The role of inhibitory neurotransmitters on macrophage function during neuroinflammation

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List of Abbreviations

7-AAD:	7-amino-actinomycin D
Aib:	Alpha isobutyric acid
Alx:	Alx 5407
Amo:	Amoxapine
ANOVA:	Analysis of variance
bp:	Base pairs
CNS:	Central nervous system
CRAC:	Ca^{2+} release activated channel
CSF:	Cerebrospinal fluid
DAPI:	4'-6-Diamidino-2-phenylindole
DHR-123:	Dihydrorhodamine 123
DiI:	1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
DMSO:	Dimethyl sulfoxide
EAE:	Experimental autoimmune encephalomyelitis
EDTA:	Ethylenediaminetetraacetic acid
EPSP:	Excitatory postsynaptic potential
ER:	Endoplasmatic reticulum
FCS:	Foetal calf serum
GABA:	γ-aminobutyric acid
GABA-R:	GABA receptor
GABA-T:	GABA-transaminase
GABDH:	Glyceraldehyde-3-phosphate dehydrogenase
GAD:	Glutamate decarboxylase
GAT:	GABA transporter
Gly-R:	Glycine receptor
GLYT:	Glycine transporter
HOCl:	Hypochlorous acid
IgG:	Immunoglobulin G
IL:	Interleukin
iNOS:	Inducible nitrix oxide synthase
IPSP:	Inhibitory postsynaptic potential
LPS:	Lipopolysaccharide
M1:	Classically activated macrophages
M2:	Alternative activated macrophages
MS:	Multiple sclerosis
MTT:	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NO:	Nitric oxide
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction

PFA:	Paraformaldehyde
PMA:	Phorbol 12-myristate 13-acetate
ROS:	Reactive oxygen species
RR:	Relapsing-remitting
SNAT:	System A Na^+-coupled neutral amino acid transporter
T_A:	Annealing temperature
TauCl:	Taurine chloramine
TNF-α:	Tumor necrosis factor alpha
tPA:	tissue plasminogen activator

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Abstract

Gamma-aminobutyric acid (GABA) and glycine, the main inhibitory neurotransmitters of the mammalian central nervous system (CNS), also participate in the communication between the mammalian nervous and the immune system. In that respect, glycine (Gly-R) and GABA receptors (GABA-R) are expressed in various peripheral tissues and immune cells such as T-cells, neutrophils and macrophages. Moreover, glycine and GABA are protective in different animal models of inflammation. Although glycine and GABA levels are changed in multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE), the exact role in the disease process is unclear. Therefore, the **purpose of this study** was to determine the modulatory effect of GABA and glycine on macrophage functions playing a role in the disease process. Rat peritoneal macrophages clearly expressed the $GABA_A$-R, Gly-R and the Gly-R associated molecule gephyrin. Also glycine transporters 1 & 2 were detected. Furthermore, glycine dose-dependently increased the nitric oxide (NO) production and myelin phagocytosis of peritoneal rat macrophages, whereas ROS production was not affected. The Gly-R antagonist strychnine was not able to reverse the effect on NO production evoked by 1 mM glycine. This suggests that glycine modulates peritoneal rat macrophage function in a receptor-independent manner. The use of transporter blockers counteracts the effect of glycine, whereas the main substrate of glycine synthesis L-serine mimics it. This suggests that glycine exerts its effect through a yet unknown intracellular pathway. *In vitro* cultures of peritoneal rat macrophages pre-treated with GABA showed that GABA blunted increases in NO production, myelin phagocytosis and ROS production. Furthermore, this effect could be reversed by gabazine and saclofen, antagonists of the $GABA_A$-R and $GABA_B$-R respectively, indicating a role of the GABA-R in the GABA-mediated effects.

It can be concluded that GABA has an anti-inflammatory role and down regulates important macrophage effector functions involved in MS such as the NO synthesis, myelin phagocytosis and ROS production. In contrast, glycine leans more towards a pro-inflammatory response. Because neuroinflammation is a vital part in the pathogenesis of MS/EAE, the high concentration of neurotransmitters present in the CNS could influence the course of MS/EAE.

1 Introduction

Neurotransmission is a process that occurs when a neuron releases substances which transmit a signal to another neuron or target cell across a synapse. In this way cells can communicate with each other to regulate several functions such as sensation, perception and control of movement (1). In a chemical synapse neurotransmission is accomplished by diffusible neurotransmitter molecules and occurs in several steps (figure 1).

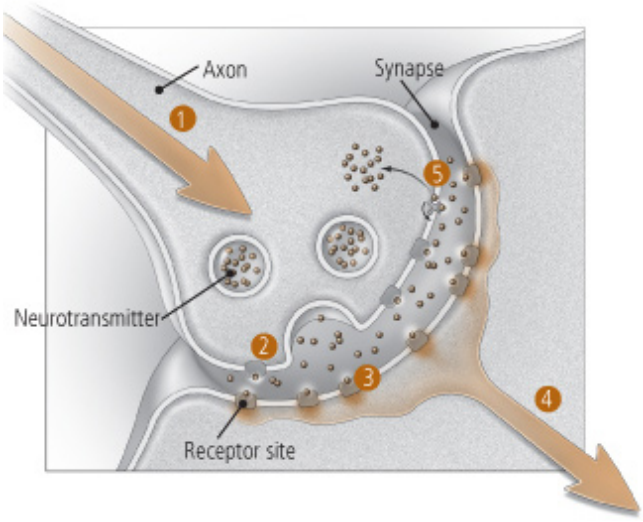

Figure 1: A schematic view of a chemical synapse. At step 1, an action potential arrives at the axon terminal. Next, vesicles containing neurotransmitters fuse with the presynaptic membrane due to an increase of intracellular Ca^{2+} occurs (step 2). Step 3 and 4 involves binding of the neurotransmitter to specific receptors on the postsynaptic cell membrane and transduction of signals to the cytoplasm. Lastly, transmission is stopped due to degradation, reuptake or diffusion of the neurotransmitter away from the synapse (step 5) (2, 3).

When an action potential arrives at the presynaptic terminal, a depolarisation of the nerve ending takes place. This results in the opening of voltage-gated calcium (Ca^{2+}) channels and an increase in intracellular Ca^{2+} concentration. Subsequently, vesicles containing the neurotransmitter fuse with the presynaptic membrane and the transmitters are released into the synaptic cleft, after which they diffuse across the synaptic cleft and bind to their receptors expressed on the postsynaptic membrane. Binding of the neurotransmitter can have an excitatory, inhibitory or modulatory effect on the target cell. Neurotransmission is terminated by enzymatic destruction of the transmitter, reuptake of the neurotransmitter in the presynaptic terminal or neighbouring cells or diffusion of the transmitter out of the synapse (2).

Neurotransmitters fall into three categories: amino acids, monoamines or neuropeptides (1). Besides the use of amino acids as an energy source, small amino acids such as glutamate, γ-aminobutyric acid (GABA) and glycine serve essential messenger functions within the central nervous system (CNS). The outcome of a stimulus depends on the neurotransmitter that is released. For example, glutamate is required for fast excitatory transmission by generating an excitatory postsynaptic potential (EPSP) due to opening of glutamate-gated channels. The opposite also exist by which neurotransmitters like GABA and glycine rather cause an inhibitory postsynaptic

potential (IPSP) than an EPSP. The excitability of neurons thereby depends on the balance between excitation and inhibition, because most central neurons have excitatory as well as inhibitory synapses (2).

1.1 Inhibitory neurotransmission

Glutamate is used as a neurotransmitter in most excitatory synapses. However, the level of excitation is under regulatory control of inhibitory neurotransmitters like GABA and glycine (4). GABA is used as a neurotransmitter in at least 40% of all inhibitory synaptic processes and is established as the most important inhibitory neurotransmitter in the mammalian CNS (5).

1.1.1 GABA

In the adult CNS, GABA is involved in inhibitory neurotransmission that regulates the activity of glutamate. Furthermore GABA plays a role in the expression of higher brain functions such as memory, learning and anxiety. In the immature CNS, GABA controls brain morphogenesis which includes cell proliferation and migration, formation of synapses, axonal growth, steroid-mediated sexual differentiation and cell death (6).

GABA is an amino acid that is synthesized from glutamate by the enzyme glutamate decarboxylase (GAD). By this process, the principle excitatory neurotransmitter glutamate is converted into the main inhibitory neurotransmitter GABA (4). In the nervous system, GABA is present in high concentrations in synaptic terminals. However in the extracellular space the concentration varies from 0 to 1 µM. Outside the brain, GABA is also found in several tissues and has a plasma concentration of 100 µM in healthy individuals. This concentration is in line with the GABA levels surrounding CNS neurons (7). It is suggested that the plasma levels of GABA reflect the GABA concentrations or activity in the brain. However the precise source of GABA in the plasma is not known yet (8).

When GABA is released at the presynaptic membrane, it binds to postsynaptic GABA receptors (GABA-R) which can be subdivided into $GABA_A$, $GABA_B$ and $GABA_C$ receptors. As illustrated in figure 2A, $GABA_A$ and $GABA_C$ receptors are ionotropic receptors, composed of several subunits which assemble into a heteropentameric receptor. $GABA_A$-R are build out of a combination of $\alpha_{1\text{-}6}$, $\beta_{1\text{-}4}$, $\gamma_{1\text{-}4}$, δ or π subunits. In contrast, only $\rho_{1\text{-}3}$ subunits can be found in the $GABA_C$-R. Despite the presence of a number of subunits, certain subunits are principally expressed. For example, the $GABA_A$-R composed of two α_1, two β_2 and one γ_2 subunits is mainly expressed in the CNS (9). Each subunit has a large extracellular N-terminus and a short extracellular C-terminus. In between there are four membrane spanning regions and a large cytoplasmic loop flanked by the third and fourth membrane spanning region. Five subunits together form a chloride channel that allows the flux of chloride into the cell thereby causing hyperpolarisation (9).

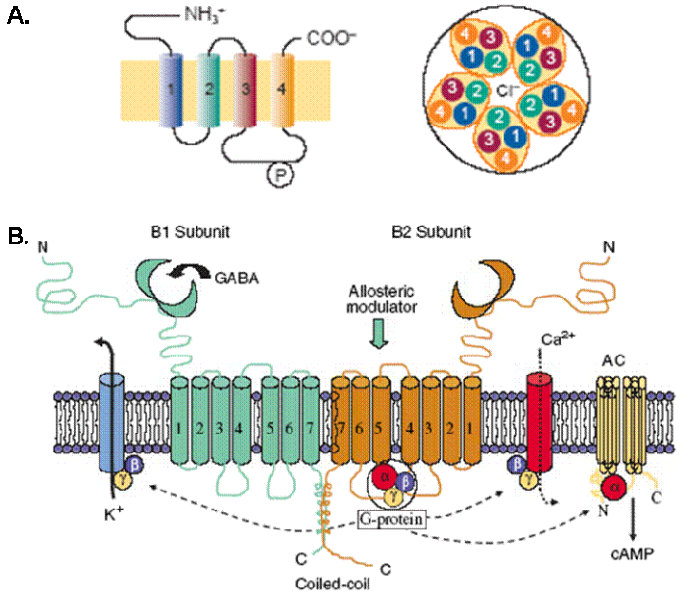

Figure 2: Schematic view of the different GABA-R. (A) $GABA_A$ and $GABA_C$ receptors are composed of five subunits forming a chloride channel. Each subunit consists out of 4 transmembrane spanning regions and a cytoplasmic loop for posttranslational modifications and interactions with different proteins. (B) $GABA_B$ receptors are composed of two subunits B1 and B2 coupled via a coiled-coil of their C-termini. The B1 subunit contains the GABA-binding site whereas the B2 subunit provides the G-protein coupling to adenylate cyclase (AC), K^+ and Ca^{2+} channels and a site for allosteric modulation (5, 10).

Besides the binding of GABA to ionotropic receptors, GABA can also interact with a metabotropic receptor (5). This $GABA_B$-R is very distinct from the $GABA_A$-R and $GABA_C$-R as seen in figure 2B. The receptor is produced as two separated subunits B1 and B2 which consist both of 7-transmembrane spanning monomers. In the endoplasmatic reticulum the C-terminus of the two subunits form a coiled-coil which allow their combined transport for insertion into the plasma membrane. Each subunit has it own function. $GABA_{B1}$ contains the GABA-binding domain whereas the $GABA_{B2}$ subunit is involved in the G-protein coupling of the receptor to adenylate cyclase, K^+ and Ca^{2+} channels. The $GABA_{B2}$ subunit also contains an allosteric site which modulates the response to an agonist (5). $GABA_B$-R are expressed throughout the CNS with the highest level at the thalamic nuclei, cerebellum, cortex, hippocampus, basal ganglia and the dorsal horn of the spinal cord. They act postsynaptically to cause a long-lasting hyperpolarisation and presynaptically to inhibit neurotransmitter release (11).

