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Datum: 14.12.2009

***Glycine enhances microglial calcium fluxes
A possible role for neutral amino acid transporters***

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Eindverhandeling voorgedragen tot het bekomen van de graad
master in de biomedische wetenschappen klinische moleculaire
wetenschappen



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Abbreviations

<p>[Ca²⁺]_i = Intracellular Ca²⁺ concentration</p> <p>AD = Alzheimer's disease</p> <p>AIB = α-amino-isobutyric acid</p> <p>ANOVA = analysis of variance</p> <p>AP = Acid Phosphatase</p> <p>APC = Antigen presenting cell</p> <p>ATP = Adenosine triphosphate</p> <p>CNS = Central nervous system</p> <p>CSF = Cerebrospinal fluid</p> <p>DMEM = Dulbecco's Modified Eagles Medium</p> <p>DMSO = Dimethylsulfoxide</p> <p>ELISA = Enzyme-linked immunosorbent assay</p> <p>F = Fluorescence signal</p> <p>F_b = Baseline fluorescence</p> <p>FCS = Fetal calf serum</p> <p>FLIPR = Fluorescence imaging plate reader</p> <p>F_{peak} = Maximum peak fluorescence</p> <p>Fu = Fluorescence units</p> <p>GlyR = strychnine-sensitive glycine receptor</p> <p>GlyT = Glycine transporter</p> <p>HIV = Human immunodeficiency virus</p> <p>IHC = Immunohistochemistry</p> <p>iNOS = inducible nitric oxide synthase</p> <p>K_{ca} = Ca²⁺ sensitive potassium channel</p> <p>LBP = LPS binding protein</p>	<p>LDH = Lactate dehydrogenase</p> <p>LPS = Lipopolysaccharide</p> <p>MAPK = Mitogen activated protein kinase</p> <p>MeAIB = α-(Methylamino)-isobutyric acid</p> <p>MS = Multiple sclerosis</p> <p>NF-κB = Nuclear factor kappa-light-chain-enhancer of activated B cells</p> <p>NMDAR = N-methyl-D-aspartate receptor</p> <p>NMDG = N-methyl-D-glucamine</p> <p>NO = Nitric oxide</p> <p>O²⁻ = Superoxide</p> <p>P/S = Penicillin/streptomycin</p> <p>PBS = Phosphate buffered saline</p> <p>PFA = Paraformaldehyde</p> <p>PMC = Primary cultured microglia</p> <p>PNI = Peripheral nerve injury</p> <p>RT-PCR = reverse transcription polymerase chain reaction</p> <p>S.E.M. = Standard error of the mean</p> <p>SAT = System A neutral amino acid transporter</p> <p>SERCA = Sarco/endoplasmatic reticulum Ca²⁺ ATP'ase</p> <p>SES = Standard extracellular solution</p> <p>TLR-4 = Toll like receptor 4</p> <p>TSG = Thapsigargin</p> <p>VGCC = Voltage gated Ca²⁺ channels</p>
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Abstract

Microglia are the resident immune cells of the brain and there is evidence for involvement of microglia in multiple disease processes, such as: viral infection, neuropathic pain, multiple sclerosis and Alzheimer's disease. In this study we investigate the role of non-synaptic glycine communication in regulating the microglial immune response. Studies of this nature can, in the long term, contribute to a better understanding of microglial behavior in the central nervous system environment. Preliminary data had already shown that glycine can enhance nitric oxide production of LPS-stimulated microglia in a glycine receptor (GlyR)-independent fashion. This contrasts most results obtained on peripheral immune cells, where glycine has been shown to inhibit immune function in a GlyR-dependent manner. Based on recent electrophysiological data, we hypothesized a possible role for neutral amino acid transporters in glycine modulation of microglial activity. Since it has been demonstrated that calcium (Ca^{2+}) signals correlate to microglial activity, Ca^{2+} signal modulation was investigated. A new method was developed for measuring Ca^{2+} signals, using a fluorescence plate reader with the Fluo-3 Ca^{2+} indicator. Forthcoming results showed that glycine enhanced microglial Ca^{2+} signals induced by ATP, LPS and thapsigargin. These effects were GlyR-independent and could be blocked by the competitive amino acid transporter substrate AIB (α -amino-isobutyric acid). Glycine immunostaining also showed an AIB-sensitive uptake of glycine in microglial cells. Together, these results provide strong arguments for transporter involvement in glycine enhancement of microglial Ca^{2+} signals. Additional results obtained using ELISA assays, showed enhanced TNF- α production in glycine conditions. This further confirms that the immune function of microglial cells is enhanced by glycine and that the glycine effects on microglial cells are opposite to the effects of glycine on peripheral immune cells.

Introduction

First some general information on the role of glycine and the glycine receptor (GlyR) in neurotransmission is given. Then the focus shifts to glycine and its effects on cells of the immune system. This is narrowed down to the relationship between glycine and microglial cells, leading to the preliminary research and the current research aims. Background information on the current research aims is given in the following sections. To emphasize the importance of microglial research, the final paragraph deals with the role of microglia in several pathological conditions.

1. Glycine and the Glycine Receptor

Glycine is the smallest of 20 amino acids commonly found in proteins and has long been known to be an inhibitory neurotransmitter in the spinal cord, brainstem and retina. Glycine-mediated inhibitory neurotransmission is essential for startle responses, voluntary motor control and sensory signal processing in the spinal cord. Glycine exerts its inhibitory actions by binding its receptor which is largely localized in postsynaptic neuronal membranes. Inhibitory postsynaptic signals oppose the depolarizing action of excitatory/stimulatory neurotransmission by increasing chloride permeability across the postsynaptic neuronal membrane (1). The identity of glycine as an inhibitory neurotransmitter was originally proposed by Aprison et al. (2) and Davidoff et al. (3), who described in detail the distribution of glycine throughout the central nervous system. Autoradiographic studies with radiolabeled glycine demonstrated that glycine is localized in spinal cord synaptic regions (4). Functional studies later demonstrated that glycine hyperpolarizes postsynaptic motor neurons by increasing chloride conductance (5-7) thus, the GlyR is often referred to as a glycine-gated chloride channel. Inhibitory neurotransmission by glycine was shown to be selectively blocked by strychnine, a plant alkaloid, which enabled further characterization of glycine action in the nervous system (8, 9). With the use of the high-affinity inhibitor strychnine, the GlyR was purified from membrane fractions of the adult rat spinal cord. The subunit composition and binding sites of the receptor and the amino acid sequence of many of the subunits have been characterized (10).

2. Effect of glycine on white blood cells

The inhibitory effects of glycine on immune cells, including macrophages (11), monocytes (12), neutrophils (13) and T-lymphocytes (14), have been established in several studies. These studies have demonstrated that millimolar concentrations of glycine reduce agonist-stimulated Ca^{2+} (Ca^{2+}) signaling, phagocytic activity and cell proliferation and attenuate the generation of reactive oxygen species and cytokines. It has been confirmed that binding of glycine to the GlyR causes hyperpolarisation in these peripheral immune cells.

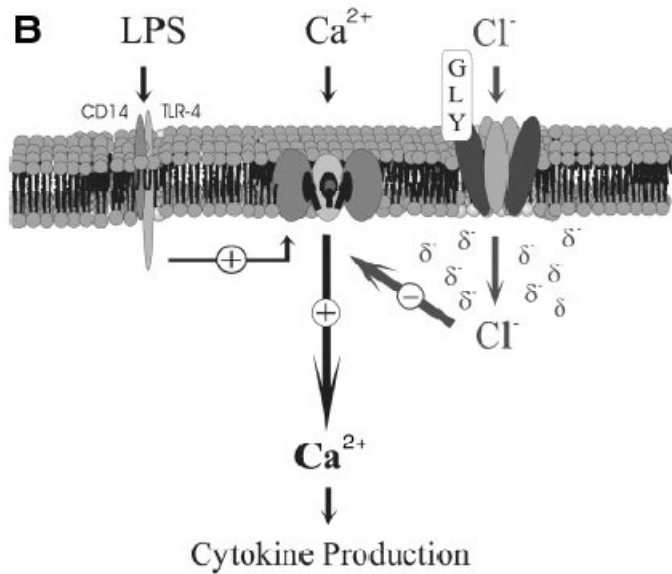


Figure 1. Proposed mechanism for glycine modulation of Kupffer cells (11). LPS = lipopolysaccharide

Based on these findings it is hypothesized that this hyperpolarisation, due to Cl⁻ influx, blunts the depolarisation that occurs when the cells are activated by lipopolysaccharide (LPS, Fig. 1). This lack of depolarisation would inhibit the activation of voltage-gated Ca²⁺ channels (VGCC) and prevent a rise in intracellular Ca²⁺ required for the activation of the inflammatory cytokine cascade (1).

