Rs and mediated by intracellular D-serine metabolism. These effects showed serum-dependency (with the exception of D-serine) and were very susceptible to changes in other amino acids, suggesting an essential role of transporter-mediated uptake. These findings need to be correlated to uptake experiments and to a molecular characterization of the transporters involved. The exact identification of these targets may provide new therapeutic tools in different pathologies (*e.g.* neuro-inflammatory and -degenerative diseases) were microglia or macrophages are known to be involved.

4. CONCLUSION

The main aim of this chapter was to correlate the findings in microglial cells to peripheral macrophages. In RAW 264.7 macrophages it was shown that glycine enhances ATP- and thapsigargin-induced calcium signals and demonstrated that this enhancement was strychnine-insensitive and hence GlyR-independent. These findings are contradicted by other studies on macrophages, showing that glycine activates GlyRs, leading to a hyperpolarization-induced blunting of calcium fluxes. Although not shown in this chapter, we did find mRNA and protein molecular evidence for α2-containing GlyR expression, similar to microglial cells and some other macrophages (Froh et al., 2002). Contrary to the findings in microglia, the enhancement was less pronounced (at least for TG-induced calcium transients) and largely insensitive to AIB, correlating to AIB-insensitive glycine uptake, as demonstrated by our immunofluorescence experiments. These findings highly suggest that glycine modulatory properties are not completely microglia-specific and, at least partially dependent on the extracellular environment in macrophages, although the precise modulation seems to depend on cell-specific transporter expression.

The same approach could be used for cell culture experiments in which we would expect that, when using the same experimental conditions as we did in microglial cells, we would also find enhancing effects on the production of inflammatory mediators such as superoxide, nitric oxide and TNFα. This research is currently under investigation in our laboratory and is part of another PhD thesis, studying the effects of inhibitory neurotransmitters on macrophages.

In this chapter we furthermore showed that both glycine and L-serine in low micromolar concentrations could enhance macrophage viability and in high millimolar concentrations caused a morphological bipolar differentiation. Both effects were inhibited by serum and were sensitive to concentrations of other amino acids in the extracellular environment (*e.g.* interaction between glycine and L-serine, block of the effects by cationic amino acids arginine and lysine). D-serine was shown to have opposite effects on viability at higher millimolar concentrations. This was shown to be independent on NMDARs, and probably reflects an intracellular metabolic effect requiring cellular uptake of D-serine.

Given the crucial role of amino acid uptake mechanisms, we summarize the main systems in Figure IV.13. System A and N neutral amino acid transporters (together called sodium-coupled neutral amino acid transporters or SNATs) transport neutral amino acids (*e.g.* glycine, serine, alanine). Both are secondary active transport systems as they are coupled to sodium (Hyde et al., 2003). Another way of transporting neutral amino acids, mostly larger hydrophobic ones (*e.g.* leucine, isoleucine, valine), is by means of the system L neutral amino acid transporters. These transporters are actually exchangers, and are dependent on the intracellular availability of other neutral amino acids, taken up by other transporters, meaning that system L amino acid transporters are a form of tertiairy active transport (shown in purple in Figure IV.13) (Hyde et al., 2003; Hundal and Taylor, 2009). Other exchangers for neutral amino acids include the sodium-independent alanine-serine-cystein (asc) exchanger or the ASC-like (ASCT) sodium-dependent exchanger (where type 2 was also reported to be coupled to countertransport of potassium) (Arriza et al., 1993; Utsunomiya-Tate et al., 1996; Nakauchi et al., 2000; Chairoungdua et al., 2001; Helboe et al., 2003). Uptake of (mostly

larger) neutral amino acids can also be coupled to the exchange of cationic amino acids (*e.g.* arginine, lysine) as in the case of the sodium-dependent y^+L transporter. These cationic amino acids can also be taken up by means of the cationic amino acid transporter called y^+ (Closs et al., 2006). Furthermore a lot of these transporters are able to invert, depending on the concentration gradients of the transported amino acids. Besides transporting amino acids, these transporters are coupled to the co-transport or exchange of sodium, protons, potassium or charges (for the electrogenic transporters), which means they can lead to changes in intracellular sodium- or potassium concentrations, in pH and/or in membrane potential, depending on the precise system used and the amino acid concentration differences. The signaling role of these types of solute co-transporters is further discussed in chapter V (as part of "mechanism 2", see Figure V.1). These findings, together with the fact that these transporters have different Km and V_{max} values for their substrates makes the interpretation of transporting signaling challenging.

What can we say at this moment in light of our findings? As we already discussed system A, N, L and type 1 ASC transporters are sensitive to AIB, which pointed to a crucial role of System A SNATs to explain the findings in chapter III. In this chapter, we reported higher glycine uptake, for the larger part independent of AIB, which correlated to a less pronounced modulation of calcium signals, confirming a role of AIB-sensitive transporters in calcium signal modulation. Furthermore, the basic amino acids arginine and lysine were able to reduce not only the glycine and L-serine protective, but also the D-serine toxic effects on macrophages viability. The precise explanation at this point remains speculative at most. A direct or indirect interaction between basic and neutral amino acid transporters seems tempting, although this could not be confirmed in our uptake experiments, showing that arginine did not block glycine uptake (data not shown). The crucial signaling role these transporters seem to fulfill necessitates a thorough transporter characterization in macrophages and microglia.

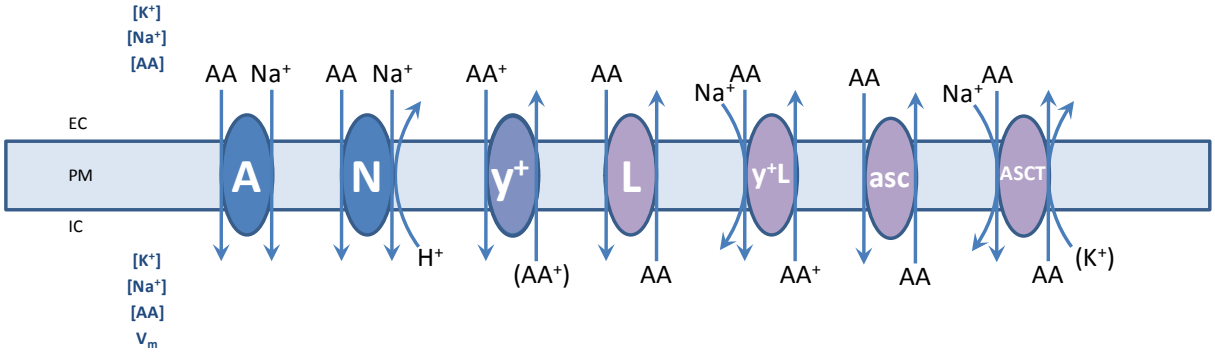

Figure IV.13 Main amino acid transport systems responsible for uptake of neutral and cationic amino acids.