After activating its receptor, neurotransmission is ended by reuptake of GABA via high affinity transporters and degradation by GABA-transaminase (GABA-T). Various GABA transporters (GAT) exist such as GAT-1, GAT-2, GAT-3 and GAT-4 which are all highly dependent on Na^+ and less on Cl^-. GAT-1 and GAT-4 are located on presynaptic neurons and surrounding astrocytes. However, GAT-1 is more abundantly expressed than GAT-4. GAT-2 has a more dendritic localization and GAT-3 has the highest density in extra-synaptic regions. In the brain GAT-1 and GAT-4 account for the largest uptake of GABA (4).

1.1.2 Glycine

Another important inhibitory neurotransmitter is glycine which is involved in reflex responses, voluntary motor control and sensory signal processing in the CNS. Glycine is known to be the main inhibitory neurotransmitter in the gray matter of the spinal cord and brain stem. However lower levels of glycine and its receptor are also found in the midbrain, hypothalamus and thalamus but are more or less absent in the spinal cord white matter and the higher brain (12-14).

Glycine is a non-essential amino acid that is mainly synthesized from the amino acid serine by the enzyme serine hydroxymethyltransferase (15). When glycine is released at the presynaptic membrane, it binds to specific glycine receptors (Gly-R) expressed at the postsynaptic membrane. This receptor consists of three distinct polypeptides, an α subunit, β subunit and gephyrin. Several isoforms of the α subunit exist and are termed respectively α_{1-4}, but only one β subunit is known (5, 12). The β subunit associates with gephyrin, which in turn links the receptor complex to the cytoskeleton via attachment to tubulin (5, 12). Functional Gly-R can be either homomeric or heteromeric, composed of five α subunits or a combination of two α and three β subunits, respectively. A receptor composed of only β subunits is not functional. Because of the possibility of different subunit arrangements, functional properties of the receptor can be somewhat different. Furthermore, subunit composition is regulated by the region of expression or development stage (5, 12, 13). In that respect, fetal Gly-R are homomeres composed of only α_2 subunits, whereas adult receptors form a heteropentameric receptor of α_1 and β subunits (5, 12).
As is the case with the $GABA_A$ and $GABA_C$ receptor, each subunit of the Gly-R has a extensive extracellular N-terminus, four membrane spanning regions and a short extracellular C-terminus. A large cytoplasmic loop is present between the third and fourth membrane spanning region (see figure 3). Five subunits together form a chloride channel that allows the flux of chloride into the cell thereby causing a hyperpolarisation (12). Gly-R activity can be regulated by several substances such as taurine and β-alanine which function as agonists, and strychnine which blocks the glycine receptor.

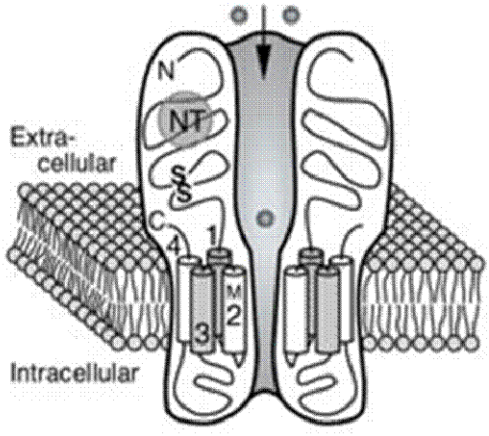

Figure 3: Schematic view of the Gly-R. Gly-R are composed of five subunits forming a chloride channel. Each subunit consists out of 4 transmembrane spanning regions (M1-M4) and a cytoplasmic loop for posttranslational modifications and interactions with different proteins. Figure adapted from Paas et al. (16). NT, neurotransmitter binding site.

The synaptic action of glycine ends by uptake of glycine through Na^+ and Cl^--coupled transporters that are located in neuroglia and neurons. The transport of one glycine molecule involves the transport of one Cl^- ion and two or three Na^+ ions dependent on which of the two glycine transporters (GLYT) are expressed, GLYT1 or GLYT2, respectively. Other differences between the two transporters exist in their location and distribution throughout the CNS. Although both the GLYT1 and GLYT2 are expressed in caudal regions of the brain which confirms the association with the inhibitory glycinergic neurotransmission, GLYT2 expression is more restricted to spinal cord, brain stem and cerebellum. Moreover, GLYT1 is mostly present in astrocytes whereas GLYT2 is virtually only present on axons and presynaptic membranes (14).

1.2 Inhibitory neurotransmitters and the immune system

GABA and glycine are the main inhibitory neurotransmitters of the mammalian CNS. Besides their role in mediating fast inhibitory neurotransmission within the CNS, these neurotransmitters participate in the communication between the mammalian nervous and the immune system.

1.2.1 GABA and the immune system

The presence of GABA at sites of inflammation, where it could modulate the ongoing immune responses, indicates a possible role of GABA in the interaction between both systems (17). Outside the CNS, GABA, GAD and GABA-R are expressed in various tissues such as the adrenal gland and pancreas (18-20). Also immune cells like T-cells and macrophages express subunits of the $GABA_A$-R (21, 22). Moreover, different animal models show clear anti-inflammatory effects of GABA (21, 23).

In that respect, Han et al. reported that GABA accelerates the healing process by suppressing inflammation and stimulating reepithelialization. *In vitro*, GABA significantly inhibited the mRNA expression of inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) in lipopolysaccharide (LPS)-stimulated mouse macrophage RAW 264.7 cells. *In vivo*, cutaneous open wounds of rats were less inflamed and exhibited reduced wound areas when treated with GABA as compared to a control group (23). From this study it can be concluded that GABA reduces inflammation, both *in vitro* and *in vivo*, which is also confirmed by other groups. In a mouse model for type 1 diabetes, administration of GABA inhibited autoreactive T cell responses and retarded disease progression in NOD mice which had already established autoimmunity. Moreover, GABA reduced proliferation of autoimmune T-cells *in vitro* (21). This was mimicked by $GABA_A$-R agonists and blocked with $GABA_A$-R antagonists which suggests a $GABA_A$-R mediated effect. This was confirmed by RT-PCR, demonstrating the presence of the $GABA_A$-R on $CD4^+$ T cells (21).

Another study identified the expression of the $GABA_A$-R on peritoneal mouse macrophages. When GABA was added to mouse macrophage cultures, it decreased IL-6 and IL-12 production (24). Moreover, the group of Stankevicius et al. showed that a non-competitive antagonist of the $GABA_A$-R (picrotoxin) increased the respiratory burst after phorbol 12-myristate 13-acetate (PMA) or Staphylococcus aureus stimulation of mouse macrophages (25).

1.2.2 Glycine and the immune system

As is the case with GABA, glycine plays an important role in the communication between the CNS and the immune system. This fact is supported by several studies that show the presence of the glycine receptor on Kupffer cells (26, 27), alveolar macrophages (27, 28) and neutrophils (27, 29). Even other cells in the periphery and immune cells express glycine receptors as is demonstrated by the group of Yamashina et al. which detected glycine receptors on bovine endothelial cells (30). Moreover, different *in vitro* and *in vivo* studies show a protective role of glycine during inflammation. *In vitro* studies revealed that treatment of rat alveolar macrophages with glycine blunts the production of pro-inflammatory mediators such as TNF-α and superoxide (28). This protective role of glycine is also confirmed in rat neutrophils (29) and the resident macrophages of the liver, the Kupffer cell (13), where glycine also suppressed pro-inflammatory cytokines and superoxide production. Further confirmation about the influence of glycine on immune cells came from Spittler et al. They showed a glycine-induced inhibition of TNF-α and IL-1 production and an increase in IL-10 production in isolated blood monocytes (31). Also T cells are sensitive to glycine which was proven by the group of Stachlewitz. In this case glycine dose-dependently reduced the proliferation of rat T lymphocytes with a maximum suppression of 60%. Suppression was reversed when 1 μM strychnine, a Gly-R antagonist, was used (32).

In several *in vivo* studies the beneficial effect of glycine was proven. In general, dietary glycine administration causes an elevation of blood levels of glycine from basal levels of 0,1-0,2 mM to 1 mM. This increase in glycine concentration improved the recovery from shock, alcoholic liver injury and nephrotoxicity due to certain drugs (13). In a mouse model where liver damage was induced by intraperitoneal injection of LPS, mice treated with dietary glycine had improved liver function and diminished systemic inflammation compared to LPS treated controls. Moreover, morphological changes induced by the injection of LPS were significantly reduced when mice were given a dietary supplementation with glycine. Lastly, Kupffer cells isolated from the liver of these mice showed a reduction in TNF-α and an increase in IL-10 production after glycine treatment (33). This modulating effect of glycine on cytokine production further confirms that glycine plays an anti-inflammatory role in Kupffer cells.

Besides the clear role of glycine on inflammation, a study done by the group of Yamashina et al. has revealed glycine as a potent anti-angiogenic nutrient for tumor growth by preventing the proliferation and migration of endothelial cells in mice (34). Moreover, dietary glycine also diminished the growth of B16 myeloma cells transplanted in mice (34). These facts prove that glycine not only modulates inflammation but also has a role in the control of carcinomas.

Although glycine has a clear protective effect on various immune cells and animal models, controversy remains about the involved mechanism. Although the majority of scientists support the hypothesis that glycine mediates the effect in a glycine receptor-dependent way, as is proven by several studies done with the Gly-R antagonist strychnine (13, 27-29), other studies elucidate alternative pathways such as the down regulation of Toll like receptors (33, 35).

Multiple studies have proven that GABA and glycine participate in the communication between the nervous and immune system. The expression of GABA and glycine receptors on various immune cells and tissues, confirm this interaction. Moreover GABA and glycine play an immunomodulatory

role on various immune cells and animal models of inflammation. This might be of importance for neuroinflammatory diseases such as multiple sclerosis.

1.3 Multiple sclerosis

MS is a chronic inflammatory autoimmune disease of the CNS. It is characterized by inflammation, demyelination, axonal degeneration and gliosis (scarring). MS affects 1 out of 1000 people in the Western world and it leads to chronic disability in mostly young adults. The disease begins mainly at the age of 30 and can follow different clinical courses (36, 37).
The most common clinical course is relapsing-remitting (RR) MS usually followed by a chronic progressive form. At the first stage inflammatory lesions arise in the nerve tissue. These lesions consist of multiple cell types such as T cells, macrophages and B cells. Due to the immune infiltrate and accompanied inflammation process, the myelin producing cells or the myelin, which surrounds and insulates axons, are damaged. This results in a disturbance of the pulse propagation between neurons. Between various periods of inflammation and demyelination also episodes of recovery take place. Later on, the RR-phase proceeds into a chronic progressive form, where the emphasis lies more on neurodegeneration and not on inflammation. In this phase there is a further accumulation of the damage that was previously caused by inflammation (36-38).
In a minority of patients, the disease is progressive from the beginning although relapses and remissions could be superimposed (36).

MS is a complex disease with many participating factors such as T cells, macrophages, B cells and cytokines, but the etiology and pathology behind the disease remain unknown. Although in the classic model, T helper cells play a crucial role in the initiation of MS (39), infiltrating macrophages are the main effector cells. This is proven by the fact that a depletion of macrophages in acute experimental autoimmune encephalomyelitis (EAE), the animal model of MS, leads to a complete suppression of clinical symptoms (40). During MS, monocytes are activated in the periphery where after they migrate through the blood-brain barrier and differentiate into macrophages. Subsequently these macrophages start to produce inflammatory mediators that damage the myelin sheats and induce axonal loss ultimately leading to a conduction block and neurodegeneration (41). The role of soluble mediators was confirmed by a study done by Alcazar et al. where they induced axonal damage *in vitro* by adding cerebrospinal fluid (CSF) from MS patients to axonal cell cultures (42).

Macrophages produce various pro-inflammatory cytokines like TNF-α, IL-1 and IL-6 which are neurotoxic *in vitro*. For example TNF-α causes oligodendrocyte apoptosis which lead to an increase in demyelination and a rise in the vulnerability of axons to other factors (43).
Another group of mediators produced by macrophages are reactive oxygen species (ROS) and nitric oxide (NO). Although cells have several antioxidant mechanisms against low concentrations of ROS, macrophages produce these radicals at a high concentration during inflammation. In this manner, antioxidant mechanisms cannot compete with the abundance of radicals and therefore ROS cause oxidative damage to proteins, lipids and nucleic acids (41). Moreover, superoxide radicals are increased in the serum of MS patients (44) and ROS were shown to induce DNA damage in MS patients leading to neurodegeneration (45, 46). Also, NO is detrimental during CNS

inflammation. It is very reactive and forms products such as peroxynitrite and 3-nitrotyrosine which are capable of damaging myelin and axons. This idea is supported by a study showing a decrease in axonal necrosis after treatment of EAE animals with a NOS inhibitor (47). Furthermore, NO products are present in serum and CSF of MS patients (48) and in active MS and EAE lesions, iNOS and NO products are mainly present in macrophages and microglia (41). This confirms the role of macrophages as the most important effector cells in MS.