3. Glycine and microglia

Microglia are the resident immune cells of the brain and are able to recognize the onset of a pathological condition. They can respond by acquiring features that they use in the defense of the integrity of the tissue e.g. migration towards recruitment sites, proliferation, antigen presentation, secretion of pro- or anti-inflammatory substances and phagocytosis of the products of tissue degeneration or infecting agents (15, 16). However, in some circumstances they can contribute to the exacerbation of pathological conditions by secreting inflammatory mediators implicated in brain damage (17). Glycine inhibition of white blood cells suggests that it may also suppress the sometimes detrimental immune function of microglia. In this context, studies specific for microglial cells show that the addition of glycine to microglial cultures increases their activity. This is manifested by an elevation in the generation of superoxide anion (O²⁻) and an increased production of acid phosphatases (AP) and inducible nitric oxide synthase (iNOS) (18, 19). Also the nature and function of VGCCs described in the hypothesis for white blood cells (see above) remains elusive for microglia (20). This clear opposition in cellular modulation between white blood cells and microglia has warranted further investigation into the effect of glycine on microglia and the mechanism at its core.

4. Preliminary research

Preliminary research started by investigating the effects of glycine on nitric oxide (NO) production. BV-2 cells from the murine microglial cell line and primary cultured microglial cells (PMC) were incubated with or without glycine and stimulated with LPS. By means of the Griess reaction, which measures nitrite production, the NO response was determined at 72 hours. Cells in the glycine (0,01 – 1 mM) condition showed a significantly higher response (35%) compared to control conditions. This finding confirmed the stimulating effects of glycine on immune cell properties of microglia. The preliminary research first focussed on the GlyR involvement. The presence of the receptor and its anchoring protein (Gephyrin) was demonstrated on BV-2 cells and PMCs by means of western blot, reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC). Further cell culture experiments measuring NO production were carried out to determine the functionality of the GlyR. The first set of experiments showed that blocking the GlyR with different antagonists (strychnine, picrotoxinin) failed to prevent the rise in NO response seen with glycine administration (Fig. 2A). Following experiments proved that administering GlyR agonists (taurine, β -alanine), did not yield the same effect as glycine on the NO response, concluding that the effects were GlyR-independent (21). The negative results for GlyR involvement lead to the investigation of the N-methyl-D-aspartate receptor (NMDAR), for which glycine is a co-agonist. Presence of this glutamate-gated receptor has been demonstrated on microglia and these cells have been shown to secrete glutamate under culture conditions (22, 23). The hypothesis behind the role of this receptor, was that glycine might modulate a paracrine effect of glutamate secretion. The same cell culture experiments conducted for the GlyR were conducted for the NMDAR. Blocking of the NMDAR with antagonists (MK-801, APV) again did not influence the glycine effect on NO response and neither did using a co-agonist similar to glycine (D-ser) mimic it (unpublished results). This result ruled out the NMDAR involvement and ended the investigations into receptor-ligand interaction. Next to receptor interactions, glycine can also be transported across the cell membrane through different transporters. This transport is electrochemically coupled and may induce certain direct or indirect changes. There are two main subtypes of glycine transporters (GlyT) in the central nervous system (CNS) known to regulate uptake of glycine from the extracellular space into the cytosol (24). GlyT1 is mainly found on glial cells and has an ion/substrate stoichiometry of $2\text{Na}^+/\text{Cl}^-/\text{glycine}$. GlyT2 is present only in glycinergic neurons, where it is juxtaposed to GlyRs containing postsynaptic specializations and it has an ion/substrate stoichiometry of $3\text{Na}^+/\text{Cl}^-/\text{glycine}$. Because only GlyT1 is expressed on microglial cells preliminary research investigated the role of this transporter in the glycine mediated increase in NO response. The presence of GlyT1 was confirmed by RT-PCR, but there was no reversal of glycine effects on NO response when a blocker for GlyT1 (ALX) was applied (unpublished results).

Patch clamp recordings were also done to verify the results in an electrophysiological setting. Results show small currents (5 pA) evoked by glycine when administered to microglia. These currents were unaltered when strychnine was added and were shown to be Na^+ -dependent. Because microglial cells show high membrane resistance (25), these small currents can be translated into much larger changes in membrane potential (10 mV). To detect whether this depolarisation influenced NO production, cell culture experiments were conducted in which glycine was replaced by an elevation of extracellular potassium (K^+), to mimic a depolarisation. In this condition cells also showed a higher NO production, confirming a possible role for depolarisation.

A last clue from cell culture experiments came from the fact that L-serine could mimic the effect on NO production seen with glycine (Fig. 2B). L-serine and glycine are closely related from a metabolic view, but L-serine itself is only a very weak agonist of the GlyR. Combined, all findings from the preliminary research provide great support for the GlyR-independent hypothesis, but are limited in giving information on the underlying mechanism.

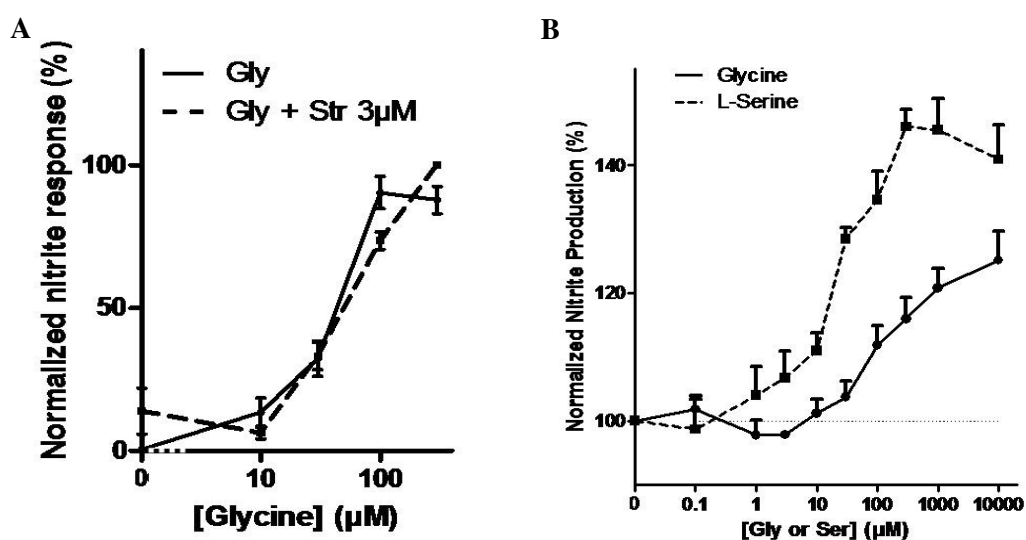


Figure 2. Results from the preliminary research measuring LPS stimulated nitrite response as a measure for NO production. **A:** Preliminary results show no inhibition of the glycine mediated enhancement of the normalized nitrite response by strychnine. **B:** Preliminary results show an even more potent enhancement of the normalized nitrite response by L-serine.

5. Research aims

Following the extensive preliminary research, the primary aim of this study was pointed towards discovering the mechanism behind modulation of microglial activity by glycine. Since it has been demonstrated that calcium (Ca^{2+}) signals correlate to microglial activity (26), Ca^{2+} signal modulation was investigated. Secondary to this, there was also further characterization of the glycine modulation of microglial immune function, carried out by measuring the microglial production of tumor necrosis factor alpha ($\text{TNF-}\alpha$) under glycine conditions.

6. The mechanism of GlyR-independent modulation

To investigate the underlying mechanism of GlyR-independent modulation of microglial cells by glycine and L-serine, the main focus of this study was Ca^{2+} homeostasis in microglial cells. This ion has been described as a central element in the regulation of executive functions in activated microglia (26). The intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) influences multiple cellular functions, including enzyme or release activities. Transient $[\text{Ca}^{2+}]_i$ increases serve the intracellular signaling of numerous plasma membrane receptors (27). In microglia, receptors for classical neurotransmitters as well as those for immune system mediators are linked to the Ca^{2+} signaling machinery (15, 20, 28). Ca^{2+} may serve as an integrator of their cytosolic consequences to control microglial behaviour under resting and activated conditions. In this regard, transient changes in $[\text{Ca}^{2+}]_i$ evoked by LPS, adenosine triphosphate (ATP), thapsigargin (TSG) were examined and the influence of glycine on these Ca^{2+} signals determined. All three stimuli generate an increase in $[\text{Ca}^{2+}]_i$, but the mechanisms influenced to achieve this effect are different.

The by far most frequently used model stimulus to study microglial activation and inflammatory signaling is the Gram-negative bacterial cell-wall component **LPS**. LPS associates with the soluble LPS binding protein (LBP) and CD14, which is anchored in the outer leaflet of the plasma membrane. Signal transduction across the plasma membrane is made possible through the interaction of the LPS-CD14 complex with the transmembrane Toll-like receptor-4 (TLR-4) and the extracellular accessory protein MD-2. This leads to activation of kinases of various intracellular signaling pathways and upregulation of gene transcription for a variety of proinflammatory factors and free radical-generating enzymes (29). LPS also induces Ca^{2+} transients in cultured microglial cells, possibly resulting from caffeine-sensitive Ca^{2+} release (30) and/or dependent on external Ca^{2+} influx (31).