Amino acid uptake can be secondary active (uptake coupled to electro-chemical gradients of solutes; shown in blue) or tertiairy active (amino acid exchangers, shown in purple). See text for explanation. AA, neutral amino acid (*e.g.* glycine, serine, alanine); AA^+, cationic amino acid (*e.g.* lysine, arginine); A, system A amino acid transporter; N, system N amino acid transporter; L, system L amino acid transporter; y^+, cationic amino acid transporter; y^+L neutral amino acid transporter requiring exchange of cationic amino acids; asc, alanine-serine-cysteine transporter; ASCT, ASC–like transporter; EC, extracellular; IC, intracellular;PM, plasma membrane.

CHAPTER V. SUMMARY AND GENERAL DISCUSSION

The central nervous system (CNS) is an integration center for signal processing, receiving signals from the different sensory systems and transmitting signals to the motor system. The main cells conducting signals are neurons, and for the largest part of the 20th century most attention of neuroscientist was focused on neurons. A role of glial cells, for a long time considered as passive connective tissue elements, in normal physiology and pathophysiology is now becoming increasingly appreciated. Different neurotransmitter receptors, transporters and enzymes for neurotransmitter synthesis and recycling have been described on glial cells, allowing them to communicate with neurons. This non-neuronal and non-synaptic function of neurotransmitters is mainly studied for glutamate and GABA, the most important excitatory and inhibitory neurotransmitters respectively. This work focused on non-neuronal functions of glycine, another inhibitory neurotransmitter acting mainly in the spinal cord and brainstem, with a special interest in immune cells and its potential role in neuro-immune communication. The starting point of this thesis was the study of "the inhibitory neurotransmitter glycine". This naming already holds an assumption that is clearly challenged in this work. Indeed glycine working as an inhibitory neurotransmitter implies the amino acid to act on glycine receptors (GlyRs), which is clearly not always the case when discussing non-neuronal functions.

In **chapter I** the reader was introduced to the neuronal, synaptic function(s) of glycine. Glycine was introduced as an inhibitory neurotransmitter, acting on GlyRs, ligand-gated ion channels belonging to the superfamily of cys-loop receptors. These are pentameric proteins, in the case of glycine composed of 4 different α(1-4) and 1 β-subunit, forming heteromeric $\alpha_1\beta$ receptors in the mature and homomeric α_2 receptors in the immature CNS. GlyRs are chloride channels and, depending on the intracellular chloride concentration, glycine gating will lead to chloride influx or efflux. In the inhibitory synapse, GlyR activation leads to chloride influx, resulting in a hyperpolarization (or clamping) of the postsynaptic membrane and inhibitory postsynaptic potentials (IPSPs). This hyperpolarization will decrease the likelihood of reaching the action potential threshold in the soma or axon hillock, explaining inhibitory neurotransmission. Although glycine is mainly considered as an inhibitory neurotransmitter, it can also function as a co-agonist at excitatory glutamate-gated NMDA receptors. We performed an extensive review, focused on the current findings of glycine and glycine receptor signaling in non-neuronal cells. Glycine effects were described on neuroglial cells (astrocytes, oligodendrocytes and microglia), immune cells (macrophages, neutrophils, monocytes and lymphocytes), renal cells, hepatocytes, endothelial cells and some other cell types (*e.g.* sperm cells and cardiomyocytes). When reviewing the literature it became apparent that a distinction can be made between two main kinds of effects: a modulatory one and a cytoprotective one. Concerning the modulatory effects, glycine was shown to modulate different cellular effector functions (*e.g.* proliferation, migration, apoptosis, cytokine production). Clear molecular and functional evidence for GlyR expression is present, which, after glycine gating, leads to chloride fluxes and changes in membrane potential. This alterations of membrane potential influences calcium signaling and hence effector functions. The role of GlyRs in the cytoprotective effect of glycine, which is mainly present under ischemic conditions, is less clear. Molecularly, most studies could only demonstrate β-subunits (which are not supposed to form functional GlyRs) and functional evidence is lacking. The general hypothesis is that under ischemic conditions cells become increasingly

permeable to macromolecules because of the formation of a multimeric channel, leading eventually to cell death. Glycine somehow stabilizes this channel, and although GlyR(s) (subunits) seem to be involved, the precise link is not clear yet.

As discussed in the review, glycine (immune)modulatory effects were best studied in macrophages, but also in neutrophils and monocytes. However, molecular evidence was lacking in T lymphocytes and some discrepancies existed in the functional findings, which was the starting point for **chapter II**, where glycine effects in human T-lymphocytes were examined. In this chapter, molecular evidence for GlyR expression in human T-lymphocytes is provided, although these receptors were not found to be functionally located at the plasma membrane as was obvious from confocal imaging and electrophysiological findings. GlyR α4-subunit expression was found, which was not described in any specific human tissue before, but actually represents a pseudogene, as confirmed from our experiments. Furthermore the existence of an unknown subunit is suggested. This is currently under investigation in our lab. Anti-apoptotic effects, that were suggested by few studies could not be attributed to GlyRs, although a more general mechanism, with glycine acting on amino acid transporters could not be excluded.

In **chapter III** the attention was shifted to microglial cells. In terms of neuro-immune communication, glycine modulation of microglial cells is very relevant, both in physiological and in pathophysiological conditions, because of their resident presence in the CNS. Although glycine effects on macrophages were extensively studied before, data on microglial cells were scarse. Molecular evidence for α1, α2 and β-subunit containing GlyRs was found, although the location of the receptors seemed to be mainly cytoplasmic as illustrated by microscopic images and the lack of GlyR-gated currents in patch clamp experiments. The lack of functionality was confirmed in cell culture experiments, where a clear enhancing role of glycine on nitric oxide (NO) and TNFα production was found, which could not be pharmacologically linked to GlyRs. This raised two questions: 1) How can GlyRs be activated and what is their functional meaning? 2) What is the mechanism behind the modulating properties on microglial cells? Considering the first question, there seems to be an important analogy with macroglial cells, which only have functional GlyR-mediated currents when cultured in slice preparations, as discussed in chapter I. Because electrophysiology of microglia in slice preparations is technically challenging, this was not part of this PhD thesis. It will be investigated as part of one of the upcoming (PhD) research projects in our lab, studying microglia in brain slices. We rather focused on the second question. A role for NMDA receptors, which was suggested because of the agonistic effects of (L-) serine in our cell culture system, was pharmacologically ruled out. As most (immune)modulatory glycine effects so far were shown to be correlated with a modulation of calcium signaling, we optimized a fluorescent platereader-based assay to measure calcium signals, induced by different agents. Surprisingly LPS, which is used as an inducer of fast transient calcium signals in almost all reports studying glycine modulation of calcium signals in macrophages so far, was not found to induce calcium transients by it self. Calcium signals were rather found to be secondary to small amounts of rat serum, questioning the existence of LPS-induced calcium transients that were reported previously. When using ATP or thapsigargin as calcium signal inducers, it was found that glycine could modulate calcium signals, but contrary to other reports on macrophages an enhancing role of glycine was found, which was