Glutamate, which in large quantities causes excitotoxicity to neurons resulting in cell death, is also secreted by macrophages (49). Moreover, clearance of glutamate is suppressed by different macrophage products such as TNF-α, ROS and arachidonic acid (41). Lastly, the protease tissue plasminogen activator (tPA) is secreted by macrophages and has been reported to induce neuronal apoptosis *in vitro* (50, 51). Furthermore, Cuzner et al. reported an increase of tPA in active plaques of MS patients (52).

In addition to the release of various mediators, macrophages can directly damage myelin. Macrophages are capable to phagocytose and degrade myelin (53). Macrophages that are present in the lesions are filled with myelin which was shown by histological stainings done by Brück et al. (54). However, it needs to be mentioned that macrophages have a dual role in MS and also possess anti-inflammatory properties. Besides the classically activated macrophages or M1, also alternative activated macrophages (M2) exist. M1 macrophages play a pro-inflammatory role whereas M2 macrophages are involved in the scavenging of debris, promoting tissue remodelling and repair. Moreover these M2 macrophages express anti-inflammatory molecules. This was supported by findings of Boven et al. which demonstrate that myelin-laden (foamy) macrophages express several markers involved in anti-inflammatory responses. Furthermore these macrophages were inhibited to react to pro-inflammatory responses (55).

It can be concluded from aforementioned studies that infiltrating macrophages are the most important effector cells in MS. This makes the macrophage an interesting target for therapy to reduce the damage resulting from myelin phagocytosis and soluble pro-inflammatory mediators during neuroinflammation.

1.4 The effect of inhibitory neurotransmitters on macrophage function during neuroinflammation

It is well established that there are bidirectional interactions between the mammalian nervous and immune system. GABA and glycine, both important inhibitory neurotransmitter of the CNS, play a significant role in this interaction as is proven by their presence outside the CNS. The Gly-R and GABA-R are expressed in various peripheral tissues and immune cells such as T-cells, neutrophils and macrophages. Moreover GABA and glycine have an anti-inflammatory effect in different animal models of inflammation. Such an immunomodulatory role might be of importance for neuroinflammatory diseases like MS. In that respect, glycine levels are increased in the spinal cord and brain stem of rats during EAE (56). This is also demonstrated in the plasma and CSF of MS patients. In contrast, conflicting results are published concerning the concentration of GABA in MS patients and EAE animals. Some studies reveal a decrease in GABA levels in the spinal cord and brain stem of EAE rats and in the CSF of MS patients (56, 57), while others find an elevation of

GABA levels in MS patients related to the presence or exacerbation of spinal cord lesions (58). The differences in glycine and GABA concentrations between healthy controls and MS patients/EAE animals indicate a possible role of these inhibitory neurotransmitters in the disease pathogenesis.

Although glycine and GABA levels are changed in MS/EAE, the exact role in the disease process is unclear. Because macrophages are the main effector cells in MS, the **aim** of this study is to determine the **modulatory effect** of GABA and glycine on macrophage functions related to neuroinflammation. First, the presence of the GABA and glycine receptor/transporter on primary peritoneal rat macrophages is investigated by means of immunocytochemistry. Moreover, polymerase chain reactions (PCR) are performed on the cDNA of peritoneal macrophages for various Gly-R subunits. Next, peritoneal rat macrophages are cultured *in vitro* and treated with different concentrations of GABA or glycine to examine the effect on various macrophage functions, like the phagocytosis of fluorescent-labelled myelin, the formation of ROS and the production of NO. Secondly, cell cultures are performed with receptor agonists and antagonists to investigate if the effect on macrophage functions is receptor-dependent or not.

In this manner, this study tries to elucidate the effect of GABA and glycine on macrophages functions important in the disease process of MS. More generally, this study also contributes to the insights already obtained in the field of the interaction of inhibitory neurotransmitters and immune cells. Moreover, macrophages are involved in various inflammatory processes. Therefore, knowledge acquired from this study could be applied to other inflammatory diseases. Ultimately, GABA and glycine and its mechanism could be exploited as a new approach for pharmaceutical companies in developing new therapies for inflammatory diseases.

2 Materials and methods

This study was aimed at examining the modulatory effect of inhibitory neurotransmitters like GABA and glycine on macrophage functions during neuroinflammation. In the following sections the various methods that were applied will be explained.

2.1 Macrophage isolation and cultures

Macrophages were isolated by intraperitoneal injection of phosphate buffered saline (PBS, Lonza, Belgium) supplemented with 5 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, Belgium) in 9 week old Wistar rats (Harlan, The Netherlands). After 5 minutes of incubation with EDTA, the solution was removed and centrifuged for 5 minutes at 1400 t/min. Subsequently macrophages were counted and dissolved at 1 x 10^6 cells/ml into the culture medium that consisted of MEM (GIBCO, Belgium) supplemented with 1% Penicilline-streptomycin (GIBCO, Belgium), 20 mM L-glutamate and 10% foetal calf serum (FCS, Hyclone; Belgium). Cells were plated out into a 24 or 96 well plate according to the experiment that followed. After two hours of incubation cells were washed and various concentrations of GABA or glycine (both Sigma-Aldrich, Belgium) were added. Co-incubation experiments were done with 1 mM GABA or 1 mM glycine in combination with diverse receptor antagonists or transporter blockers (see table 1).

Table 1: Overview of the receptor agonist, antagonist and transporter blockers used in the co-incubation experiments with 1 mM GABA or 1 mM glycine.

	Concentration	Function
Gabazine	30 µM	$GABA_A$ receptor antagonist
Bicuculline	30 µM	$GABA_A$ receptor antagonist
Saclofen	30 µM	$GABA_B$ receptor antagonist
Strychnine	1 µM	Glycine receptor antagonist
Taurine	1 mM	Glycine receptor agonist
β-alanine	1 mM	Glycine receptor agonist
L-serine	1 mM	Substrate for glycine synthesis
Alx 5407	10 nM	Glycine transporter 1 blocker
Amoxapine	10 µM	Glycine transporter 2 blocker
Alpha isobutyric acid	1 mM	General transporter blocker

2.2 Fluorescent Immunocytochemistry

Fluorescent immunocytochemistry was applied to examine the expression of GABA-R and Gly-R, gephyrin (a Gly-R associated molecule) and GLYT1&2 on peritoneal rat macrophages. Coverslips were coated with poly-L-lysine (Sigma-Aldrich, Belgium) for 30 minutes before use. Peritoneal rat macrophages were cultured at a density of 3 x 10^5 cells/coverslip. After two hours of incubation, coverslips were washed to remove unattached cells. Twenty-four hours later, fluorescent immunocytochemistry was performed at room temperature. First, coverslips were washed with PBS

and fixed in methanol/acetone (90/10). Next, Cells were blocked with 3% goat serum (Chemicon, Belgium) or 3% donkey serum (Chemicon Belgium) in PBS for 30 minutes. Subsequently, cells were washed with culture medium and incubated with primary antibodies (1/200; Invitrogen, Belgium) against the $\alpha_{1\text{-}4}/\beta$ subunits of the Gly-R, $\beta_2\text{-}\beta_3$ subunit of the $GABA_A$-R, GLYT1&2 and gephyrin, a Gly-R associated molecule for one hour. Then cells were washed and incubated with secondary Alexa-488 or Alexa-555 antibody (1/200; Invitrogen, Belgium) staining for another one hour. Table 2 shows an overview of the primary and secondary antibodies that were used. Afterwards nuclear staining was performed with 4'-6-Diamidino-2-phenylindole (DAPI; Invitrogen, Belgium) and macrophages were analysed and photographed with the Nikon eclipse 80i microscope. For the experiments with the $GABA_A$-R immunostainings on ice and at room temperature, cells were fixed and permeabilized after adding the primary and secondary antibodies. In this case 4% paraformaldehyde (PFA) was added for 10 minutes in the fixation step and macrophages were permeabilized with 0,2% Triton X-100 in PBS for 10 minutes.

Table 2: Overview of the primary and secondary antibodies (Invitrogen, Belgium) used in the immunocytochemistry stainings of the Gly-R, $GABA_A$-R, glycine transporter 1&2 and gephyrin. IgG, immunoglobulin G; Gly-R, glycine receptor; $GABA_A$-R, $GABA_A$ receptor; GLYT1&2, glycine transporter 1&2.

	Primary antibody	**Secondary antibody**
$\alpha_{1\text{-}4}/\beta$ subunits of the Gly-R	Mouse anti-rat IgG	Goat anti-mouse Alexa 488
$\beta_2\text{-}\beta_3$ subunit of the $GABA_A$-R	Mouse anti-rat IgG	Goat anti-mouse Alexa 555
GLYT1	Goat anti-rat IgG	Donkey anti-goat Alexa 488
GLYT2	Sheep anti-rat IgG	Donkey anti-sheep Alexa 488
Gephyrin	Mouse anti-rat IgG	Goat anti-mouse Alexa 488

2.3 RNA isolation and cDNA synthesis

Peritoneal rat macrophages were cultured for 24 hours and RNA was isolated from 2,5 x 10^6 cells with Rneasy Plus (Qiagen, The Netherlands) according to the manufacturer's manual. Next, cDNA was synthesized using a cDNA synthesis kit (Promega, The Netherlands). Hereby 10 µl of RNA was added to a reagens mix containing 4 µl 25 mM $MgCl_2$, 2 µl 10x reverse transcriptase buffer, 2 µl 10 mM dNTP, 0,5 µl rRNAse inhibitor, 0,5 µl AMV reverse transcriptase and 1 µl oligo dT primer. Samples were incubated for 60 minutes at 42°C, 5 minutes at 90°C and 10 minutes on ice followed by a purification of the cDNA by adding a mixture of nuclease-free water, fenol and chloroform/isoamylalcohol (24/1). Afterwards cDNA was washed with chloroform/isoamylalcohol (24/1) followed by a overnight precipitation step at 20°C with sodium acetate (3M, pH = 5,2) and cold 100% ethanol. The next day, pellets were washed with cold 70% ethanol followed by air drying the pellets for 15 minutes. Pellets were resuspended in nuclease-free water and stored at -20°C. Concentration of the RNA and cDNA was determined with the Nanodrop spectrophotometer (Isogen, The Netherlands).

2.4 Polymerase chain reaction

A PCR was performed in the first place to check the cDNA synthesis using a PCR for the household gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR program used in the BioRad

iCycler contained a denaturation phase of 5 minutes at 95°C followed by 35 cycles containing a next denaturation phase of 20 seconds at 95°C, an annealing phase of 20 seconds at 60°C and an elongation phase of 40 seconds at 72°C. After the 35 cycles, an extra elongation phase followed 6 minutes at 72°C after which the samples were stored at 4°C. Next, a PCR was performed for the various subunits of the Gly-R and the primer sequences that were used are shown in table 3. The mastermix contained 24 µl per 1 µl sample: 10% 10x Mg buffer (Roche, Belgium), 4% forward primer (Eurogentec, Belgium), 4% reverse primer (Eurogentec, Belgium), , 1% dNTP's (Roche, Belgium) and 0,6% Taq DNA polymerase (Roche, Belgium) dissolved in MilliQ. The PCR program used in the BioRad iCycler for the amplification of Gly-R subunits or gephyrin contained a denaturation phase at 5 minutes at 95°C followed by 35 cycles containing a next denaturation phase of 20 seconds at 94°C, an annealing phase of 20 seconds with different annealing temperatures for each primer set as seen in table 2 and an elongation phase of 40 secondes at 72°C. After the 35 cycles, an extra elongation phase followed of 6 minutes at 72°C after which the samples were stored at 4°C. As a positive control cDNA from rat spinal cord was used.

Table 3: Overview of Gly-R subunits and gephyrin with their primer sequences, length of amplification product and annealing temperature (T_A, °C). bp, base pairs.