The expression of **ATP**-sensitive purinergic receptor subtypes P2X and P2Y has been well documented in microglia. Purinoreceptors control several properties of microglia, including the motility of their fine processes, the release of cytokines, migration and phagocytosis (23). ATP, which binds P2-receptors, induces a rapid microglial activation in response to local brain injury in vivo (32). It also triggers a rapid physiological response, the activation of cationic and K^+ conductance and an increase in $[\text{Ca}^{2+}]_i$ (33). The P2X receptors are nonselective cation channels and are Ca^{2+} -permeable providing a pathway for Ca^{2+} influx. The G-protein coupled metabotropic receptors of the P2Y family are classical 7-transmembrane G-protein coupled proteins which are known to activate phospholipase C-driven inositol 1,4,5-triphosphate (InsP_3 , IP_3) production and subsequent Ca^{2+} release from internal stores (28). Release of Ca^{2+} from internal stores can also be achieved by blocking sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps, which are responsible for re-uptake of Ca^{2+} in the lumen of the endoplasmic reticulum. This blockage of SERCA can be obtained by administering **TSG**, which

is a non-physiologic substance frequently used to study Ca^{2+} transients (28, 34, 35). To study the evoked Ca^{2+} transients, a Fluo-3 based Ca^{2+} measurement platform for plate reader technology. This was done to create a new platform for Ca^{2+} measurements, which offers a higher temporal resolution and a higher measurement throughput, compared to available fluorescence microscopy.

The fact that the stimulation of NO production is also GlyR-independent is in line with an electrophysiological study performed by Schilling et al. (36). This electrophysiological study shows that currents evoked by glycine in BV-2 cells are not Cl^- but Na^+ dependent and can be blocked by the System A neutral amino acid transporter (SAT) substrate α - (methylamino)-isobutyric acid (Me-AIB, Fig. 3). Based on these findings a possible role for neutral amino acid transporters in glycine modulation of microglial activity was hypothesized. To further examine the role of glycine transport as a possible mediator for glycine effects, a correlation was made between glycine modulation of Ca^{2+} signals and glycine uptake in BV-2 cells. Glycine uptake was determined in BV-2 cells using fluorescence immunostaining of intracellular glycine.

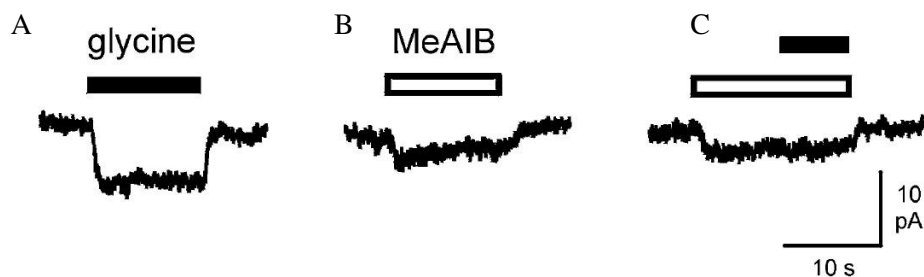


Figure 3. Patch clamp data concerning the glycine-evoked currents in BV-2 microglia (36). **A:** Inward current induced by 10 mM of glycine. **B:** Effect of 10 mM MeAIB (α -methylamino-isobutyric acid) on BV-2 microglial cells. **C:** During application of 10 mM MeAIB, inward currents could not be augmented by 10 mM glycine. Holding potential of all cells was -60 mV.

7. Production of $\text{TNF-}\alpha$

Next to NO production, cytokine secretion is an important property of microglial activation. Among the cytokines secreted from microglia, $\text{TNF-}\alpha$ is a central cytokine in the inflammatory process. It induces the expression of cell adhesion molecules in endothelial cells and astrocytes followed by infiltration of other leukocytes, resulting in inflammatory brain destruction (37). In multiple sclerosis (MS), a neuro-inflammatory disorder associated with the upregulation of $\text{TNF-}\alpha$ (38), it has been shown that $\text{TNF-}\alpha$ potentially induces myelin destruction and apoptosis of the myelin-producing oligodendrocytes, making a role for microglial $\text{TNF-}\alpha$ production in the pathogenesis of MS feasible (39, 40). Elevated $\text{TNF-}\alpha$ levels have also been associated with Alzheimer's disease (AD) and animal models of idiopathic Parkinson's disease (IPD), indicating a role for $\text{TNF-}\alpha$ not only in neuroinflammatory, but also in neurodegenerative diseases (29, 41).

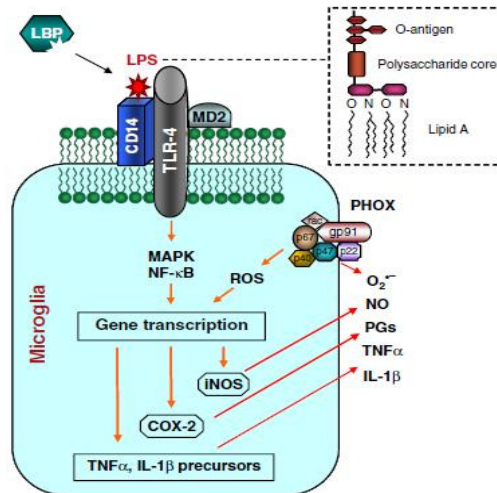


Figure 4: Schematic representation of LPS-induced glial activation (29).

Looking into research concerning the effects of glycine on TNF- α production, the paradox between experiments on peripheral immune function and microglia surfaces again. In the periphery, both in vitro and in vivo studies show suppression of TNF- α production through glycine administration (42, 43). Studies investigating the effect of glycine on TNF- α production in microglia are scarce, but it has been shown that L-serine increases TNF- α production in LPS-stimulated microglia (44). For detection of TNF- α in this study, enzyme-linked immunosorbent assays (ELISA) were applied on PMCs activated with LPS. LPS activation of TLR-4 on microglia leads to an initiation of downstream signaling events involving mitogen-activated protein kinases (MAPK) and transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B). Subsequent upregulation of gene transcription leads to the production and release of cytokines such as TNF- α (Fig.4).

8. The role of microglia in disease

As the major defenders of the brain parenchyma, microglia are called upon to perform a pivotal defense function against a broad array of pathogens that target the CNS. However, as mentioned before, in some conditions microglia can contribute to the exacerbation of pathological conditions. As an example, infectious agents like HIV (Human Immunodeficiency Virus) and tuberculosis are known to infiltrate microglia for the purpose of reproduction. In the case of HIV-1 pathogenesis, it is also becoming increasingly clear that neurotoxic mediators (e.g. NO, TNF- α , glutamate) released from microglia play an important role in CNS abnormalities that occur in HIV-associated dementia. Activation of microglia is also one of the hallmarks of neuropathic pain (17). Neuropathic pain typically develops when there is peripheral nerve injury (PNI), such as through surgery, bone compression in cancer, diabetes or infection. The main focus in neuropathic pain research has been investigating neurons and neuronal functioning. Though underappreciated, there is also evidence for participation of spinal cord microglia in the pathogenesis of PNI-induced neuropathic pain. One of the hypotheses is that activated molecular pathways lead to signaling from microglia to neurons within the

dorsal horn and ultimately change the properties of the spinal pain-processing network to bring about PNI-induced pain hypersensitivity. The P2X4 receptor and p38 MAPK have already been identified as intermediaries, but how exactly spinal cord microglia become activated or what effects the signaling microglia have on neurons is still under investigation (45). Open questions like these emphasize the importance of research on microglial activation, but also of research concerning communication between different cell types of the CNS.

Another field of pathology where interactions between microglia and the CNS-environment are important, is that of the neuroinflammatory and neurodegenerative diseases. The original idea behind the role of activated microglia in these diseases, was in a supportive function as scavengers of dead cells and debris. Today however, a neuropathological role for microglia has been postulated in most neuroinflammatory/neurodegenerative diseases. For neurodegenerative diseases, AD was the first of classic neurodegenerative diseases in which activated microglia were considered to be of neuropathological importance (17). The primary hypothesis for the role of microglia in AD, is the amyloidcascade/neuroinflammation hypothesis. This hypothesis proposes a linear sequence of events, where excessive amounts of beta-amyloid (A β) protein, deposited extracellularly as senile plaques, serve as a chronic inflammatory stimulus to activate microglial cells. As a result, the microglia produce neurotoxins that bring about neurodegenerative changes, such as neurofibrillary tangles and loss of synapses (46). Reports suggest that chemokine-like properties of A β protein are responsible for the pathophysiological changes in AD microglia and that they are Ca²⁺- and G-protein coupled receptor-dependent (47, 48).