again GlyR-independent. As the calcium modulation could be mimicked by some neutral amino acids (*e.g.* serine, alanine, glutamine and valine) and could be blocked by AIB (2-amino isobutyric acid), a substrate antagonist of system A sodium-coupled neutral amino acid transporters (SNATs), a crucial role for these transporters in the modulating properties of glycine was suggested. This was further evidenced by correlating glycine uptake with the calcium experiments, which clearly showed SNAT-dependent glycine uptake in microglial cells.
Because these results, showing an enhancing role on calcium signaling, were conflicting with the literature findings on peripheral macrophages, the central question at this point was whether these findings were microglia-specific or represented some general mechanism in macrophages, where the differences might be explained by cell culture or other experimental conditions. This question was addressed in **chapter IV** where it was demonstrated that the GlyR-independent positive modulation of glycine on calcium signaling could also be found in a peripheral macrophage model. Compared to microglia this modulation was less pronounced, and the effect could not be significantly blocked by AIB. This was correlated with immunocytochemical glycine uptake experiments which showed an extensive glycine uptake, which, contrary to microglial cells, could only partially be blocked by AIB, suggesting that other transport mechanism exist in these cells. Some other unexpected but certainly interesting findings in chapter IV are the serum dependent protective roles of glycine and L-serine on macrophage viability in low concentrations and on their morphology at higher concentrations, and the NMDA receptor-independent cytotoxic activity of the glycine NMDAR site-agonist D-serine.

The findings of this thesis are actually discrepant with what was known about glycine signaling in immune cells. While we found a transporter-induced depolarization, an enhancement of calcium signaling and a production of immune-mediating agents (*e.g.* nitric oxide and TNFα), in other macrophages opposite findings were observed and were shown to be GlyR-dependent (see also Table III.4) As we did find molecular evidence for non-functional cytoplasmic GlyRs, we hypothesize that under certain (yet unknown) conditions these receptors might traffick to the membrane, leading to an overruling of the transporter-dependent enhancing effects of glycine, and making the inhibiting effects predominate. This trafficking and furthermore the potential factor determining its regulation are currently unknown, but there seem to be striking similarities with macroglial cells, where functional receptors were only found when studying brain slices. In this work it was also shown for the first time that glycine can enhance calcium signals. As we proved a role for neutral amino acid transporters, this is to our knowledge also one of the first studies linking calcium signal modulation to neutral amino acid transporter signaling. Interestingly some more recent studies also provided evidence for similar calcium modulation via glutamate transporters and GABA transporters (Kirischuk et al., 2007; Doengi et al., 2009) and also leucine was shown to influence mTOR (mammalian target of rapamycine) signaling via changes in intracellular calcium (Gulati et al., 2008). The research findings of this thesis suggest a signaling role for (system A neutral) amino acid transporters in microglial cells and probably also in other macrophages. A signaling role for system A neutral amino transporters was previously suggested to explain anti-apoptotic effects of several amino acids in lymphocytes as well

(Franek and Sramkova, 1996; Franek et al., 2002). Although amino acids and their transporters are mainly considered as nutritional elements to cells, there is growing evidence for specific signaling cascades that are transporter-dependent (Hyde et al., 2003; Hundal and Taylor, 2009). In an excellent review on amino acid transporter signaling the authors suggest four general mechanisms by which these transporters may exert their signaling function, falling into two major categories: a direct response via the transporter (mechanisms 1 and 2 in Figure V.1) and an indirect response via amino acid uptake (mechanisms 3 and 4 in Figure V.1) (Hyde et al., 2003):

- **Mechanism 1:** The amino acid transporter by itself, possibly via some change in conformation, initiates cellular signaling, independent of the specific substrate transported. These transporters are also called transceptors, because of their dual function as a transporter and as a receptor. This is mainly studied for system A neutral amino acid transporters, where starvation (and hence amino acid deprivation) was suggested to lead to the activation of MAP kinases (*e.g.* ERK1/2 and JNK), leading to transporter upregulation (Franchi-Gazzola et al., 1999; Hundal and Taylor, 2009).
- **Mechanism 2:** The signaling is initiated because the co-transported solute leads to changes in membrane potential or pH. An example of this system can be found in glycine effects on macroglial cells, as discussed in chapter I. Belachew found that, besides GlyRs, also GlyTs contributed to a membrane depolarization in oligodendrocyte progenitors, leading to calcium fluxes via voltage-gated calcium channels (Belachew et al., 2000). In chapter III we mainly hypothesized that this mechanism (sodium influx and depolarization) were to be important.
- **Mechanism 3:** The amino acid is transported inside the cells where it acts on an intracellular receptor/sensor. This mechanism has been very well studied for the regulation of the mTOR signaling pathway (van Sluijters et al., 2000; Lynch, 2001). It was found that when leucine is injected intracellularly into *Xenopus* oocytes, mTOR is activated. This effect was not present when leucine was just present in the extracellular environment, unless there was an overexpression of system L amino acid transporters (LAT), suggesting an intracellular initiated signaling pathway for leucine (Christie et al., 2002). This mechanism was also shown to be relevant in neurons (Ishizuka et al., 2008). Interestingly, mTOR was reported to be activated by glycine as well (Christie et al., 2002), and there seems to be a crucial role for this pathway in hypoxia-induced upregulation of iNOS and survival in microglial cells (Lu et al., 2006; Chong et al., 2007). Very recently, mTOR was also shown to be involved in LPS- and cytokine-induced upregulation of iNOS and COX as well as regulation of proliferation and cell viability of microglial cells (Dello Russo et al., 2009). Some other interesting reports, in light of our findings, point to the fact that leucine-, thapsigargin- or ionomycine-induced calcium concentration changes can activate the mTOR pathway (Conus et al., 1998; Gulati et al., 2008).
- **Mechanism 4:** The amino acid (*e.g.* glycine or glutamate) is acting on its specific receptor, but the transporter is responsible for the regulation of the extracellular amino acid concentration and hence their action on their receptors. An example of this system can be found in the role of astrocytic GlyT1 in the regulation of glycine concentrations at glycinergic synapses.