Subunit	Forward primer	Reverse Primer	Length	T_A
Glycine receptor α_1	CCGTCTCGCCTACAATGAAT	TCCATCGGGAAATTCTTCAG	231 bp	53
Glycine receptor α_2	ATGGARGTCCAGACCTGTACAATG	GCAGTGACCCATCCCATAACCGCT	600 bp	51
Glycine receptor α_3	GGCTGAAGGACTCACTTTGC	TACCCGAGCCGGAGCTGCAT	226 bp	58,7
Glycine receptor α_4	CCACGTCTGGCCTACCGAGAA	GTAGCCCATCTGCCGCTCC	457 bp	58,7
Glycine receptor β	GAAGAACACTGTGAACGGCA	GGCTTCTTGTTCTTTGCCTG	228 bp	55
Gephyrin	CCATGGGGGAAAAGGACTAT	GGATTCCCTGGTAGTGCAAA	160 bp	55

PCR products (sample and 5 µl Orange G) were analysed on a 1% agarose (Invitrogen, Belgium) gel, Gel electrophorese ran at 120 V in a TAE buffer with ethidium bromide. The agarose gel was analysed with the program Quantity one.

2.5 Griess assay and MTT assay

A Griess assay was performed on the supernatant of cultured macrophages to assess NO production. After a 24 hours pre-treatment of macrophages with GABA (0mM-1mM) or glycine (0mM-1mM) alone or co-incubation of 1mM GABA/glycine and receptor antagonists or transporter blockers (see table 1), macrophages were stimulated for another 24 hours with 10 ng/ml LPS (Calbiochem, United Kingdom). Subsequently NO measurements were carried out on the cell supernatant according to the manufacturer's instructions (Griess reagent system; Promega, The Netherlands). This assay measures not directly the NO but determines the concentration of a stable and non-volatile breakdown product, nitrite. The nitrite concentration is a representation of the actually NO level present in the culture medium.

2.6 MTT assay

After removing the supernatant for NO measurements, the macrophages were used in a 3-(4,5-

Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to assess cell viability. Hereby 100 µl culture medium with 0,5 mg/ml MTT (Sigma-Aldrich, Belgium) was added to the macrophages for 4 hours. During this time, the MTT will be reduced by mitochondrial reductase enzymes to a purple formazan in living cells. Next, the supernatant was removed and the formazan was made soluble by adding a solution containing 150 µl Dimethyl sulfoxide (DMSO; VWR, Belgium) and 25 µl 0,1 M glycine (Sigma-Aldrich, Belgium) per well. Lastly, the absorbance of the colour reaction was quantified by a spectrophotometer (Biorad Benchmark, Belgium) at a wavelength of 550 nm compared to a reference wavelength of 630 nm.

2.7 Myeline phagocytosis assay

To define the myelin phagocytosing capacity of peritoneal rat macophages, myelin was isolated from adult rat brains by the method of Norton and Poduslo (59) and labelled with the fluorescent dye 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Sigma-Aldrich, Belgium). Myelin was incubated for 30 minutes with DiI (12,5 µg DiI for each mg of myelin), after which excess DiI was removed by washing with PBS. Stocks were made at a final myelin concentration of 10 mg/ml and stored at -20°C.

After pre-treatment of macrophages for various time points with different concentrations of GABA or glycine, DiI-labelled myelin (20 µg/ml) was added to the cultures for 90 minutes. Fluorescence intensity (FL-2 channel), which represents the amount of myelin phagocytosed by macrophages, was analysed by flow cytometry (FACSCalibur; Becton Dickinson, Belgium).

2.8 7-AAD assay

A 7-amino-actinomycin D (7-AAD; BD Pharmingen, Belgium) staining was performed to determine the amount of cell death. 7-AAD intercalates into double-stranded DNA of dying or dead cells and is excluded by viable cells. First peritoneal rat macrophages were incubated for 20 minutes with 7-AAD (1/25 diluted in FACS buffer) after which cells were washed with FACS buffer containing PBS, 2% FCS and sodium azide. Fluorescence intensity (FL-3 channel) was analysed by flow cytometry.

2.9 Dihydrorhodamine assay

Macrophages pre-treated for various time points with 1 mM GABA or glycine were stimulated for 15 minutes with 100 ng/ml PMA (Sigma-Aldrich, Belgium) to induce ROS production. Next, cells were incubated for 15 minutes with 5 µM dihydrorhodamine 123 (DHR-123; Sigma-Aldrich, Belgium) for another 15 minutes. During this time, the DHR-123 will be oxidised to rhodamine, a fluorescent substance, by intracellular ROS and especially hydrogen peroxide. The fluorescence intensity (FL-2 channel), which is a reflection of the amount ROS produced, was measured by flow cytometry.

2.10 Statistical analysis

Analysis of variance (ANOVA) and student T test were applied to evaluate the effect of GABA and glycine on the different effector functions of peritoneal rat macrophages. Statistical significance was set at a p-value of 0,05 and indicate by one star in the figures. Statistical significance of $p<0,01$ or $p<0,001$ were respectively noted as two or three stars.

3 Results

In this study, the modulatory effect of GABA and glycine on macrophage functions important in neuroinflammation were determined. First, the presence of the $GABA_A$-R, Gly-R and GLYT on peritoneal rat macrophages was investigated by means of PCR and immunocytochemistry. Next, we tested if GABA or glycine influenced macrophage functions, such as the phagocytosis of myelin, the formation of ROS and the production of NO. Moreover, various receptor agonists, antagonists and transporter blockers were used to examine if the observed effects were receptor-dependent or not.

3.1 Peritoneal rat macrophages express $GABA_A$-R, Gly-R, gephyrin and GLYT1&2

To investigate the expression of the Gly-R and the Gly-R associated molecule gephyrin on mRNA level, a PCR for the various Gly-R subunits and gephyrin, was performed on peritoneal rat macrophages. Rat spinal cord was used as a positive control. In rat spinal cord, all α subunits (α_1-α_4), the β subunit and gephyrin were expressed (figure 4). In contrast, macrophages only showed the presence of the α_4 subunit and gephyrin. While there was a band for the α_3 subunit, this did not have the correct length and therefore was aspecific.

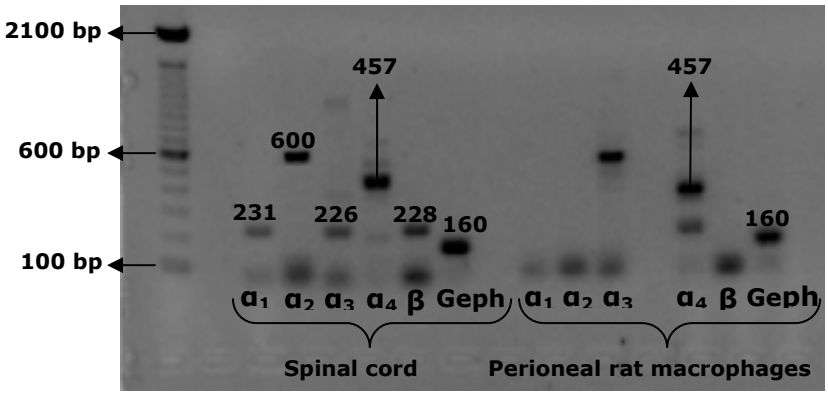

Figure 4: Gly-R subunits and gephyrin mRNA expression of peritoneal rat macrophages and spinal cord. All subunits of the Gly-R (α_{1-4}, β) and gephyrin were present in rat spinal cord (positive control), whereas in the peritoneal rat macrophages only the α_4 subunit and gephyrin were detected. bp, base paires; Geph, gephyrin.

Next, fluorescent immunocytochemistry with antibodies directed against the $GABA_A$-R, Gly-R and gephyrin was carried out on peritoneal rat macrophages. As shown in figure 5B&D, the $GABA_A$-R and Gly-R were clearly expressed. Moreover, gephyrin, involved in the anchoring of the Gly-R to the cytoskeleton, was also present (Figure 5C). Furthermore, the expression of GLYT1&2 was examined on the macrophages. As with the Gly-R, also GLYT1&2 were clearly detected on rat peritoneal macrophages on protein level (Figure 5E&F).

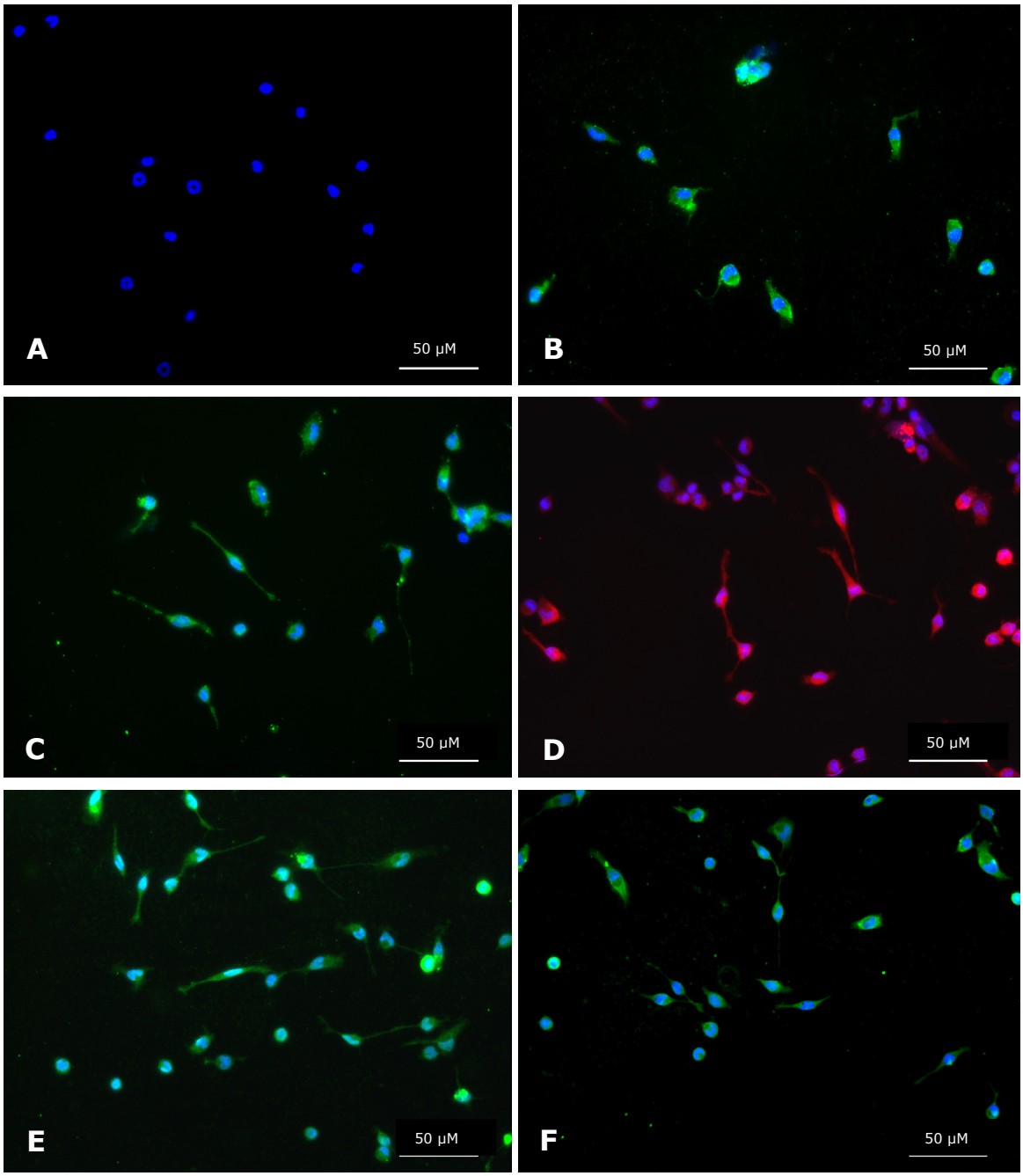

Figure 5: Expression of $GABA_A$-R, Gly-R/gephyrin and GLYT1&2 on peritoneal rat macrophages by immunofluorescence. Primary antibodies against the $\alpha_{1\text{-}4}/\beta$ subunits of the Gly-R, β_2-β_3 subunit of $GABA_A$-R, GLYT1&2 and gephyrin were used, followed by secondary Alexa-488 (green) or Alexa-555 (red) antibody staining. Nuclei of macrophages were stained with DAPI (blue). Peritoneal rat macropages showed a clear expression of the Gly-R (B), gephyrin (C), β_2-β_3 subunit of the $GABA_A$-R (D), GLYT1 (E) and GLYT2 (F) compared to a negative control (A; only staining with secondary antibody). Magnification (40X).

Previous studies have shown that the GABA-R can undergo receptor internalization (9). Therefore, because it could not be confirmed that the $GABA_A$-R is either expressed at the plasma membrane or somewhere inside the cell (figure 5D), the immunostaining procedure was repeated on viable (non fixated) macrophages. Moreover, the antibody incubation was performed at room temperature and on ice. Since on ice the metabolic activity of cells is suppressed, no receptor internalization will occur. Hence, it could be determined if the receptor was specifically present on

the plasma membrane. The differences between both conditions can be seen from figure 6. On ice, the peritoneal rat macrophages showed a clear plasma membrane staining of the $GABA_A$-R whereas at room temperature vesicular-like spots were present throughout the cell, which might indicate a possible $GABA_A$-R internalization.