A disease in which the role of microglia is also under careful study is the neuroinflammatory disease MS. Microglia have been shown to be active participants throughout the inflammatory disease process. They are functional as antigen presenting cells (APCs), initiate and propagate the immune response, phagocytose dying cells and cell debris and produce toxic innate effectors. These disease-related functional properties are in turn modulated by interactions with stranger/danger signals, infiltrating immune cells or their products, and neural cells such as injured or dying oligodendrocytes (49). These complex interactions lead to the idea of different functional levels regulated by feed-back signals which contribute in directing microglia from a detrimental to a non-detrimental-protective level, according to the tissue environment needs (50). With this in mind, studying the interactions between microglia and the CNS environment may reveal important information on modulation of microglial function. Significantly increased glycine levels in cerebrospinal fluid (CSF) and blood have been reported in patients presenting with acute MS (51, 52). Discovering how glycine influences microglia can thus prove important in understanding the functional levels of these cells in the injured CNS.

Materials & Methods

The materials and methods section is a summation of the different assays carried out during the research. For the most part, it contains standard protocols and refers to assay kits. The materials and method applied for the Ca^{2+} measurements however, were self-developed and optimized. A report of the development process of this assay is included in the results section.

1. Cell culture

BV-2 cells – Cells from the murine microglial cell line (53, 54) were kept in culture on Dulbecco's Modified Eagles Medium (DMEM) with L-glutamine and a high glucose content (4500 mg/ml) without pyruvate (Invitrogen, Merelbeke, Belgium) supplemented with 10% Fetal Calf Serum (FCS; Gibco, Paisley, UK), 100 U/ml Penicillin and 100 $\mu\text{g/ml}$ Streptomycin (1% P/S; Life Technologies, Paisley, UK). 24 hours before experiments cells were transferred to experiment dependent carries and put on Minimum Essential Medium (Gibco) supplemented with 10% FCS, 1% glutamine (Gibco) and 1% P/S (serum-MEM). For use in experiments medium was removed and replaced by a Standard Extracellular Solution (SES) containing 130 mM NaCl, 5 mM KCl, 2 mM CaCl, 1 mM MgCl_2 , 10 mM HEPES and 10 mM glucose (Sigma Aldrich, Bornem, Belgium).

Primary microglial cells – Primary cultures were isolated from one day old Whistar rats (P1-P2). After decapitation, rat forebrains were isolated and mechanically dissociated. The cell homogenate was cultured for 10–12 days in poly-L-lysine-coated (5 $\mu\text{g/ml}$) cell culture flaks (Nunc, Roskilde, Denmark) in DMEM with L-glutamine and a high glucose content (4500 mg/ml) without pyruvate supplemented with 10% FCS, 1% extra glutamine and 1% P/S. Incubation occurred at 37°C and 5% CO_2 . Microglial cells were then collected after a shake off (180 rpm, 37°C, 1 hour) and transferred to 96-well plates (Nunc) on serum-MEM. For use in Ca^{2+} measurements medium was removed and replaced by a SES identical to the one for BV-2 cells.

2. Cell viability assay

Cell viability was determined using the DHL™ Cell viability and proliferation assay kit. In this kit, resazurin is used as a sensitive fluorogenic indicator of lactate dehydrogenase activity (LDH), correlating to total cell viability. Resazurin is reduced to the strongly fluorescent resorufin (Ex: 560 nm Em: 590 nm) by mitochondrial LDH. Component A assay solution was diluted (1/6) in serum-MEM and 120 $\mu\text{l/well}$ was added to cells plated into F-bottom 96-well plates from which the supernatants had been removed. Measurements were done at one and two hours incubation and values from the first measurement were subtracted from the second measurement to determine the cell viability over one hour.

3. Ca²⁺ measurements

Ca²⁺ measurements were carried out according to optimized protocols using the Fluostar optima plate reader (Isogen Life Science, De Meern, The Netherlands). Cells were plated at a density of 50.000 (BV-2) and 100.000 (PMC) cells/well into U-bottom 96-well plates (Nunc). After plating, cells were allowed to incubate 24 hours (37°C, 5% CO₂) in serum-MEM. After 24 hours medium was removed and cells were rinsed twice in physiological SES buffer containing 2,5 mM Probenecid (Sigma Aldrich). Prior to loading with Fluo-3 AM (Invitrogen) a measurements was made to establish the background signal later subtracted from the actual fluorescence measurement. 100 µl of dye-loading buffer (SES) containing a final concentration of 5 µM Fluo-3 AM, dissolved in dimethylsulfoxide (DMSO) with 20% pluronic acid (Sigma Aldrich), was added to each well and cells were incubated for 30 minutes at 37°C and 5% CO₂. Once dye-loaded, the cells were washed thoroughly with assay buffer to remove any unincorporated dye. Exactly 100 µl of assay buffer was left in each well. The assay buffer consisted of SES buffer to which different products were added to test their acute effects on Ca²⁺ signals (see results). For determining chronic effects the different test products were added to the serum-MEM during the 24 hour incubation. Products used for the different conditions (glycine, L-serine, Strychnine, N-methyl-D-Glucamine (NMDG), α -amino-isobutyric acid (AIB), Valine, Leucine and Mannitol) were all purchased from Sigma Aldrich.

Before measuring cells were rested for 15 minutes, which allowed modulation of the cells by the test products and ensured full de-esterification of the Fluo-3 dye inside the cells. Measurements were performed by first establishing the baseline fluorescence (F_b), followed by injection of the Ca²⁺ signal eliciting stimulus (10 µl) and a follow up of the response. The different wells were measured sequentially (well mode) or simultaneously (plate mode) depending on the stimulus. The different stimuli were dissolved in SES buffer and diluted 1/11 (100 µl assay buffer + 10 µl injection) upon injection. For LPS, addition of 5% rat serum containing LBP, was required to elicit a Ca²⁺ signal. ATP, TSG, LPS and rat serum were all purchased from Sigma Aldrich.

Table 1. Summary of the different stimuli and the applied measurement parameters.

Ca ²⁺ signal elicitor	ATP	LPS	TSG
Final concentration	≈ 90 µM	≈ 9 µg/ml	≈ 450 nM
Measurement interval	1 interval = 1 s	1 interval = 1 s	1 interval = 15 s
Baseline	10 intervals	10 intervals	4 intervals
Follow up	90 intervals	90 intervals	56 intervals
# Wells	12	12	16-24
Scan mode	Well mode	Well mode	Plate mode
Tot. meas. time	1200 s	1200 s	15 minutes

4. Analysis of Ca²⁺ measurements

For analysis of the fluorescent traces, the fluorescence signal (F) was normalized to the F_b resulting in a F/F_b ratio. The F_b was defined by the mean fluorescence value of all the baseline measurements. The final parameter for analysis consisted of the ratio between the peak fluorescence value (F_{peak}) and the F_b (F_{max}/F_b). The F_{peak}/F_b parameter for each conditioned well was normalized to the mean of the controls. Conditioned wells were defined as wells exposed for 15 minutes to assay buffer conditioned with different products described in the results and the controls were defined as wells exposed for 15 minutes to assay buffer consisting only of SES buffer. Each condition, including control, was present in triplet for each individual experiment. Normalized TSG traces (Fig. 5A) required correction for a drift in fluorescence that occurred over the 15 minutes of the measurement (in contrast to the 100 s of ATP and LPS traces). Measuring this drift was done by quantifying the change in fluorescence seen in time on the normalized traces of non-injected controls (triplet). The F_b was subtracted from the mean F of the normalized non-injected controls and the subtracted result represented the drift in fluorescence (Fig. 5B). The final correction of the TSG traces was achieved by subtracting the drift from the normalized TSG signals (Fig 5C). Further analysis of these corrected traces was done as described earlier. For quantification, the maximum intracellular Ca²⁺ signal was measured (15 minutes, 60 s intervals) by removing the assay buffer and adding SES with 10 μ M ionomycin (Sigma Aldrich) and 5 mM CaCl. Subsequently the minimum intracellular Ca²⁺ signal was measured (15 minutes, 60 s intervals) by removing the buffer and adding SES with 10 μ M ionomycin, 10 mM EGTA (Sigma Aldrich) and 10 μ M BAPTA (Sigma Aldrich). The absolute $[Ca^{2+}]_i$ was calculated according to Merritt et al. (55).