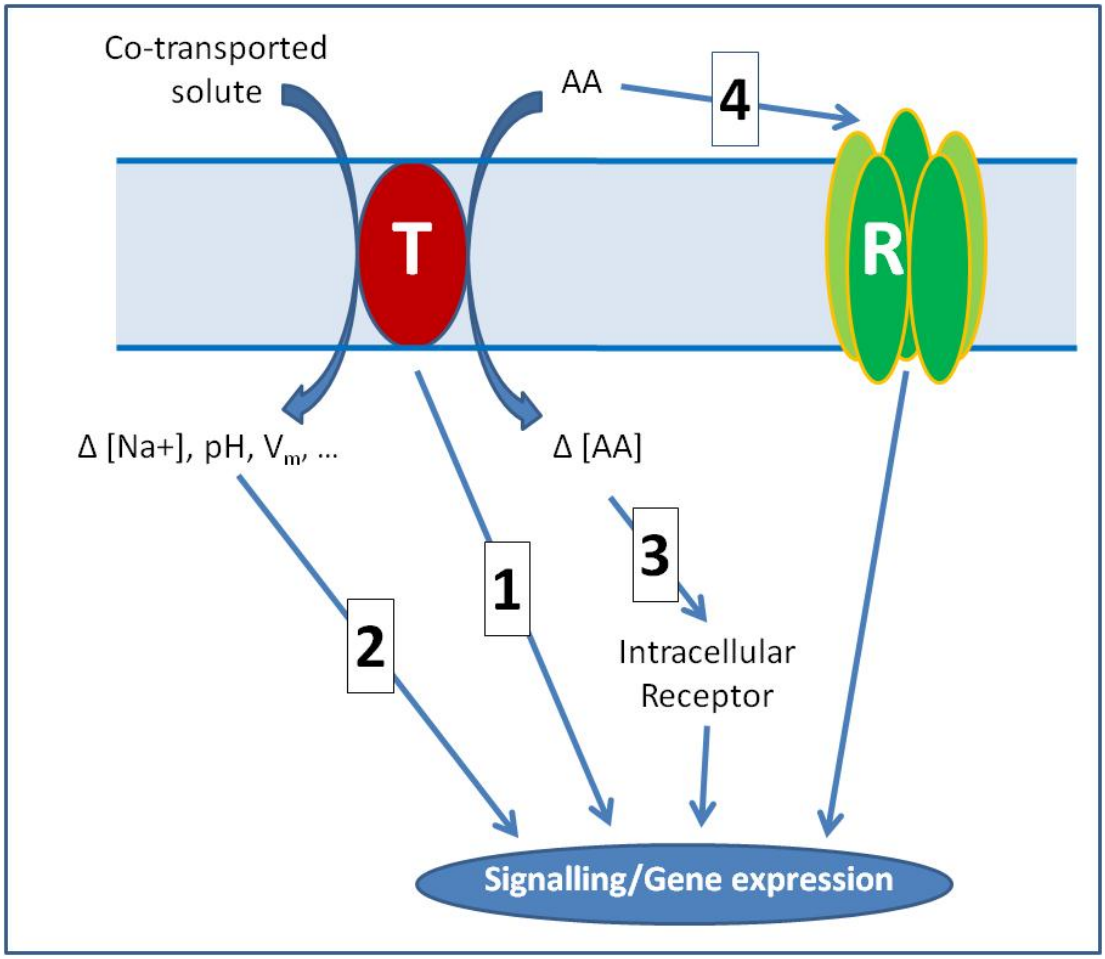

Figure V.1 Suggested potential mechanisms explaining amino acid transporter dependent signaling.
See text for explanation.

Considering these four mechanisms, the question then becomes which one is the most likely candidate to explain the glycine effects on microglial cells as reported in this thesis. Mechanism 4 does not seem to be very likely as a functional role of GlyRs (or NMDARs) was ruled out. Although mechanism 1 is hard to exclude or to confirm the most likely candidates seem to be mechanisms 2 and 3. Although most findings seem to point to a role of sodium influx and changes in the membrane potential, we cannot exclude that glycine uptake by itself is necessary for the effects to take place, and that the specific transporter it uses is less important. This would mean that glycine acts on a specific intracellular receptor, as described in mechanism 3. Further evidence for this mechanism comes from the fact that most effects reported in this thesis (*e.g.* NO production, viability and morphology) could be mimicked by L-serine, which is metabolically very related to glycine. To definitely differentiate between mechanism 2 and 3 future research has to focus on the precise correlation between effects on calcium and changes in intracellular sodium or membrane potential. A fluorimetric technique using dual labeling of calcium and sodium/membrane potential may offer the best way to achieve this goal. Given the role of the transporters there also seems to be a need to a precise characterization of these systems, which may offer more insights in the mechanism as well. These topics are part of a PhD project that was recently started. Irrespective of the precise mechanism, the role of amino acid transporters in glycine

signaling is clear. This also means that substrate competition for the transporter exists and that the precise amino acid composition of the extracellular environment, together with the characteristics of the transporters (Km and Vmax values), determines which substrates are transported and which ones are not. This insight points to a critical role for the selection of the appropriate cell culture conditions when investigating amino acid effects, as exemplified at different stages in this thesis, where both serum conditions and certain amino acids were shown to critically modulate the different effects.

The brain has long been considered as an "immune privileged organ". It has become clear now that microglia are rapidly activated upon CNS injury, undergoing characteristic activational changes. This leads to activation of other neuroglial cells, potential recruitment of peripheral immune cells and finally neuronal cell death or neuroprotection. This view has become increasingly appreciated as it has become clear that microglia and neuro-inflammatory factors have an important role in different neuropathologies (*e.g.* neuropathic pain, Alzheimer's disease, ALS). This work demonstrated that glycine, considered as an inhibitory neurotransmitter, can modulate this activation process. As this modulation depends on the local glycine concentrations, one expects it to be mainly important near glycinergic neurons. However, the fact that it has been shown that not only neurons, but also astrocytes can secrete glycine, points to a role of glycine as intercellular glial messenger (or a "gliotransmitter") as well. The identification of the different messengers and their cellular targets (in the case of glycine the glycine receptor or different transporters) is crucial to understand the complex interglial interaction that takes place in the CNS. Indeed the insights that glial cells are critical for the maintenance of the normal precare homeostatic balance of neurons, have shifted the long hold "neurocentric" view towards a view where neuropathologies are more and more considered as primary gliopathologies (Nedergaard et al., 2009). Furthermore, calcium is one of the main second messengers regulating both glial as immune cell intracellular signaling. Disturbances in normal glial calcium signaling have been implied in different neuropathological conditions as well (*e.g.* brain ischemia, epilepsy and neurodegenerative disease) (Verkhratsky, 2006; Nedergaard et al., 2009). As this work shows that glycine and structurally related amino acids can modulate calcium signals, the findings may be relevant in pathologies were local concentrations of glycine (or other amino acids) are elevated in the millimolar concentration ranges. Although the concentrations needed are rather high (at least for the calcium modulation) and not likely to be reached in the normal neural micro-environment (normal CSF glycine concentrations are around 10 µM), one has to realize that the concentration of neurotransmitters inside the presynaptic neuronal cytosol can reach 10 mM and in synaptic vesicles may even reach 100 mM, meaning that near synapses and certainly after degeneration of glycinergic neurons microglial calcium signaling may indeed be modulated in the immediate environment. The insights in the role of the transporters in this modulation may offer potential targets for new therapies for different diseases were microglia have been shown to play a crucial pathogenic role.