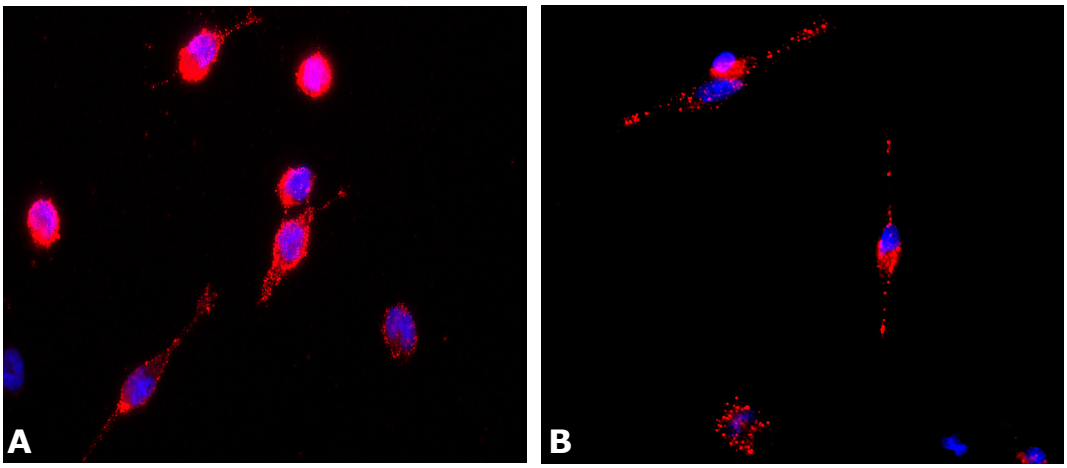

Figure 6: Expression of the $GABA_A$-R on peritoneal rat macrophages when fluorescent immunocytochemistry was performed on ice (A) or at room temperature (B). On ice peritoneal rat macrophages showed plasma membrane staining whereas at room temperature vesicular-like spots throughout the cell were present. $GABA_A$-R (red), nuclei (DAPI, blue). Magnification (40x).

3.2 Glycine modulates peritoneal rat macrophage functions

Although macrophages expressed the Gly-R and its associated molecule gephyrin at the protein level, the question remained what the effect of glycine would be on macrophage function. Therefore, peritoneal rat macrophages were cultured and treated with glycine *in vitro* and different macrophage effector functions important in MS were investigated.

3.2.1 Glycine increases NO production

To determine whether glycine had an effect on NO production, macrophages were pre-treated with an increasing dose of glycine (0µM-1mM) for 24 hours, where after LPS (10 ng/ml) was added for another 24 hours to stimulate NO production. Glycine dose-dependently increased NO production, compared to macrophages stimulated with LPS alone (figure 7A). This rise was significant when macrophages were pre-treated with a glycine concentration of 100 µM or higher. Moreover the effect of glycine on NO production was not due to differences in cell viability (figure 7B).

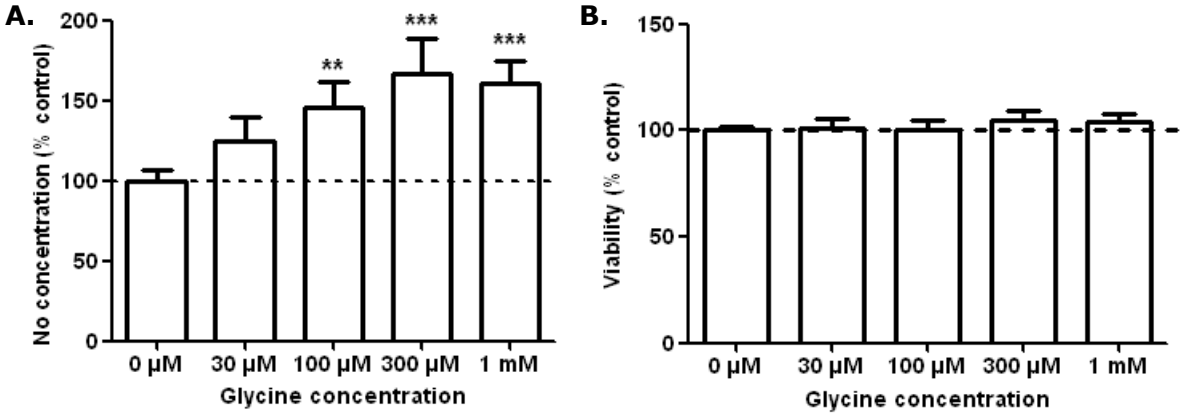

Figure 7: Glycine dose-dependently increased the NO production in peritoneal rat macophages. Macrophages were pre-treated for 24h with different concentrations of glycine and subsequently stimulated for 24h with 10 ng/ml LPS. (A) Griess assay was performed to determine NO levels in cell culture supernatans. Glycine caused a dose-dependent increase in NO production. (B) Cell viability was assessed by a MTT assay. Glycine did not affect macrophage viability. LPS-treated macrophages were used as a control and set to 100% (n = 15 - 48; ** = p<0,01; *** = p < 0,001).

Next, the possible involvement of the Gly-R in mediating the rise in NO production was addressed. Hereby, peritoneal rat macrophages were co-incubated with 1 mM glycine and 1 µM strychnine, which is a potent Gly-R antagonist. Strychnine (1 µM) was not able to reduce the NO production, evoked by 1 mM glycine, back to control levels, which indicated that the effect of glycine is not receptor mediated (Figure 8A). Cell viability remained constant among different conditions (figure 8B).

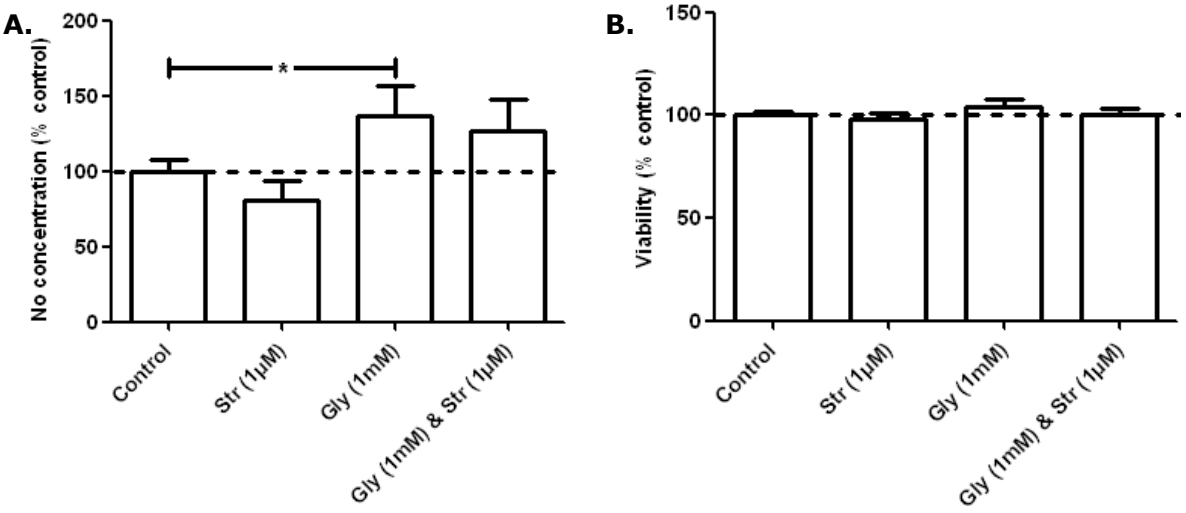

Figure 8: Glycine effect on NO production was not Gly-R dependent. (A) Griess assay was performed to determine NO production. Peritoneal rat macrophages were pre-treated for 24h with 1 mM glycine, 1 µM strychnine or 1 mM glycine together with 1 µM strychnine after which 10 ng/ml LPS was added for 24h. Glycine enhanced the NO production which could not be reversed by strychnine (B) Cell viability was assessed by a MTT assay and remained constant throughout the experiment. LPS-treated macrophages were used as a control and set to 100% (n = 12 - 24; * = p<0,05). Gly, glycine; Str, strychnine.

To confirm that the effect of glycine was receptor-independent, as shown in figure 8A, macrophages were pre-treated with 1 mM taurine or 1 mM β-alanine which both are Gly-R

agonists. Macrophages were also incubated with 1 mM L-serine, the main substrate for glycine synthesis, to investigate whether glycine could mediate its effect on NO production via other intracellular mechanisms. As in the previous experiments, pre-treatment of macrophages with 1 mM of glycine caused a significant increase in NO production. The opposite was detected when 1 mM taurine was added, which significantly reduced NO production. In contrast, β-alanine showed no effect. Moreover, 1 mM L-serine more than doubled the NO production compared to the LPS-treated control. Because L-serine can be intracellular converted to glycine by the enzyme serine hydroxymethyltransferase, it is suggested that it mediates its effect on NO production via an intracellular pathway. The cell viability was determined with a MTT assay and remained constant in all conditions except for 1 mM L-serine (figure 9B). However, in this case the viability significantly increased but could not explain the large increase in NO production when macrophages were pre-treated with 1 mM L-serine.

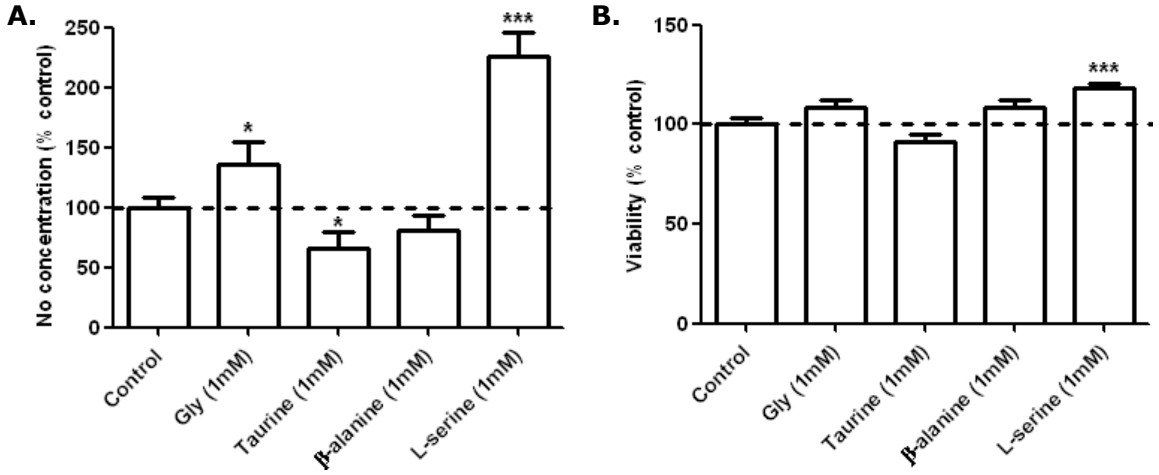

Figure 9: L-serine enhanced the NO production suggesting that glycine affects NO production via an intracellular mechanism. (A) A Griess assay was performed to determine NO production. Peritoneal rat macrophages were pre-treated with 1 mM glycine, taurine, β-alanine or L-serine for 24h followed by a 24h stimulation with 10 ng/ml LPS. 1 mM taurine reduced NO production whereas 1 mM glycine or L- serine caused an increase. (B) Cell viability was assessed by a MTT assay, viability was significantly increased in the conditions pre-treated with 1 mM L-serine. LPS-treated macrophages were used as a control and set to 100% (n = 9 - 15; * = p<0,05; *** = p<0,001). Gly, glycine.

Because of the fact that glycine had a significant effect on NO production which was not receptor-mediated, it was suggested that glycine modulated the macrophage function by other means. Since macrophages expressed the GLYT1&2 on protein level (figure 5E&F), it was hypothesized that glycine could enter the cell via GLYT to exert its intracellular effect on NO production or influence the ion homeostasis by activating these transporters which ultimately leads to an increased NO production. To examine the involvement of transporter systems, macrophages were co-incubated with 1 mM glycine and 10 nM Alx 5407 or 10 μM amoxapine (Amo) which are blockers of GLYT1&2, respectively. Furthermore, the influence of 1 mM alpha isobutyric acid (Aib) on NO production was tested with and without 1 mM glycine to determine whether system A transporters are involved (60).