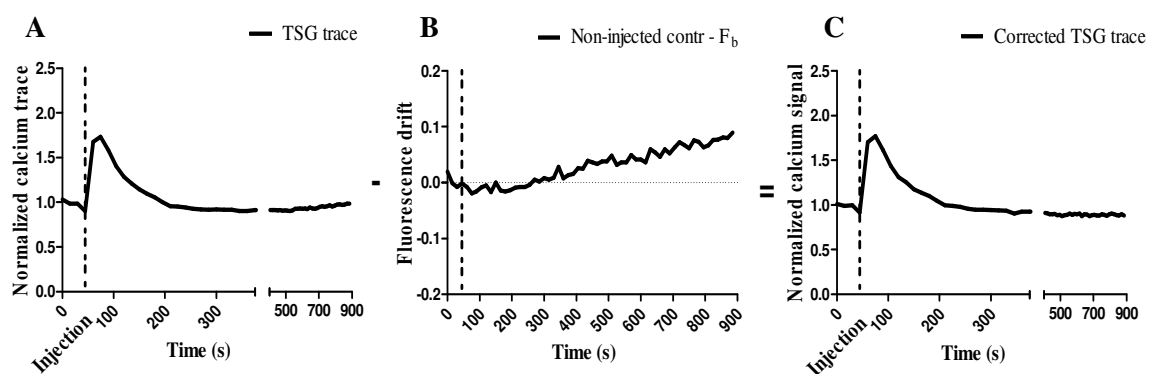


Figure 5. Processing of the normalized TSG Ca²⁺ traces (A) required a correction for the drift in fluorescence occurring over the 15 minute measuring period (B). This correction was achieved by subtracting the drift in fluorescence from the normalized TSG Ca²⁺ traces (C). The resulting trace represented the Ca²⁺ changes induced by TSG without interference of the drift in fluorescence. TSG: thapsigargin, F_b : fluorescence baseline

5. Glycine immunohistochemistry

BV-2 cells were transferred onto glass coverslips in S-MEM at a density of 25.000 cells/glass and allowed to incubate for 24 hours (37°C, 5% CO₂). After washing with SES buffer, cells were exposed to different conditions diluted in SES buffer described in the results. They were incubated for half an hour (37°C, 5% CO₂) to allow the cells to take up glycine. Following incubation, cells were immediately fixated for 5 minutes in 4% paraformaldehyde (PFA). Triton 0,2% was then applied for 10 minutes to ensure permeabilization, followed by blocking for 30 minutes with 3% filtered goat serum. For glycine detection a 1/1000 dilution of the primary polyclonal rat anti-glycine antibody (ImmunoSolution, Queensland, Australia) was added to the cells. Detection of the primary antibody was done by adding Alexa Fluor® 555 goat anti-rat IgG (1/500; Molecular Probes, Eugene, OR, USA). Phosphate buffered saline (PBS) was used for dilution of PFA, Triton, goat serum, primary and secondary antibody and for thorough washing of the cells between each step. After this procedure, the coverslips were mounted on glass slides using ProLong® Gold antifade reagent (Invitrogen).

Microscopical imaging was carried out on the Zeiss LSM 510 META one-photon confocal laser-scanning microscope (Jena, Germany). Images were made at 63x magnification and quantification was done using ImageJ software (National Institute of Health, Bethesda, USA). The background signal was subtracted from each image, regions of interest were manually drawn around individual cells and the average cellular fluorescence intensity of each condition was determined.

6. TNF- α assay

Primary cultured microglia were plated at a density of 100.000 cells/well into F-bottom 96-well plates (Nunc) on serum-MEM. Different conditions, described in the results, were also incorporated into the medium to determine their effects on TNF- α production. After overnight incubation at 37°C and 5% CO₂ half of the wells from each condition (triplets) were stimulated with 1 μ g/ml LPS. Supernatants were collected at different time points (6 and 10 hours) and TNF- α concentration was determined using the Quantikine® rat TNF- α immunoassay (R&D systems, Abingdon, UK) according to the kit manual. Absolute TNF- α concentrations were normalized to the stimulated control.

7. Statistical analysis

Data was analyzed using GraphPad Prism 5.01 software (GraphPad software Incorporated, La Jolla, USA). Statistical analysis was done by means of the student's t test or One-way analysis of variance (ANOVA) with Tukey (compare all pairs of columns) or Dunnett (compare all columns versus control column) post hoc tests. Results are presented as means \pm standard error of mean (S.E.M.) of the stated number of independent experiments and were considered significant when the p-value was below 0,05 (* p-value < 0,05; ** p-value < 0,01; *** p-value < 0,001).

Results

This section describes the relevant results obtained in the current study on the effects of glycine on microglial activity. Most results were derived from the extensive study of Ca^{2+} signals on BV-2 cells. This includes the optimization which was required to create a successful assay capable of measuring Ca^{2+} signals and a visualization of the strategy used to analyze the forthcoming data. The final segment contains the results from the TNF- α assays together with the control for cell viability.

1. Development of the Fluo-3 based Ca^{2+} measuring protocol

Ca^{2+} is one of the key elements in intracellular signaling, thus studying Ca^{2+} homeostasis forms a cornerstone of research into intracellular signaling pathways. The most conventional way of studying this parameter, is through Fura-2 AM based fluorescence microscopy (13, 26, 56). A labor intensive strategy based on fluorescence visualization of intracellular Ca^{2+} . Another way is through a Fluorescence Imaging Plate Reader (FLIPR) module, which allows a highly automated and high throughput screening of intracellular Ca^{2+} (57, 58). The downside of this assay being that it requires advanced plate reader technology to apply. The incentive for creating our own Ca^{2+} assay, came from a lack of conventional equipment and the curiosity to discover the options for less advanced plate reader technology.

1.1 Selecting a suitable Ca^{2+} tracer

The first obstacle in creating our new Ca^{2+} assay was to select the appropriate Ca^{2+} tracer. The ratiometric dye Fura-2 AM, which would be the most conventional choice, did not meet the requirements imposed by the available means. Due to its UV light excitability, it was not suited to be used with transparent plastic 96-well plates and available plate reader technology lacked the appropriate capacities for ratiometric excitation. Instead our preference went out to Fluo-3 AM, which had excitation and emission wavelengths more suited to the available plate reader. Next to a more convenient excitation wavelength, Fluo-3 AM had several other advantages over Fura-2 AM. The K_d of Fluo-3 is higher (864 nm at 37 °C), which allowed more sensitive measurement at higher $[\text{Ca}^{2+}]_i$ and Fluo-3 is highly fluorescent: binding of Ca^{2+} increases dye fluorescence by 40-fold. The great disadvantage to this tracer, is that it does not exhibit a spectral shift on binding of Ca^{2+} , which makes it a non-ratiometric tracer. To avoid any issues with calibration using Fluo-3 (Fig. 6), although technically feasible (55), research focused on measurements ratio's instead of absolute $[\text{Ca}^{2+}]_i$. Calculating measurement ratio's made the results cell number independent, as would be the case using the Fura-2 AM F340/F380 ratio. Information on the selected measurement ratio's will be provided after a description of the measurement optimization.

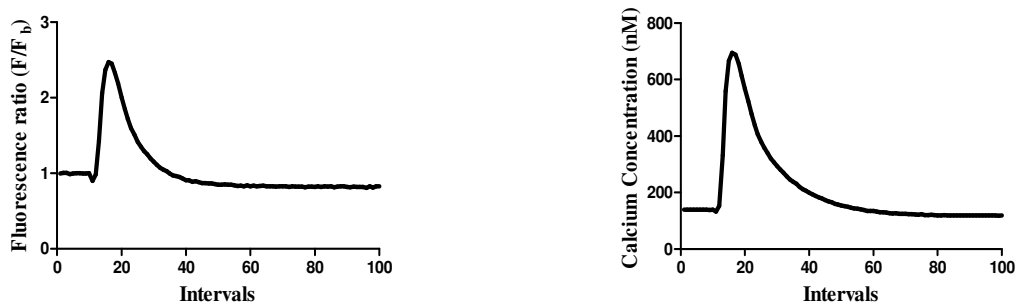


Figure 6. Left: Representation of an ATP-induced Ca^{2+} signal by normalizing fluorescence to the baseline level (F/F_b). **Right:** The same ATP-induced Ca^{2+} signal represented by the calculated Ca^{2+} concentration using the formula for Fluo-3 according to Merrit et al. 1990 (55).

1.2 Devising a measurement strategy

In devising our own methodology, different items found in literature were incorporated. This included adding 2,5 mM of probenecid to all buffers in order to block tracer leakage and dissolving the Fluo-3 AM dye in DMSO containing 20% pluronic acid to enhance dye solubility and uptake. To ensure a sensitive fluorescence signal, a loading time of half an hour was established. This resulted in a fluorescence signal which was approximately three times larger than the background (Fig. 7 Left). Once loaded and washed, cells were allowed to rest for 15 minutes. Besides allowing modulation of the cells by the conditions of the wash buffer, this period also allowed development of a stable signal and full de-esterification of Fluo-3 dye taken up by the cells. Because tracer leak at low amounts is essentially inevitable, a limited measuring time of 20 minutes was imposed in which stable measurements could be made (Fig. 7 Right). As a final measure, conditions in each separate experiment were spread randomly over all available wells. This was done to avoid artifacts due to variation caused by location on the plate or measurement order.