In conclusion, this PhD thesis examined the immunomodulating role of glycine and of GlyRs on human T lymphocytes, microglial cells and macrophages. Although the receptors were expressed in all cell types investigated, we could not demonstrate any functional role for these

receptors. We did find a crucial role for amino acid transporters in determining the immunomodulating potential of glycine and some related amino acids (*e.g.* L-serine) and suggested a modulation of calcium signaling in determining the effects on the immune effector functions. This work sheds a whole new light on glycine signaling (and by extension amino acid signaling) in immune cells that only received no or minimal attention by the scientific community until now. These insights may be important to offer new potential drug targets, but also raise important other research questions, making this thesis a basis for further research.

SAMENVATTING & ALGEMENE CONCLUSIE

Het centraal zenuwstelsel (CZS) is een integratiecentrum voor signaalverwerking, dat enerzijds signalen ontvangt vanuit de verschillende sensorische systemen en anderzijds signalen verstuurt naar het motorisch systeem. De belangrijkste signaalgeleidende cellen zijn neuronen en in de 20e eeuw was bijna alle aandacht binnen het neurowetenschappelijk onderzoek gericht op deze cellen. Gliacellen werden lang beschouwd als passieve steunelementen. Recent gaat echter meer en meer aandacht uit naar de rol van deze cellen in de fysiologie en pathofysiologie van CZS. Verschillende neurotransmitter receptoren, transporters en enzymes nodig voor de synthese en recyclage werden beschreven op gliacellen, dewelke deze cellen in staat stelt om te communiceren met neuronen. Deze niet-neuronale en niet-synaptische functie van neurotransmitters werd voornamelijk bestudeerd voor GABA en glutamaat, de voornaamste inhiberende respectievelijk exciterende neurotransmitters. Dit werk richtte zich voornamelijk op de niet-neuronale functie van glycine, de andere inhiberende neurotransmitter, dewelke voornamelijk actief is in het ruggenmerg en de hersenstam. Bijzondere aandacht ging hierbij uit naar immuuncellen en de potentiële rol van glycine in termen van neuro-immuuncommunicatie. Het startpunt van deze thesis was de studie van "de inhiberende neurotransmitter glycine". Het beschouwen van glycine als inhiberende neurotransmitter houdt reeds een veronderstelling in die duidelijk wordt uitgedaagd in deze thesis. We gaan er hierbij immers vanuit dat glycine werkt via glycine receptoren (GlyR), wat zeker niet steeds het geval is wanneer niet-neuronale functies bestudeerd worden.

In **hoofdstuk I** werd de lezer geïntroduceerd in de neuronale, synaptische functie(s) van glycine. Glycine werd geïntroduceerd als een inhiberende neurotransmitter, werkende via GlyR, ligand-geactiveerde ionkanalen die tot de familie van de cys-loop receptoren behoren. Dit zijn pentamere proteïnen, dewelke in het geval van de GlyR samengesteld zijn uit 4 verschillende α(1-4) en 1 β-subeenheid. Deze subeenheden kunnen heteromere $\alpha_1\beta$ receptoren vormen in het mature CZS of homomere α_2 receptoren in het immature CZS. GlyR zijn chloorkanalen en, afhankelijk van de intracellulaire chloorconcentraties kan glycine activatie leiden tot chloorinflux of –efflux. In de inhiberende synaps leidt glycine tot chloorinflux, resulterend in een hyperpolarisatie van de postsynaptische membraanpotentiaal en dus inhiberende postsynaptische potentialen. Deze potentialen verminderen de kans op het bereiken van de actiepotentiaal drempel in het cellichaam of axonheuvel, leidend tot inhiberende neurotransmissie. Alhoewel glycine veelal beschouwd wordt als inhiberende neurotransmitter, kan het ook fungeren als een co-agonist op exciterende glutamaat-geactiveerde NMDA receptoren. In hoofdstuk I werd verder een uitgebreide review verricht, gericht op de huidige bevindingen van glycine en GlyR signaaltransductie op niet-neuronale cellen. Glycine effecten werden beschreven op neurogliacellen (astrocyten, oligodendrocyten en microglia), immuuncellen (macrofagen, neutrofielen, monocyten en lymfocyten), niercellen, levercellen, endotheelcellen en enkele andere celtypes (bv. spermacellen en hartspiercellen). Uit deze review werd duidelijk dat een onderscheid kan gemaakt worden tussen 2 verschillende effecten: een modulerend en een cytoprotectief effect. Met betrekking tot het modulerend effect werd aangetoond dat glycine verschillende effectorfuncties van cellen kan moduleren (bv. proliferatie, migratie, apoptose en cytokineproductie). Er is duidelijk moleculair en functioneel bewijs voor GlyR expressie, dewelke, na activatie door glycine, leidt tot chloorstromen en veranderingen in membraanpotentiaal. Deze

potentiaalveranderingen beïnvloeden calciumsignalen en uiteindelijk effectorfuncties. De rol van GlyR in het cytoprotectief effect, hetwelk voornamelijk aanwezig is in ischemische condities, is minder duidelijk. Moleculair lijken de meeste studies enkel β-subeenheden aan te tonen (dewelke niet verondersteld worden op zichzelf functionele GlyR te vormen) en functioneel zijn geen aanwijzingen voor GlyR aanwezig. De algemene hypothese die naar voor wordt geschoven veronderstelt dat onder ischemische condities cellen progressief doorlaatbaar worden voor macromoleculen, tengevolge van de vorming van een multimeer kanaal, uiteindelijk leidend tot celdood. Glycine zou op de één of andere manier dit kanaal stabiliseren, en, alhoewel GlyR (subeenheden) betrokken lijken te zijn, is de precieze link onduidelijk.

Zoals duidelijk werd uit de review werden de immuunmodulerende effecten van glycine voornamelijk bestudeerd op macrofagen, maar ook op neutrofielen en monocyten. In T cellen is echter geen moleculair bewijs voor GlyR aanwezig en er bestaan tegenstrijdigheden in de functionele bevindingen, dewelke het startpunt vormden voor **hoofdstuk II**, waar glycine effecten op humane T lymfocyten werden bestudeerd. In dit hoofdstuk wordt de moleculaire aanwezigheid van GlyR aangetoond. Confocale beeldvorming, samen met elektrofysiologisch onderzoek toonden echter aan dat de gevonden GlyR niet functioneel aanwezig waren op de celmembraan. Verder werd de expressie aangetoond van de GlyR α4-subeenheid, dewelke nooit eerder op een specifiek menselijk weefsel werd beschreven. We bevestigden dat deze subeenheid in feite een pseudogen is bij de mens. Verder veronderstelden we de aanwezigheid van een onbekende GlyR subeenheid, wat op dit moment verder wordt onderzocht in ons labo. We konden de anti-apoptotische effecten, die door sommige studies werden gesuggereerd, niet verklaren door GlyR activatie, alhoewel een meer algemeen mechanisme, waarbij glycine op aminozuurtransporters zou ingrijpen niet uitgesloten kon worden.