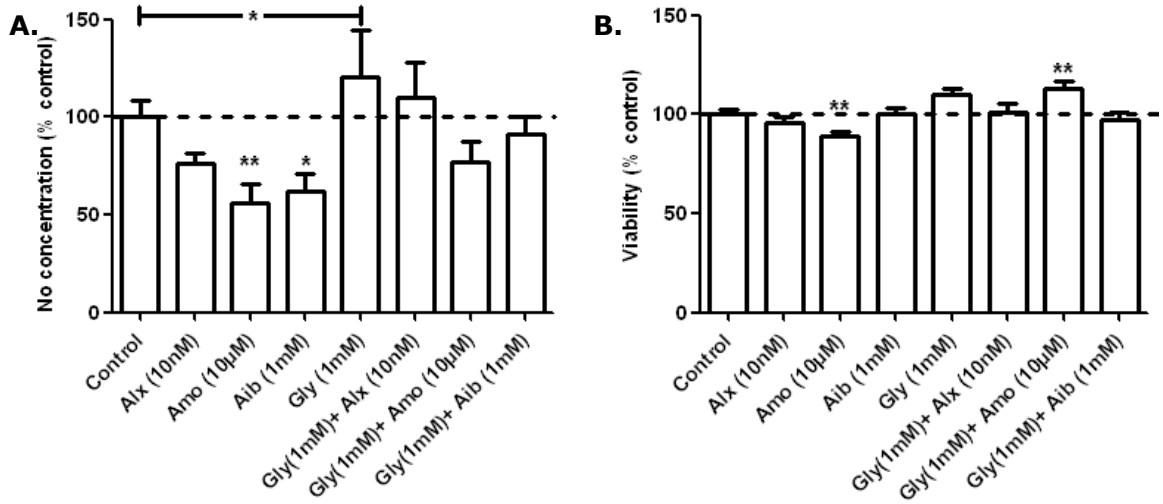

Figure 10: A transporter system is probably involved in the effect of glycine on NO production. (A) A Griess assay was performed to determine NO production. Peritoneal rat macrophages were co-incubated with 1 mM glycine and 10 nM Alx, 10 µM Amo or 1 mM Aib followed by a 24h stimulation with 10 ng/ml LPS. In addition experiments were performed with transporter blockers alone. Glycine significantly increased NO production, an effect that was partially reversed by 10 µM Amo and 1 mM Aib, however this was not significant. (B) Cell viability was assessed by a MTT assay and significantly increased in the condition of 1 mM glycine together with 10 µM Amo. In the condition with 10 µM Amo alone, cell viability was decreased. LPS-treated macrophages were used as a control and set to 100% (n = 9 - 21; * = p<0,05; ** = p<0,01). Gly, glycine; Alx, Alx 5407; Amo, amoxapine; Aib, alpha isobutyric acid.

In the co-incubation experiments with 1 mM glycine, only 10 µM Amo or 1 mM Aib could slightly reverse the increase in NO production due to 1 mM glycine (figure 10A). However this was not significant. In addition, the same trend in decrease was obtained with the blockers alone compared to the co-incubation experiments. However, the ideal situation would be that these values remained at control level. Furthermore, cell viability was significantly decreased in the condition pre-treated with 10 µM amoxapine and an increase was obtained with 1 mM glycine or 1 mM glycine with 10 µM Amo (figure 10B). It remains the question if these small differences in cell viability could affect NO production.

3.2.2 Glycine increases myeline phagocytosis

During MS, macrophages also lead to damage by the phagocytosis of myelin. Therefore, the effect of glycine on myelin phagocytosis was investigated by means of flow cytometry. Peritoneal rat macrophages were pre-treated for various time points (6, 12, 24, 48 and 72 hours) with 1 mM glycine and thereafter DiI-labelled myelin was added. As shown in figure 11A, 1 mM glycine significantly increased the myelin phagocytosis from 12 hours until 48 hours and reached its maximum at 24 hours. Furthermore, the cell viability was checked with a 7-AAD staining and remained constant throughout these time points except for 72 hours, where the viability of the macrophages was increased (figure 11B).

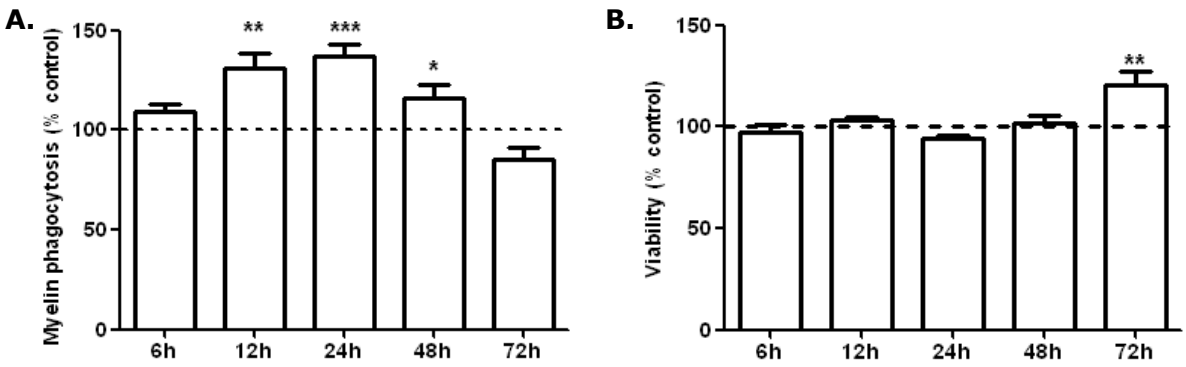

Figure 11: Effect of glycine on myelin phagocytosis was investigated by flow cytometry. (A) Rat peritoneal macrophages were pre-treated with 1 mM glycine for different time points. Phagocytosis of myelin was measured by adding DiI-labeled myelin for 90 minutes, followed by flow cytometric analysis. Pre-treatment with 1 mM glycine had a time-dependent effect on myelin phagocytosis, which was significant at 12, 24 and 48 hours. (B) Cell viability was assessed with a 7-AAD staining and was constant in all conditions except when macrophages were pre-treated for 72h, where viability increased. Non glycine-treated macrophages were used as a control and set to 100% (n = 6-9; * = p<0,05; ** = p<0,01; *** = p<0,001).

Because the above described experiment showed that glycine maximally increased phagocytosis at 24h (figure 11), this time point was used to determine concentration dependent effects. Glycine was added in a concentration range of 0-1 mM and dose-dependently increased the phagocytosis of myelin. This was significant from a concentration of 10 µM and was most pronounced at a concentration of 1 mM glycine (figure 12).

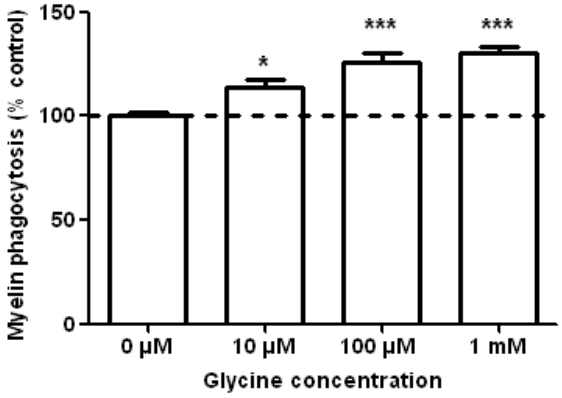

Figure 12: Glycine dose-dependently increased myelin phagocytosis. Rat peritoneal macrophages were pre-treated for 24h with several concentrations of glycine. Phagocytosis of myelin was measured by adding DiI-labeled myelin for 90 minutes, followed by flow cytometric analysis. Glycine had a dose-dependent effect on myelin phagocytosis. Non glycine-treated macrophages were used as a control and set to 100% (n = 9; * = p<0,05; *** = p<0,001).

3.2.3 Glycine has no effect on ROS production

A last effector function studied, was the capability of macrophages to produce ROS. Peritoneal rat macrophages were pre-treated for various time points (24, 48 and 72 hours) with 1 mM glycine followed by an incubation for 15 minutes with 100 ng/ml PMA to maximally induce the ROS production. Afterwards ROS production was determined by a DHR-123 assay. Glycine (1 mM) did not affect the ROS production at any time point, compared to the PMA-treated control (figure 13).

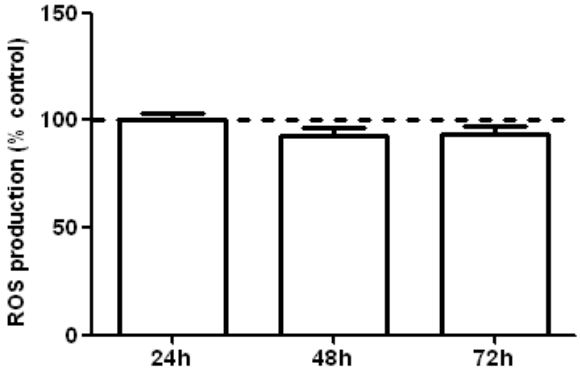

Figure 13: Glycine did not have an effect on ROS production. Rat peritoneal macrophages were pre-treated for several time points with 1 mM glycine, followed by 15 min of PMA (100 ng/ml) and 15 min DHR-123 (2 µM). The fluorescence intensity, which is a reflection of the amount of ROS produced, was measured by flow cytometry. 1 mM glycine did not have an effect on the ROS production compared to control. PMA-treated macrophages were used as a control and set to 100% (n= 12).

In conclusion, glycine dose-dependently decreased the NO production and myelin phagoyctosis of peritoneal rat macrophages through a Gly-R-independent way. Evidence provided by using transporter blockers and L-serine suggests that glycine could mediate its effect via an intracellular pathway.

3.3 GABA influences peritoneal rat macrophage functions

Peritoneal rat macrophages express the $GABA_A$-R (figure 6A). Therefore, we investigated if GABA could modulate macrophage functions important in the pathogenesis of MS.

3.3.1 GABA decreases NO production

To examine the effect of GABA on NO production, peritoneal rat macrophages were pre-treated with an increasing dose of GABA (0mM-1mM) for 24h, followed by LPS-stimulation for 24h to induce NO production. A dose-dependent decrease of NO production could be detected compared to the LPS-stimulated control (figure 14A), which was significant at a concentration of 1 mM GABA. Furthermore, cell viability was constant throughout the different conditions and therefore could not explain the observed effect on NO production (figure 14B).

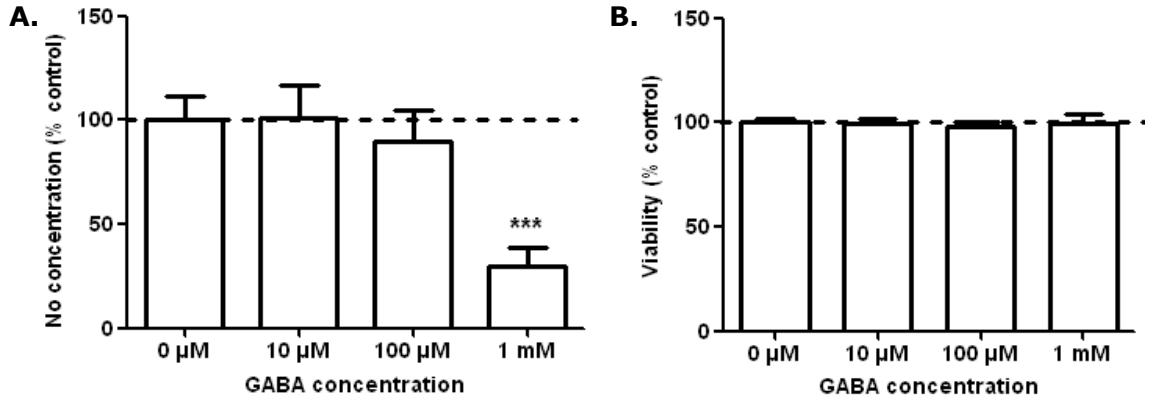

Figure 14: GABA dose-dependently decreased the NO production of peritoneal rat macrophages. Macrophages were pre-treated for 24h with different concentrations of GABA and were stimulated for 24h with 10 ng/ml LPS. (A) Griess assay was performed to determine NO production. GABA dose-dependently decreased the NO production which was significant with 1 mM GABA. (B) Cell viability was assessed by a MTT assay. GABA effect on NO production was not the result of differences in cell viability. LPS-treated macrophages were used as a control and set to 100% (n = 6; *** = p < 0,001).

Subsequently, it was addressed whether the GABA-R was involved in the observed decrease in NO production. Herefore, co-incubation experiments were performed with 1 mM GABA and 30 µM gabazine or saclofen. Gabazine is a $GABA_A$-R antagonist whereas saclofen is a $GABA_B$-R antagonist. The effect of 1mM GABA was significantly reversed by adding 30 µM saclofen (figure 15A). Although 30 µM gabazine showed a trend in counteracting the 1 mM GABA effect, it was not significant. Cell viability was not affected (figure 15B).