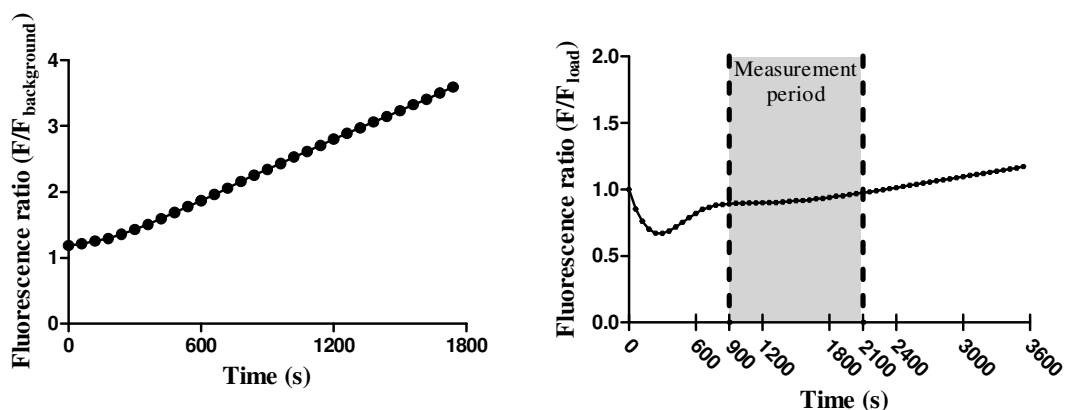


Figure 7. Left: Fluorescence ratio between loading and background fluorescence, clearly showing a more than threefold increase in fluorescence over a half hour loading period. **Right:** Graph showing the evolution in fluorescence signal represented by the ratio of the fluorescence signal to the final loading fluorescence. The measurement period indicates the stable portion of the baseline during which responses were measured.

The different stimuli used to elicit Ca^{2+} signals during the 20 minute measurement period, required two types of measurement strategies. Ca^{2+} responses to ATP and LPS, which elicited a short Ca^{2+} signal, were measured for 100 seconds, which was sufficient to measure the entire signal. A temporal resolution of one measurement per second was applied for these stimuli, which ensured a very accurate response measurement (Fig. 8 Left). Because of the high temporal resolution, and the limited time resolution in plate mode (10 s for a measurements of 12 wells) each well had to be measured separately (well mode). This implied that only 12 wells for either ATP or LPS (one injection system) could be measured within the 20 minutes allowed for one experiment. Combined with the demand that each condition be measured in triplicate, this allowed for a maximum of four conditions per experiment. The limitation to the number of wells in experiments measuring TSG-induced Ca^{2+} signals, which are much slower, were caused by the length of the measurement interval. The slower Ca^{2+} signal did not require a high temporal resolution, instead measurement intervals of 15 seconds were sufficient. This allowed simultaneous measurement of 24 wells within one interval (plate mode). In order to correct for a drift in fluorescence, each experiment included 3 non-injected controls, allowing a maximum of seven conditions (triplicate) in one experiment. The number of intervals was set at 60, resulting in a total measurement time of 15 minutes. This allowed full registration of the response and remained within the 20 minute measurement period (Fig. 8 Right).

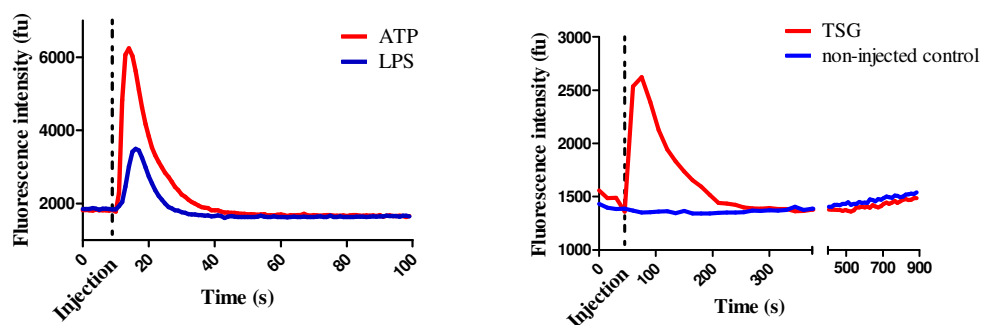


Figure 8. Fluorescence traces representing mean Ca^{2+} signals evoked by the different stimuli under control conditions. **Left:** Ca^{2+} signals evoked by ATP and LPS represented by raw fluorescence intensity over the total measurement period. **Right:** Ca^{2+} signal evoked by TSG and non-injected control. Both are represented by raw fluorescence intensity over the total measurement period. ATP: adenosine triphosphate, LPS: lipopolysaccharide, TSG: thapsigargin, fu: fluorescence units

1.3 Selecting the appropriate parameter

Once the appropriate measurement strategy was determined, an approach had to be developed for comparing the traces from different conditions. First, all traces were normalized to their baseline values (F_b) to avoid differences in raw fluorescence due to variations in cell number. The relevant parameters from the peak area of the normalized traces were extracted. These parameters were then normalized to the mean of those respective parameters in the control condition, putting the control condition at 100%. The normalized maximum was selected as the best parameter for representation of the Ca^{2+} signals because it was less prone to inter- and intra-experiment variation (Fig. 9).

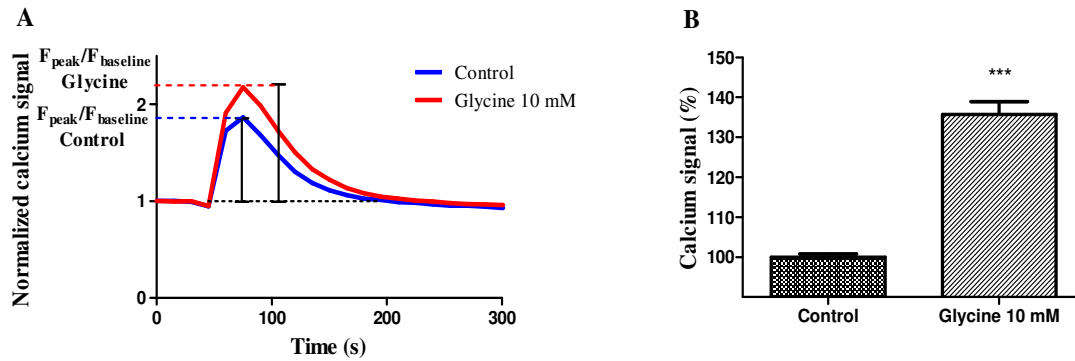


Figure 9. Parameter processing for TSG-induced Ca^{2+} signals. **A:** Partial graph of the mean normalized (F/F_b) TSG-induced Ca^{2+} signals in control and Glycine 10 mM conditions, showing the peak area of the normalized traces. The peak to baseline ratio is demonstrated for both conditions. Processing for ATP- and LPS-induced signals was identical. **B:** The peak to baseline ratio of the glycine condition is normalized to the control condition (100%). Conditions were compared using the student t-test. The glycine 10 mM condition shows significant enhancement of the Ca^{2+} signal compared to the control condition (p -value < 0,001). TSG: thapsigargin, ATP: adenosine triphosphate, LPS: lipopolysaccharide

2. Glycine enhances microglial Ca^{2+} signals

To further investigate the effect of glycine on microglia, the research focused on Ca^{2+} . Glycine and L-serine injection itself did not evoke Ca^{2+} signals in microglia (data not shown). Therefore we looked at the glycine and L-serine modulation of Ca^{2+} signals evoked by other stimuli, as it has been described for LPS in other immune cells (13). These stimuli were ATP, LPS and TSG, which are all frequently used when studying Ca^{2+} signaling. Ca^{2+} signals elicited by all three stimuli showed significant enhancement under glycine 10 mM conditions (Fig. 9-11, p -value < 0,001). After detecting this modulation of Ca^{2+} signals by glycine in an acute setting (15 minutes), several relevant issues were addressed. First 1 mM conditions were tested to determine concentration-dependence. Next it was investigated whether the Ca^{2+} signals were also modulated when cells were exposed to the same conditions for 24 hours (chronic), instead of 15 minutes (acute). Further experiments focused on the acute modulation in the 10 mM conditions and several steps were taken to determine the mechanism behind the enhancement of the Ca^{2+} signals. Based on preliminary research, the involvement of the GlyR, was the first item to be tested by means of strychnine application. This was followed up by investigating the hypothesized transporter involvement. On the one hand this was done by applying the neutral amino acid transporter substrate AIB and on the other Na^+ -free buffer was used to inhibit the Na^+ cotransport present for many of these transporters. The final experiments investigating transporter involvement focused on the amino acid specificity and osmolarity. Two other amino acids (Valine, Leucine) were selected to test for enhancement of Ca^{2+} signals and mannitol was used to check for any effects caused by changes in osmolarity. The last results from the Ca^{2+} measurements represent the first experiments towards verifying the results seen with the BV-2 cell line in primary cultured microglia.