In **hoofdstuk III** werd de aandacht verschoven naar microgliacellen. In termen van neuro-immuuncommunicatie is glycine modulatie van microglia zeer relevant, zowel in fysiologische als pathofysiologische omstandigheden, omwille van hun residente aanwezigheid in het CZS. Alhoewel glycine effecten op macrofagen reeds uitgebreid bestudeerd werden, zijn data op microglia zeldzaam. Er werd moleculair bewijs gevonden voor α1, α2 en β-subeenheid bevattende GlyR, alhoewel deze receptoren zich weer voornamelijk intracellulair leken te bevinden, zoals duidelijk werd uit onze confocale beelden en elektrofysiologische bevindingen. De niet-functionaliteit werd verder ondersteund door celkweekbevindingen. Deze toonden een duidelijke stimulerende rol van glycine aan op nitrietoxide (NO) en TNFα productie, dewelke farmacologisch niet konden worden gekoppeld aan GlyR activatie. Op dat moment stelden zich 2 vragen: 1) Hoe kunnen de gevonden GlyR worden geactiveerd en wat is hun functionele rol? 2) Wat is het mechanisme achter de modulerende eigenschappen van glycine op microgliale cellen? Met betrekking tot de eerste vraag lijkt er een belangrijke analogie te bestaan met macroglia, dewelke enkel aantoonbaar GlyR-gemedieerde stromen bevatten wanneer ze gekweekt worden in slicepreparaten, zoals aangehaald in hoofdstuk I. Omdat elektrofysiologie van microglia in slicepreparaten technisch moeilijk is maakte dit geen deel uit van deze PhD thesis. Deze vraag wordt verder behandeld in één van de toekomstige onderzoeksprojecten binnen ons labo, gericht op de studie van microglia in hersenslices. Dit werk richtte zich verder op de tweede vraag. Een rol voor NMDA receptoren werd gesuggereerd door de agonistische effecten van serine in

celkweekexperimenten, maar werd farmacologisch uitgesloten. Aangezien de meeste (immuun)modulatorische glycine effecten tot dusver gecorreleerd werden met een modulatie van calciumsignalen, werd een fluorescent plaatlezer assay geoptimaliseerd om calciumsignalen, geïnduceerd door verschillende stoffen, te meten. Tot onze verrassing vonden we dat LPS, dewelke in quasi elke studie van glycine modulatie van calciumsignalen in macrofagen gebruikt wordt als opwekker van calcium fluxen, geen calcium concentratieveranderingen kon veroorzaken. We vonden dat de beschreven calciumsignalen eerder een gevolg waren van de aanwezigheid van rat serum, dewelke een vraagteken plaatst achter het bestaan van (snelle) LPS-geïnduceerde calciumsignalen. Bij het gebruiken van ATP of thapsigargine als opwekker van calciumsignalen, werd gevonden dat glycine deze calciumsignalen kon moduleren. In tegenstelling tot eerdere rapporten op macrofagen werd een GlyR-onafhankelijke bevorderende rol gevonden. Aangezien de calciummodulatie nagebootst kon worden door structureel verwante aminozuren (vb. serine, alanine, glutamine en valine) en geblokkeerd kon worden door AIB (2-aminoisoboterzuur), een substraatantagonist van systeem A neutrale aminozuurtransporters (SNATs), werd een cruciale rol van deze transporters in de modulerende eigenschappen van glycine verondersteld. Dit werd ondersteund door de correlatie met de glycine opname experimenten, dewelke een duidelijke SNAT-afhankelijke opname aantoonden in microglia.
Omdat deze resultaten, waarbij een bevorderende rol van glycine op calcium signaaltransductie werd gevonden, tegenstrijdig zijn met de literatuurbevindingen op perifere macrofagen, was de centrale vraag op dit punt of de bevindingen microglia specifiek waren of eerder een meer algemeen mechanisme vertegenwoordigen, waarbij de verschillen verklaard zouden kunnen worden door celkweek- of andere experimentele omstandigheden. Deze vraag werd behandeld in **hoofdstuk IV**, waar kon worden aangetoond dat de GlyR-onafhankelijke positieve modulatie van glycine op calcium signaaltransdcutie ook kon worden gevonden op een perifeer macrofaagmodel. Vergeleken met microglia was de modulatie minder uitgesproken, en kon het effect niet significant geblokkeerd worden door AIB. Dit werd gecorreleerd met immuuncytochemische glycine opname experimenten, dewelke een uitgesproken glycine opname aantoonden, dewelke, in tegenstelling tot microglia, slechts zeer beperkt kon worden geblokkeerd door AIB. Enkele andere interessante bevindingen waren de serumafhankelijke beschermende functies van glycine en L-serine op macrofaagviabiliteit in lage concentraties en op hun morfologie in hogere concentraties, en de NMDA receptor-onafhankelijke cytotoxische activiteit van de NMDA receptor site-agonist D-serine.

De bevindingen in dit werk zijn in feite tegengesteld aan wat geweten is en verondersteld wordt over glycine signalering in immuuncellen. Daar waar dit werk een transporter-geïnduceerde depolarizatie en een bevordering van calcium signaaltransductie en productie van immuunmediatoren (vb. NO en TNFα) beschrijft, werden in andere macrofagen tegengestelde bevindingen gedaan, dewelke eerder wezen op GlyR-afhankelijkheid. Daar we de GlyR wel moleculair konden aantonen in het cytoplasma, is onze hypothese dat onder bepaalde, ongekende omstandigheden deze receptoren zich naar de plasmamembraan kunnen verplaatsen, dewelke leidt tot een tenietdoen van de transporterafhankelijke bevorderende effecten van glycine, leidend tot een predominantie van de GlyR-afhankelijke inhiberende effecten. Deze translocatie van GlyR en in het bijzonder de factoren die deze bepalen zijn

momenteel ongekend, maar er lijkt een belangrijke analogie te bestaan met macroglia, waar functionele receptoren enkel konden worden aangetoond in de studie van hersenslices.