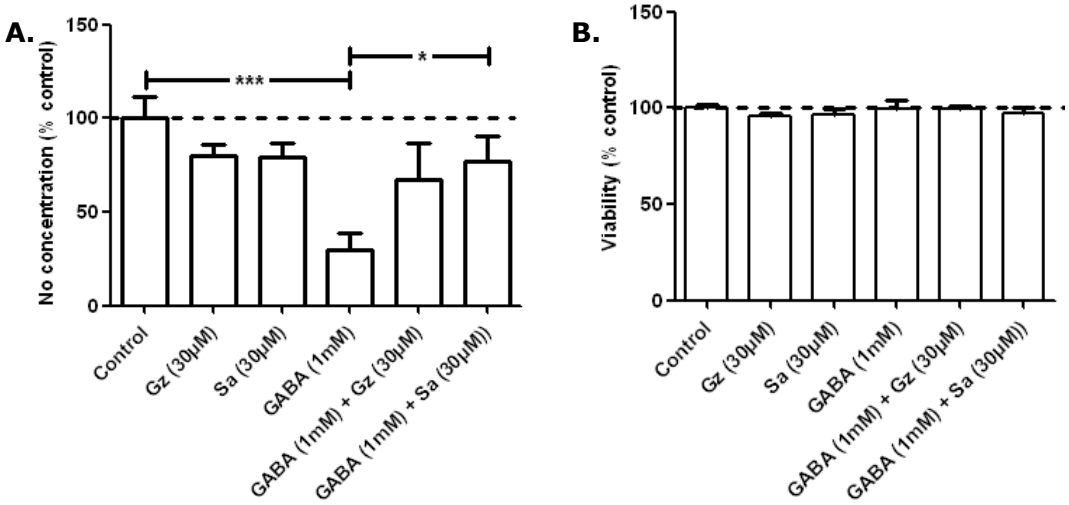

Figure 15: GABA effect on NO production was receptor mediated. Rat peritoneal macrophages were pre-treated for 24h with 1mM GABA or co-incubated with 1 mM GABA and 30 µM gabazine or saclofen followed by a 24h stimulation with 10 ng/ml LPS. (A) Griess assay was performed to determine NO production. GABA (1 mM) decreased the NO production which was reversed when a $GABA_B$-R antagonist, saclofen, was used. Gabazine ($GABA_A$-R antagonist) also showed a trend in reversing the effect of 1 mM GABA. However this was not significant (B) Cell viability was assessed by a MTT assay and no differences were detected. LPS-treated macrophages were used as a control and set to 100% (n = 6; * = p < 0,05; *** = p < 0,001). Gz, gabazine; Sa, saclofen.

3.3.2 GABA decreases myeline phagocytosis

Besides the role of GABA on NO production, it was questioned whether GABA had an effect on another macrophage effector function implicated in MS such as myelin phagocytosis. Therefore rat peritoneal macrophages were pre-treated with 1 mM GABA for several time points followed by an incubation with DiI-labelled myelin. After 48 and 72 hours of incubation, GABA significantly decreased myelin phagocytosis compared to a non GABA-treated control (dashed line, figure 16A). This decrease was not due to variations in cell viability (Figure 16B).

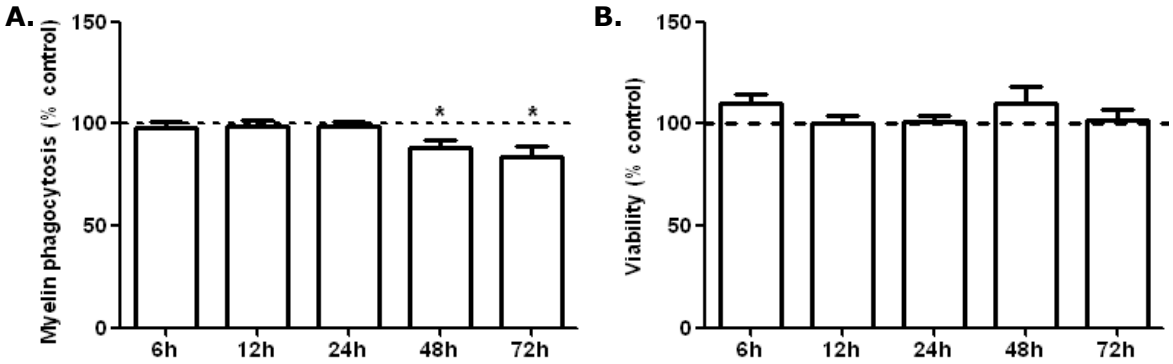

Figure 16: GABA decreased myelin phagocytosis of rat peritoneal macrophages after 48/72 hours. (A) Rat peritoneal macrophages were pre-treated with GABA for different incubation times. Phagocytosis of myelin was measured by adding DiI-labeled myelin for 90 minutes, followed by flow cytometric analysis. Pre-treatment with 1 mM GABA had a time-dependent effect on myelin phagocytosis, which was significant after 48/72 hours. (B) Cell viability was assessed with a 7-AAD staining and was constant in all conditions. Non GABA-treated macrophages were used as a control and set to 100% (n = 6-9; * = p<0,05).

3.3.3 GABA decreases ROS production

Lastly, the effect of GABA on ROS production was assessed by pre-treating macrophages for various time points (24, 48 and 72 hours) with 1 mM GABA. Afterwards 100 ng/ml PMA was added for 15 minutes and ROS production was determined by a DHR-123 assay. 72h pre-treatment with 1 mM GABA significantly decreased the ROS production of peritoneal rat macrophages compared to the PMA-treated control (figure 17).

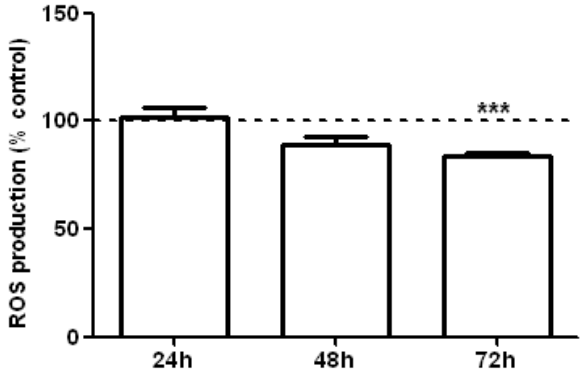

Figure 17: GABA decreased the ROS production of rat peritoneal macrophages after 72 hours. Rat peritoneal macrophages were pre-treated for 24h-48h-72h with 1 mM GABA, followed by 15 min of PMA (100 ng/ml) and 15 min DHR-123 (2 µM). The fluorescence intensity, which is a reflection of the amount ROS produced, was measured by flow cytometry. A significant decrease was obtained when macrophages were pre-treated for 72h with 1 mM GABA compared to control. PMA-treated macrophages were used as a control and set to 100% (n= 9; *** = p<0,001).

In conclusion, GABA blunted increases in NO production, myelin phagocytosis and ROS production. While the effect on NO production is GABA-R-dependent, it remains to be determined whether this is also the case for the other effects reported above.

4 Discussion

GABA and glycine are important inhibitory neurotransmitters in the CNS. Moreover, their receptors are present on immune cells such as T cells (21) and macrophage subtypes, like Kupffer cells, alveolar and splenic macrophages (13, 27). Here, glycine and GABA have anti-inflammatory effects. Since GABA and glycine levels are altered during the course of MS and its animal model EAE (56-58), this indicates a possible role of these neurotransmitters in the disease process. Therefore, we hypothesised that GABA and glycine may modulate the macrophage effector functions important during the inflammatory processes in MS.

First, it was investigated whether the ionotropic $GABA_A$-R and Gly-R were expressed on peritoneal rat macrophages. PCR experiments showed the expression of the α_4 subunit of the Gly-R and the Gly-R associated molecule, gephyrin, on the mRNA level of peritoneal rat macrophages. In rat spinal cord (positive control) all subunits were detected. A study done by the group of Froh et al. showed the expression of the Gly-R on the mRNA level of different rat macrophages subtypes such as Kupffer cells, alveolar macrophages and splenic macrophages. However, they also demonstrated the presence of the α_1, α_2 and β subunits on these macrophages in addition to the α_4 subunit (27). Our results revealed only the expression of the α_4 and gephyrin, which can be explained by the fact that we used peritoneal macrophages which was a subtype not studied by the group of Froh and therefore another Gly-R composition could be present. The detection of the α_4 subunit alone has no implications for receptor expression at the plasma membrane or functionality, since it is known that the Gly-R can form a homomeric receptor from five the same α subunits. Moreover, gephyrin, although it only binds to the β subunit, is not required for localization at the plasma membrane (12). Next, fluorescent immunocytochemistry was applied to detect the $GABA_A$-R and Gly-R on protein level. The Gly-R was clearly expressed together with its associated molecule gephyrin. However, because the used primary antibody is directed against an intracellular part of the Gly-R, until now it is unclear if the Gly-R is present only at the plasma membrane, or also somewhere inside the cell. The $GABA_A$-R was also detected on peritoneal rat macrophages. Since previous studies have shown that the $GABA_A$-R could be internalized (9), the fluorescent immunostaining protocol for the $GABA_A$-R was performed on ice or at room temperature. Because the metabolic activity of cells is suppressed on ice, no receptor internalization can occur. Hence, it could be determined if the receptor was specifically present on the plasma membrane. This experiment showed that the $GABA_A$-R underwent receptor internalization at room temperature (vesicular-like spots in figure 6B). Moreover, on ice peritoneal rat macrophages showed a clear expression of the $GABA_A$-R at the plasma membrane, which was also proven by Reyes-Garcia et al. in mouse peritoneal macrophages (24). In addition to the expression of the $GABA_A$-R and Gly-R, also GLYT1&2 were present on macrophages. GLYT1 is mainly present in astrocytes and microglia (brain macrophages) whereas GLYT2 is almost exclusively expressed in axons and presynaptic terminals (14). Until now, we are the first to describe the presence of GLYT1 and GLYT2 on peritoneal rat macrophages. The expression of the $GABA_A$-R, Gly-R, gephyrin and GLYT1&2 suggest that glycine and GABA may play a role in macrophage function.

To further investigate this possible influence of glycine and GABA on macrophage effector functions important in the pathogenesis of MS, *in vitro* cultures of peritoneal rat macrophages treated with glycine were carried out. Our results showed that glycine dose-dependently increased the NO production and myelin phagocytosis, but had no effect on ROS production.

The increase in NO production and myelin phagocytosis indicate a pro-inflammatory action of glycine. In contrast, other studies showed that glycine could suppressed the production of superoxide and TNF-α in alveolar macrophages suggesting an anti-inflammatory response (28). This controversy in glycine action could be contributed to the studied macrophage subtype, in this case peritoneal rat macrophages in stead of alveolar macrophages. Moreover, we demonstrated that the effect of glycine on macrophages was not receptor-mediated whereas Wheeler et al. described an involvement of the Gly-R. Furthermore variation could also be contributed to different culture conditions.

Like already stated above, we could not reverse the effect of glycine on NO production by using the Gly-R antagonist strychnine, which suggested a Gly-R independent way of action. This receptor-independent action of glycine was also proven by Schilling and Eder, which show that glycine-evoked currents were unaffected by strychnine in brain macrophages (60). Moreover, when the Gly-R agonist β-alanine was used no effect on NO production was observed. In contrast, another Gly-R agonist taurine did affect NO production. Because glycine had an opposite effect than taurine on NO production, this indicated that glycine acted via another mechanism. Furthermore, taurine is also known as an anti-neurodegeneration, anti-oxidation and anti-apoptotic agent (61), indicating that taurine plays a role in other mechanisms besides antagonizing the Gly-R. In that respect, taurine is abundantly present in phagocytic cells and protects against cytotoxicity caused by ROS. Hence when hypochlorous acid (HOCl), a toxic product of the myeloperoxidase system, reacts with taurine, it forms a stable and less toxic product taurine chloramine (TauCl). This TauCl on his turn, has been proven to inhibit the production of superoxide anion and NO, which could explain the observed decrease in NO production with 1 mM taurine in our experiments (62). Moreover, 1 mM L-serine was able to mimic the effect of glycine on NO production and the increase in NO production was even higher as with glycine. Because L-serine can be intracellular converted to glycine by the enzyme serine hydroxymethyltransferase (15), it is suggested that glycine mediates its effect on NO production via a yet unknown intracellular pathway. Because GLYT1&2 were expressed on the protein level, we hypothesized that transporter systems were involved in the uptake of glycine to exert the intracellular effect on NO production or that glycine influenced the ion homeostasis by activating these transporters, which ultimately leads to an increased NO production. By treating peritoneal rat macrophages with glycine together with transporter blockers, we show an involvement of transporters in mediating the glycine effect. Amoxapine, a GLYT2 blocker, was able to counteract the effect of glycine on NO production, whereas the GLYT1 blocker Alx 5407 had no effect. Furthermore Aib, which is a system A transporter blocker, could also reverse the glycine-mediated effect. These findings are in line with the group of Schilling and Eder, which showed an involvement of system A Na^+-coupled neutral amino acid transporters (SNAT) in effects mediated by glycine in brain macrophages, which suggests that glycine could exert its effect through a transporter system (60). It needs to be mentioned that the transporter blockers used in these experiments also significantly reduced NO production on their own, but this could be due to

changes in ion homeostasis when important transporter systems are blocked. Currently the role of transporters is further being tested, by repeating and optimizing the experiments with the transporter blockers. Moreover, colleagues at our lab performed Ca^{2+} measurements on peritoneal rat macrophages and found that glycine increased intracellular Ca^{2+} concentrations. Ca^{2+} is an important second messenger and affects other pathways and gene transcription. Since iNOS is a Ca^{2+}-dependent enzyme important in the LPS-stimulated NO production, this might indicate that glycine increases NO production by influencing intracellular Ca^{2+} concentrations. However, the question remains whether glycine directly interacts with a yet unknown intracellular pathway causing a rise in intracellular Ca^{2+} levels or that a depolarisation, caused by increased transport of glycine and Na^+, lead towards this Ca^{2+} increase by activating voltage-dependent Ca^{2+} channels and Ca^{2+} release activated channels (CRAC) (figure 18). The presence of these channels is already proven in microglia (63).