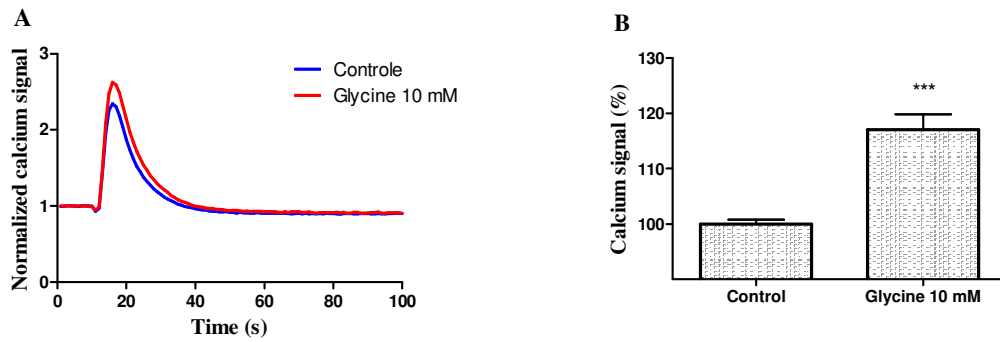


Figure 10. Glycine modulates ATP-induced Ca^{2+} signals. **A:** Graph of the mean normalized (F/F_b) ATP-induced Ca^{2+} signals in control and Glycine 10 mM conditions. **B:** The peak to baseline ratio (F_{peak}/F_b) of the glycine condition is normalized to the control condition (100%). Conditions were compared using the student t-test. The glycine 10 mM conditions shows significant enhancement of Ca^{2+} signals compared to the control condition (p -value $< 0,001$). ATP: adenosine triphosphate

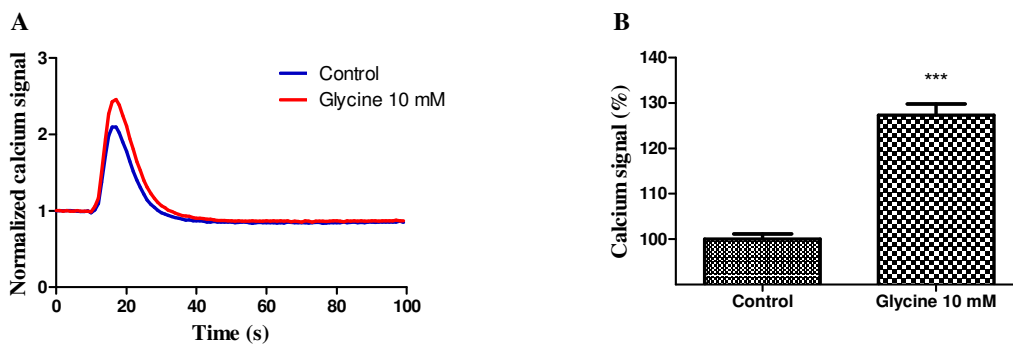


Figure 11. Glycine modulates LPS-induced Ca^{2+} signals. **A:** Graph of the mean normalized (F/F_b) LPS-induced Ca^{2+} signals in control and Glycine 10 mM conditions. **B:** The peak to baseline ratio (F_{peak}/F_b) of the glycine condition is normalized to the control condition (100%). Conditions were compared using the student t-test. The glycine 10 mM conditions shows significant enhancement of Ca^{2+} signals compared to the control condition (p -value $< 0,001$). LPS: lipopolysaccharide

2.1 Enhancement of Ca^{2+} signals occurs in an acute and concentration dependent setting

To further characterize the enhancement of microglial Ca^{2+} signals by glycine, incubation times for glycine and L-serine of 15 minutes (acute) and 24 hours (chronic) were tested. Results for TSG-induced signals show a significant increase of the Ca^{2+} signals in acute glycine and L-serine conditions compared to control (p -value $< 0,001$; Fig. 12A). In the chronic setting only L-serine shows a significant (p -value $< 0,01$) signal enhancement (Fig. 12B). When studying ATP-induced Ca^{2+} signals the acute modulation is similar to TSG (Fig. 13A). In chronic conditions, L-serine conditions show a slight enhancement compared to control (p -value $< 0,05$; Fig. 13B). This is in contrast to the chronic modulation of TSG-induced signals, but still for both stimuli, the effect of chronic conditions remains smaller than the effect observed in the acute conditions. All further results from here on out were obtained in the acute setting. Taking a closer look at these acute results reveals that at 1 mM levels only L-serine is able to significantly enhance TSG- and ATP-induced Ca^{2+} signals (p -value $< 0,001$; Fig. 12A,13A).

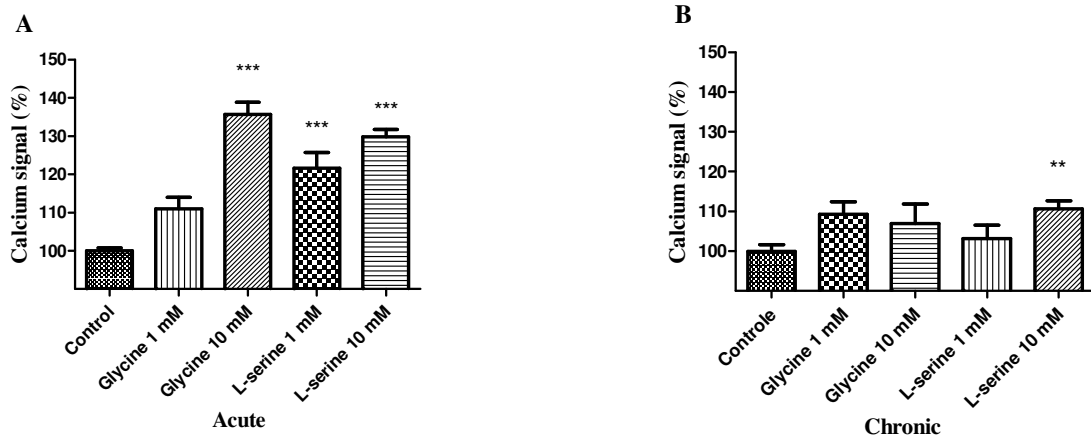


Figure 12. Modulation of TSG-induced Ca²⁺ signals by glycine and L-serine in acute and chronic conditions compared to control by ANOVA with the Dunnett post hoc test (N ≥ 3). **A:** In acute conditions, 10 mM of glycine and L-serine significantly enhance Ca²⁺ signals (p-value < 0,001). At 1 mM levels only L-serine significantly enhances the Ca²⁺ signal (p-value < 0,001). **B:** In chronic conditions the 10 mM L-serine condition is able to significantly enhance the induced Ca²⁺ signal (p-value < 0,01). TSG: thapsigargin

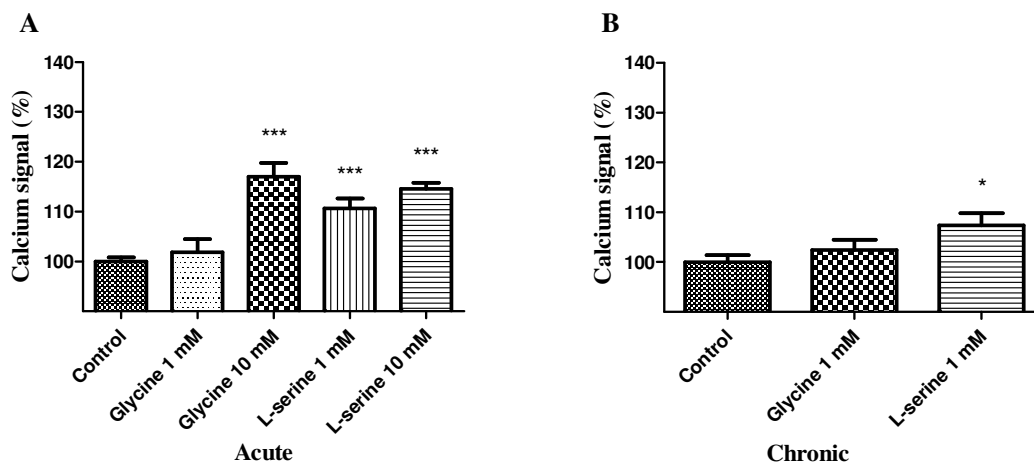


Figure 13. Modulation of ATP-induced Ca²⁺ signals by glycine and L-serine in acute and chronic conditions compared to control by ANOVA with the Dunnett post hoc test (N ≥ 3). **A:** In acute conditions, 10 mM of glycine and L-serine significantly enhance Ca²⁺ signals (p-value < 0,001). At 1 mM levels only L-serine still modulates significantly enhances the Ca²⁺ signal (p-value < 0,05). **B:** In chronic conditions 1 mM L-serine is able to significantly enhance the induced Ca²⁺ signal (p-value < 0,05). ATP: adenosine triphosphate

2.2 Modulation occurs GlyR-independently

Preliminary research showed modulation of NO production by glycine and L-serine was GlyR-independent. To investigate if this is also the case for modulation of Ca²⁺ signals, strychnine (1 μM) a high affinity competitive GlyR-blocker was applied in the glycine condition. Strychnine has no effect on the glycine modulation of the Ca²⁺ signals induced by both stimuli. No significant differences were found between the glycine and glycine + strychnine conditions and both show highly significant enhancement compared to control (p-value < 0,001; Fig. 14).