In dit werk werd aangetoond dat glycine microgliale calcium signalen kan bevorderen. Dit is tevens één van de eerste studies die een modulerende rol voor neutrale aminozuurtransporters aantoont in de calcium signaaltransductie. Enkele recente studies toonde gelijkaardige calcium signaalmodulaties aan via glutamaat en GABA trasporters (Kirischuk et al., 2007; Doengi et al., 2009), en ook van leucine werd aangetoond dat het de mTOR (mammalian target of rapamycine) signaalcascade kan beïnvloeden via veranderingen in intracellulaire calciumconcentraties (Gulati et al., 2008). De bevindingen van deze thesis suggereren een rol voor (systeem A neutrale) aminozuur transporters in microglia en waarschijnlijk ook in ander macrofagen. Een signaleringsfunctie voor deze transporters werd eerder ook reeds gesuggereerd om de anti-apoptotische effecten van verscheidene aminozuren in lymfocyten te verklaren (Franek and Sramkova, 1996; Franek et al., 2002). Alhoewel aminozuren en hun transporters voornamelijk beschouwd worden als nutritionele elementen voor cellen, is er meer en meer bewijs voor specifieke transporter-afhankelijke signaalcascades die kunnen worden beïnvloed (Hyde et al., 2003; Hundal and Taylor, 2009). In een excellente review over aminozuur transporter signalering, suggereren de auteurs 4 algemene mechanismen via dewelke transporters hun signaalfunctie kunnen uitoefenen (Hyde et al., 2003). Deze vallen in 2 categorieën: een direct effect via de transporters (mechanisme 1 en 2 in figuur V.1) en een indirect effect via aminozuur opname (mechanisme 3 en 4 in figuur V.1):

- **Mechanisme 1:** De transporter initieert zelf de signaalcascade, onafhankelijk van het getransporteerde substraat, wellicht via een verandering in transporter conformatie. Deze transporters worden ook transceptors genoemd, omwille van hun dubbele functie als receptor en transporter. Ze zijn voornamelijk bestudeerd voor systeem A neutrale aminozuurtransporters, waar een activatie van MAP kinasen (vb. ERK1/2 en JNK), resulterend in transporter opregulatie, werd gevonden na uithongering (en dus deprivatie van aminozuren) van de cellen (Franchi-Gazzola et al., 1999; Hundal and Taylor, 2009).
- **Mechanisme 2:** De signaalcascade wordt geïnitieerd door het gecotransporteerde ion, wat leidt tot veranderingen in membraanpotentiaal of pH. Een voorbeeld van dit systeem werd beschreven in hoofdstuk 1. Belachew vond dat, naast GlyR, ook glycine transporters bijdragen aan de membraandepolarisatie in oligodendrocyten progenitors, leidend tot calcium concentratieveranderingen, veroorzaakt door de activatie van voltage-geactiveerde calciumkanalen (Belachew et al., 2000). In hoofdstuk III werd dit mechanisme (natriuminflux en depolarisatie) verondersteld aan de basis te liggen van onze bevindingen.
- **Mechanisme 3:** Het aminozuur wordt in de cel getransporteerd waar het bindt op een intracellulaire receptor/sensor. Dit mechanisme werd goed bestudeerd voor de regulatie van de eerder vermelde mTOR signaalcascade (van Sluijters et al., 2000; Lynch, 2001). Er werd vastgesteld dat intracellulaire leucine injectie in *Xenopus Oöcyten* leidt tot mTOR activatie. Dit effect was niet aanwezig wanneer leucine enkele aanwezig was in de extracellulaire omgeving, tenzij system L aminozuurtransporters eerst tot expressie werden gebracht in de cellen, suggererend

dat er een specifieke intracellulair activeerbare signaalcascade aanwezig is (Christie et al., 2002). Ook in neuronen werd gevonden dat dit mechnisme relevant is (Ishizuka et al., 2008). Interessant om te vermelden is verder dat mTOR ook kan geactiveerd worden door glycine (Christie et al., 2002), en dat er een cruciale rol voor deze pathway bestaat in de hypoxie-geïnduceerde opregulatie van iNOS en COX, alsook de regulatie van proliferatie en celviabiliteit in microglia (Lu et al., 2006; Chong et al., 2007). Andere interessante rapporten wijzen op het feit dat leucine, thapsigargine en ionomycine-geïnduceerde calciumveranderingen de mTOR pathway kunnen activeren (Conus et al., 1998; Gulati et al., 2008).

- **Mechanisme 4:** In dit mechanisme, waar het aminozuur (bv. glycine of glutamaat) actief is op zijn specifieke receptor, is de transporter verantwoordelijk voor de regulatie van de extracellulaire aminozuur concentratie en dus zijn uiteindelijk effect op de receptor. Een voorbeeld is de rol van de glycine transporter type 1, aanwezig op astrocyten, dewelke de glycine concentratie kan regelen in glycinerge synapsen.

Met betrekking tot deze 4 mechanismen wordt de centrale vraag dan welk het meest waarschijnlijke mechanisme is om de bevindingen vermeld in dit werk te verklaren. Mechanisme 4 lijkt weinig waarschijnlijk daar een functionele rol van GlyR (of NMDA receptoren) werd uitgesloten. Mechanisme 1 is moeilijk volledig uit te sluiten, doch de meest waarschijnlijke kandidaten lijken mechanisme 2 en 3 te zijn. Alhoewel de meeste bevindingen lijken te wijzen op een rol van natriuminstroom en veranderingen in membraanpotentiaal, kunnen we niet uitsluiten dat de glycineopname zelf verantwoordelijk is voor de gerapporteerde effecten, en dat de specifieke transporter minder belangrijk is. Dit zou betekenen dat glycine op een specifieke intracellulaire receptor inwerkt, zoals beschreven door mechanisme 3. Dit wordt ondersteund door het feit dat de meeste effecten in deze thesis (vb. NO productie, viabiliteits- of morfologieveranderingen) nagebootst konden worden door L-serine, welk metabool nauw verwant is aan glycine. Voor een definitieve differentiatie tussen mechanisme 2 en 3 dient toekomstig onderzoek zich dan ook te richten op de precieze correlatie tussen intracellulaire natrium- en membraanpotentiaalveranderingen enerzijds en calciumconcentratie veranderingen anderzijds. Een fluorimetrische techniek, gebruik makend van een dubbelkleuring van calcium en natrium/membraanpotentiaal lijkt de beste manier om dit doel te bereiken. Gezien de rol van transporters lijkt ook een verdere karakterizatie van deze transporters zinvol te zijn. Deze topics maken deel uit van een recent gestart nieuw PhD project.

Onafhankelijk van het mechanisme is er een duidelijke rol voor aminozuur transporters in de glycine signaalcascade. Dit betekent echter ook dat er een substraatcompetitie bestaat voor de transporter en dat de aminozuursamenstelling van de extracellulaire omgeving, samen met de exacte karakteristieken van de transporters (Km en Vmax waarden), bepalen welk substraat getransporteerd wordt en welk niet. Dit inzicht wijst op een cruciale rol voor de selectie van de juiste celkweekomstandigheden bij het bestuderen van aminozuureffecten, zoals duidelijk werd in verschillende hoofdstukken van deze thesis, waar zowel serumconcentraties als het al dan niet toevoegen van bepaalde aminozuren de gevonden effecten in belangrijke mate wijzigden. De hersenen zijn lange tijd beschouwd als een "immuun bevoorrecht orgaan".