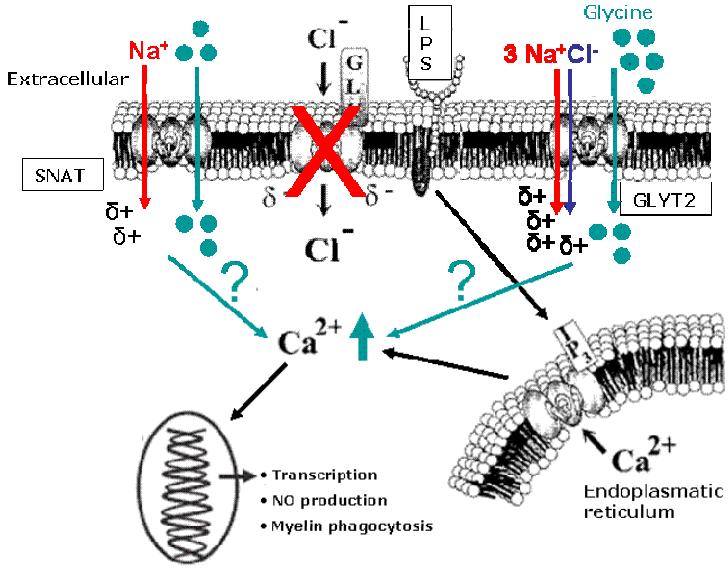

Figure 18: Proposed model for the glycine-induced increase in NO production. When peritoneal rat macrophages are treated with LPS, Ca^{2+} is released from the endoplasmatic reticulum. This results in a rise in intracellular Ca^{2+} levels, which affects transcription, NO production and myelin phagocytosis. When glycine is added to *in vitro* cultures of peritoneal rat macrophages, Ca^{2+} levels are further increased which lead to increased NO production. This increase was not due to receptor stimulation, but was transporter-mediated (GLYT2 and/or SNAT) as was suggested from the experiments with transporter blockers. However, until now it remains unclear how glycine increases intracellular Ca^{2+} concentrations. Glycine could directly interact with intracellular pathways causing this rise. Another possibility is the opening of voltage-dependent Ca^{2+} channels and CRAC channels due to a depolarisation, caused by increased transport of glycine and Na^+ into the cell. Figure adapted from Wheeler et al. (29).

During MS, macrophages may cause damage by phagocytosing myelin and producing ROS. *In vitro* experiments reported in this study, showed that glycine time-dependently increased the myelin phagocytosis, but had no effect on ROS production.

Lastly, the role of the other inhibitory neurotransmitter, GABA, on macrophages functions was addressed. We have shown that GABA decreased the NO production, myelin phagocytosis and ROS production of peritoneal rat macrophages *in vitro*. This proves the beneficial and anti-inflammatory

action of GABA seen in other studies (7, 21, 23, 24). Moreover, GABA-R antagonists could reverse the effect of GABA on NO production. Gabazine and saclofen, respectively $GABA_A$-R and $GABA_B$-R antagonists, were able to reverse the effect of 1 mM glycine, although this was not significant with gabazine. These findings suggests that the GABA-R is involved in mediating the observed effects, which is also confirmed by a study done by Reyes-Garcia et al. on mouse peritoneal macrophages. They demonstrate a decrease in IL-6 and IL-12 production when macrophages were pre-treated with GABA, which was reversed by adding picrotoxin, a $GABA_A$-R antagonist (24). This brought us to the following model concerning GABA's mechanism of action (figure 19). When GABA is increased in the extracellular matrix, it activates the $GABA_A$-R. This leads to a flux of chloride into the cell, thereby causing a hyperpolarisation. This hyperpolarisation is translated into a non-active state of the macrophage and therefore counteracts the influx of Ca^{2+} from CRAC channels and Ca^{2+} release from the endoplasmatic reticulum (ER). Consequently, peritoneal macrophages are less responsive, resulting in a decrease in NO production, myelin phagocytosis and ROS production. The mechanism by which $GABA_B$-R could mediate a Ca^{2+} decrease is yet unknown, but because the $GABA_B$-R is a G-protein coupled receptor it is assumed that G-proteins turn on an intracellular pathway that leads to a reduction in intracellular Ca^{2+} levels. On the other hand, GABA could also induce an anti-inflammatory phenotype by influencing gene expression, thereby converting M1 macrophages to M2 macrophages.

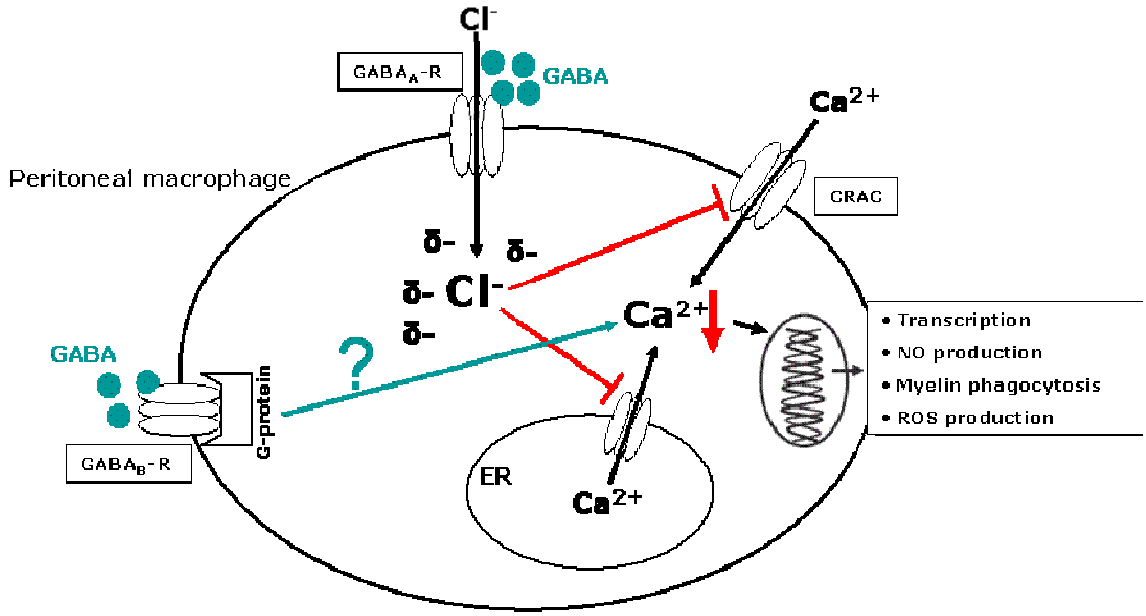

Figure 19: Proposed model for the role of GABA on peritoneal macrophage functions. GABA stimulates the $GABA_A$-R and allows the flux of chloride into the cell thereby causing a hyperpolarisation. As a consequence, intracellular Ca^{2+} concentrations are reduced and the threshold to reach an active state is higher which is translated in significant reductions in NO production, myelin phagocytosis and ROS production. Until now, the pathway behind the $GABA_B$-R remains unknown. CRAC, Ca^{2+}-release activated channels; ER, endoplasmatic reticulum.

GABA decreased the myelin phagocytosis after 48 hours, which was correlated with a decrease in ROS production after 48 hours. This suggests an interaction between these two and a possible involvement of the same mechanism behind the action of GABA. A study of van der Goes et al. demonstrated that scavenging of ROS with catalase, mannitol or lipoic acid lead to a decrease in

myelin phagocytosis (64). Moreover, they have shown that the phagocytosis of myelin induces ROS production. However, to confirm this link in our *in vitro* experiments, ROS production could be measured after myelin phagocytosis and vice versa. Furthermore, adding scavengers for ROS production to our GABA-treated cultures and looking at myelin phagocytosis could confirm the interaction between both and give us an indication that GABA influences the ROS production and myelin phagocytosis through a similar mechanism.

From this study, it can be concluded that GABA has an anti-inflammatory role and down regulates important macrophage effector functions involved in MS such as the NO synthesis, myelin phagocytosis and ROS production. Furthermore this inhibitory action was receptor-mediated. In contrast, glycine leans more towards a pro-inflammatory response. Although both the $GABA_A$-R and Gly-R are ion channels leading to influx of chloride upon activation, other effects are found on macrophage functions. This could be due to the involvement of their receptor, because the glycine effect, in contrast to GABA, was not receptor-mediated. Rather GLYT such as GLYT2 and SNAT were suggested to play an important role in mediating the effects of glycine.
Since this study demonstrates that inhibitory neurotransmitters can have significant effects on macrophage function, GABA and glycine could possible influence neuroinflammation. Moreover, GABA and glycine levels are altered in the CSF and plasma of EAE rats and MS patients indicating a possible role of these inhibitory neurotransmitters in the disease pathogenesis.

5 Conclusion

This study showed that GABA and glycine, both inhibitory neurotransmitters and neutral amino acids, could have different effects on macrophages functions in the pathogenesis of MS.

GABA had an anti-inflammatory role and suppressed important effector functions involved in MS such as NO synthesis, myelin phagocytosis and ROS production. This inhibitory action of GABA was receptor-mediated as was proven by simultaneously incubating cells with GABA and GABA-R antagonists (gabazine or saclofen). On the other hand, glycine pointed more towards a pro-inflammatory response. This was translated into a dose-dependent increase in NO production and myelin phagocytosis. In contrast to GABA, the rise in NO production was not receptor-mediated, as the Gly-R antagonist strychnine could not reverse the effect of glycine. *In vitro* experiments with L-serine, the main substrate for glycine synthesis, indicated that glycine probably acted through an intracellular pathway. Moreover, experiments done with GLYTs blockers suggested that GLYT2 and/or system A neutral amino acid transporters could play an important role in mediating the effects of glycine.

Besides looking at the role of GABA and glycine on myelin phagocytosis and production of NO and ROS, a next step would be to determine the effect of inhibitory neurotransmitters on the release of pro- and anti-inflammatory cytokines, such as TNF-α, IL-4 and IL-10. Furthermore, because autoreactive T cells play an important role in the initiation of MS, another interesting point to study would be the influence of GABA and glycine on the cytokine production and proliferation of T cells. Eventually, *in vivo* studies should be performed on healthy and EAE rats. In this case, expression of the GABA-R and Gly-R on T cells and macrophages could be compared between healthy and EAE rats. Furthermore, GABA and glycine levels in the CNS and the serum could be measured and compared between healthy and EAE rats to determine if any abnormalities exist. Because GABA had an anti-inflammatory action, EAE rats could be treated with GABA, GABA-R agonists and antagonists to evaluate what the effect would be on the disease process.

This study provides us with more insights into the interaction between neurotransmitters and the immune system. Furthermore we demonstrate that inhibitory neurotransmitters can have significant effects on macrophages functions thereby possibly influencing neuroinflammation. Because neuroinflammation is a vital part in the pathogenesis of MS, the high concentration of neurotransmitters present in the CNS could influence the course of MS. More generally, the effects provided by this study can be extrapolated to other inflammatory diseases. Ultimately, GABA and glycine and its mechanism could be exploited as a new approach for pharmaceutical companies in developing new therapies for inflammatory diseases. However, the challenge would be to modulate these mechanisms without interfering with the normal neurotransmission.

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