Meer en meer is echter duidelijk geworden dat microglia snel kunnen geactiveerd worden na hersenschade, waarbij ze karakteristieke activatie-gebonden veranderingen ondergaan. Dit leidt tot activering van andere neuroglia cellen, potentiële aantrekking van perifere immuuncellen en tenslotte neuronale celdood (of neuroprotectie). Het is meer en meer duidelijk geworden dat microglia en neuro-inflammatoire factoren een belangrijke rol spelen in verschillende neurologische aandoeningen (bv. neuropathische pijn, ziekte van Alzheimer, amyotrofe lateraalsclerose). Dit werk heeft aangetoond dat de inhiberende neurotransmitter glycine het microgliaal activatieproces kan moduleren. Aangezien deze modulatie afhankelijk is van de lokale concentratie glycine, verwacht men dat hij vooral van belang is in de buurt van glycinerge neuronen. Het is echter aangetoond dat niet alleen neuronen, maar ook astrocyten glycine kunnen secreteren, wat wijst op een rol van glycine als intercellulaire gliale messenger (of een "gliotransmitter"). De identificatie van de verschillende boodschappers en hun cellulaire targets (in het geval van glycine de glycine receptor of transporters) is van cruciaal belang om de complexe intergliale interactie die plaatsvindt in het centraal zenuwstelsel te begrijpen. De inzichten dat gliacellen cruciaal zijn voor het behoud van het normale homeostatische evenwicht van neuronen, hebben de "neurocentrische" visie verschoven naar een visie waarin neuropathologieën meer en meer beschouwd worden als primaire gliopathologieën (Nedergaard et al.., 2009). Bovendien is calcium één van de belangrijkste second messengers die zowel belangrijk is voor gliale als immuuncel intracellulaire signaaltransductie. Verstoringen in de normale gliale calcium signaaltransductie werden vastgesteld bij verschillende neuropathologieën. (bv. ischemie, epilepsie en neurodegeneratieve aandoeningen) (Verkhratsky, 2006; Nedergaard et al.., 2009). Met dit werk wordt aangetoond dat glycine en structureel verwante aminozuren calcium signalen kunnen moduleren. De bevindingen kunnen dan ook van belang zijn in pathologieën waar plaatselijk de concentraties van glycine (of andere aminozuren) sterk verhoogd zijn (millimolair). Alhoewel dit vrij hoge concentraties zijn (althans voor de calcium modulatie) die wellicht niet worden bereikt in de normale neurale micro-omgeving (normale CSV glycine concentraties zijn ongeveer 10 μM), is het toch belangrijk zich te realiseren dat de concentratie van neurotransmitters in het cytosol van de presynaptische neuronen kunnen oplopen tot 10 mM en in synaptische vesikels zelfs tot 100 mM. Dit betekent dat in de buurt van synapsen en zeker na de degeneratie van glycinerge neuronen, microgliale calcium signaaltransductie inderdaad kan worden gemoduleerd in de onmiddellijke omgeving. De inzichten in de rol van de transporters in deze modulatie kan potentiële targets bieden voor nieuwe therapieën voor verschillende ziekten waarbij is aangetoond dat microglia een cruciale pathogene rol spelen.

Concluderend kunnen we stellen dat deze PhD thesis de immuunmodulerende rol van glycine en van GlyR op humane T lymfocyten, microglia en macrofagen heeft onderzocht. Alhoewel alle onderzochte celtypes de GlyR tot expressie brengen, konden we geen functionele rol van deze receptoren aantonen. We vonden daarentegen wel een cruciale rol voor aminozuur transporters in de immuunmodulerende eigenschappen van glycine en enkele verwante aminozuren, zoals bv L-serine, waarbij via een modulatie van de intracellulaire calciumsignalen een effect op de effectorfuncties verondersteld werd. Dit werk werpt dan ook een nieuw licht op glycine (en bij extensie aminozuur) signaaltransductie in immuuncellen,

dewelke tot dusver weinig aandacht kreeg van de wetenschappelijke gemeenschap. Deze inzichten zijn belangrijp in het licht van nieuwe potentiële aangrijpingspunten van medicamenten, maar stellen ook belangrijke nieuwe vragen, dewelke van deze thesis een basis vormen voor verder onderzoek.

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CURRICULUM VITAE

Jimmy Van den Eynden was born on the 25th of May 1977 in Herentals (Belgium). In 1996, he finished his secondary grade education (mathematics and sciences) at the Sint-Jozefscollege in Herentals. In September of that year, he started studying Medicine at the "Limburgs Universitair Centrum" (now called Hasselt University) in Diepenbeek, where he got his bachelor in July 1999. He continued his Medicine studies at the Catholic University Leuven, where he graduated as a medical doctor with great distinction in June 2003. From 2003 to 2004 he worked as a physician at the nuclear medicine department of UZ Leuven. In June 2004 he obtained a teaching degree in medicine at the KULeuven and in September of 2004 he joined the group of Cell Physiology at Hasselt University where he taught Physiology at the faculty of Medicine and performed the research described in this thesis. In July 2008 he also graduated as a master in sports medicine at the KULeuven.

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- 5th Forum of European Neuroscience, Wenen, juli 2006.
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J. Van den Eynden, L. De Ceuninck, R. Dom, P Dupont, L Mortelmans, K. Van Laere. The clinical spectrum of early Parkinson's disease : a multitracer study in drug-naïve patients with and without resting tremor, in comparison to essential tremor. Society of Nuclear Medicine, 51st annual meeting, Philadelphia, juni 2004

Oral presentations

Glycine modulates microglial activity by a glycine receptor independent mechanism. EURON PhD-days, Aachen, 18 september 2008.

Glycine and L-serine modulate microglial activity. 2nd Annual Meeting of IAP network P6/31, Leuven, 7 januari 2009.

Glycine and L-serine enhance microglial calcium fluxes: a possible role for neutral amino acid transporters. 8th bi-annual meeting of the Belgian Society for Neuroscience, Luik, 11 mei 2009.

The Sentinel Node Procedure in Vulvar cancer. Scientific Meeting, Belgisch Genootschap voor Nucleaire geneeskunde, Brussel, december 2003.

Discriminant analysis of perfusion and dopamine transporter SPECT in the differential diagnosis of progressive supranuclear palsy, multiple system atrophy and dementia with Lewy bodies. Scientific Meeting, Belgisch Genootschap voor Nucleaire Geneeskunde, Brussel, 12 juni 2004.

Awards

Award best oral presentation. 12th Euron PhD Student meeting, RWTH Aachen Sept. 18-19, 2008

DANKWOORD

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