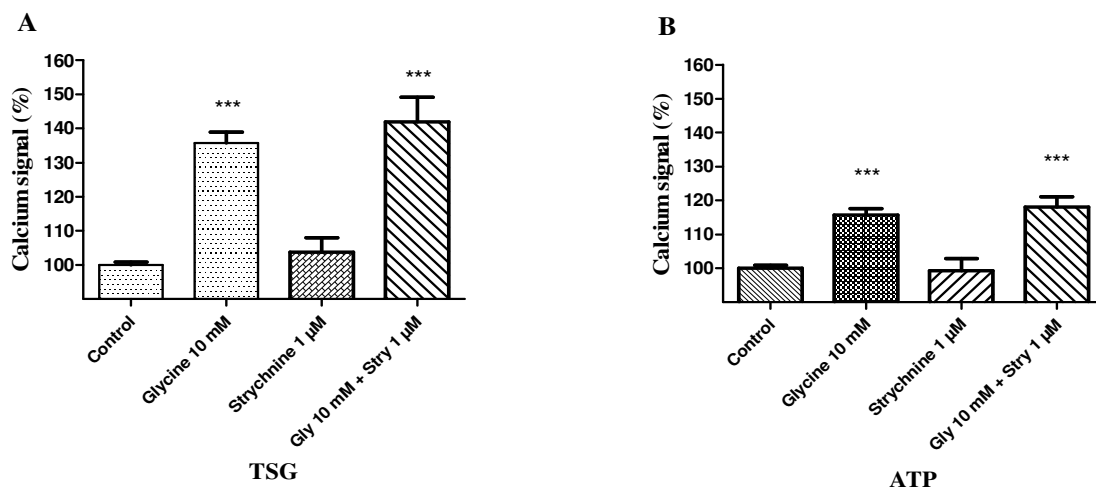


Figure 14. Enhancement of TSG- and ATP-induced Ca^{2+} signals by glycine shows no inhibition when 1 μM of strychnine is added to the condition. Conditions were compared using ANOVA with the Tukey post hoc test ($N \geq 3$). **A:** TSG-induced Ca^{2+} signals show no effect of strychnine itself and there is no significant difference between glycine and glycine + strychnine conditions. When strychnine is added, glycine still shows a significant enhancement compared to control, (p-value < 0,001). **B:** ATP-induced Ca^{2+} signals show no effect of strychnine itself and there is no significant difference between glycine and glycine + strychnine conditions. When strychnine is added, glycine still shows a significant enhancement compared to control (p-value < 0,001). Gly = glycine, Stry = strychnine, TSG: thapsigargin, ATP: adenosine triphosphate

2.3 Modulation is neutral amino acid transporter dependent

The electrophysiological study by Schilling et al. (36) showed an important role for Me-AIB in blocking glycine-evoked currents in microglia. In this study AIB, a transporter substrate for System A, L and asc neutral amino acid transporters was applied to investigate whether the glycine modulation of Ca^{2+} signals in microglia is neutral amino acid transporter dependent. Results for TSG- and LPS-induced Ca^{2+} signals indicate a significant inhibition of Ca^{2+} signal enhancement when 10 mM AIB is added to the 10 mM glycine condition (Fig. 15; TSG: p-value < 0,001; LPS: p-value < 0,05). It has been noted however that, in the AIB 10 mM control condition, there is also a small but significant enhancement of the Ca^{2+} signals compared to the control condition (Fig. 14; TSG: p-value < 0,05; LPS: p-value < 0,01). The inhibition of Ca^{2+} signal enhancement by AIB was also observed for L-serine enhancement of Ca^{2+} signals, where it showed a clear dose-dependent inhibition (Fig. 16).

An important conclusion made by Schilling et al.(36), was that glycine-evoked currents were carried by SATs and hence Na^+ -dependent. To test for the role of Na^+ in the modulation of microglial Ca^{2+} signals by glycine, the experiments were repeated without Na^+ in the extracellular environment. To this end we replaced Na^+ in the assay buffer with NMDG (or Meglumine), the most frequently used Na^+ substitute. Results show that in NMDG conditions the ATP-induced Ca^{2+} signals are no longer modulated by 10 mM glycine or L-serine (Fig. 17A). In the case of TSG-induced Ca^{2+} signals, modulation is still present under both glycine (p-value < 0,01) and L-serine (p-value < 0,001)

conditions (Fig. 17B). When comparing the NMDG conditions to conditions with Na⁺-containing assay buffer (SES), a significant enhancement of Ca²⁺ signals in NMDG control conditions compared to SES control conditions was observed (ATP: p-value < 0,01; TSG: p-value < 0,001; Fig.18).

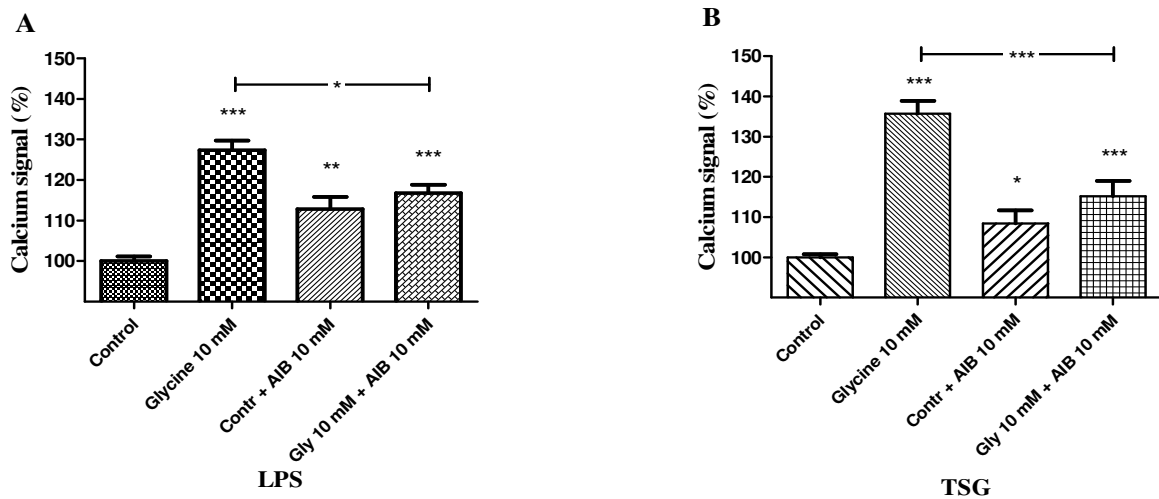


Figure 15. Modulation of TSG- and LPS-induced Ca²⁺ signals by glycine is inhibited by 10 mM of AIB. Conditions were compared using ANOVA with the Tukey post hoc test (N ≥ 3). **A:** LPS-induced Ca²⁺ signals show a significantly decreased modulation by 10 mM glycine when 10 mM AIB is added (p-value < 0,05). The condition containing 10 mM AIB without glycine shows enhanced modulation of Ca²⁺ signals compared to control (p-value < 0,01), as does 10 mM glycine + 10 mM AIB (p-value < 0,001). **B:** TSG-induced Ca²⁺ signals show a significantly decreased modulation by 10 mM glycine when 10 mM AIB is added (p-value < 0,001). The condition containing 10 mM AIB without glycine shows enhanced modulation of Ca²⁺ signals compared to control (p-value < 0,01), as does 10 mM glycine + 10 mM AIB (p-value < 0,001). Cont = Control, Gly = Glycine, AIB = α-amino-isobutyric acid, TSG: thapsigargin, LPS: lipopolysaccharide

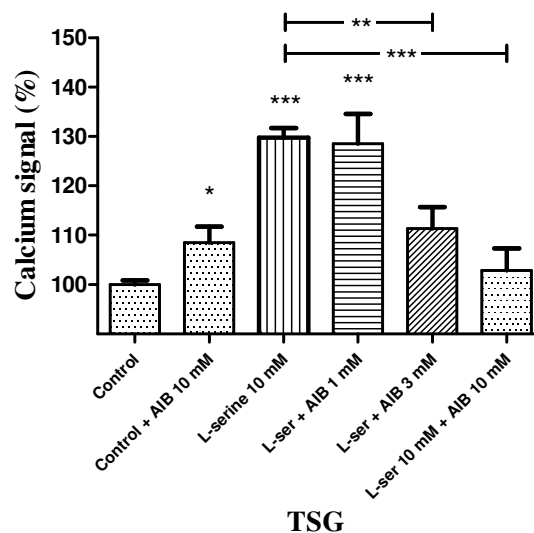


Figure 16. Enhancement of TSG-induced Ca²⁺ signals by 10 mM L-serine is inhibited by AIB. Conditions of 3 and 10 mM AIB with 10 mM L-serine show significant decrease in Ca²⁺ signal compared to 10 mM L-serine. The 1 mM AIB + 10 mM L-serine conditions is significantly enhanced compared to control, similar to the L-serine 10 mM condition (p-value < 0,001). There is no significant difference between those two conditions. Conditions were compared using ANOVA with the Tukey post hoc test (N ≥ 3). L-ser = L-serine, AIB = α-amino-isobutyric acid, TSG: thapsigargin

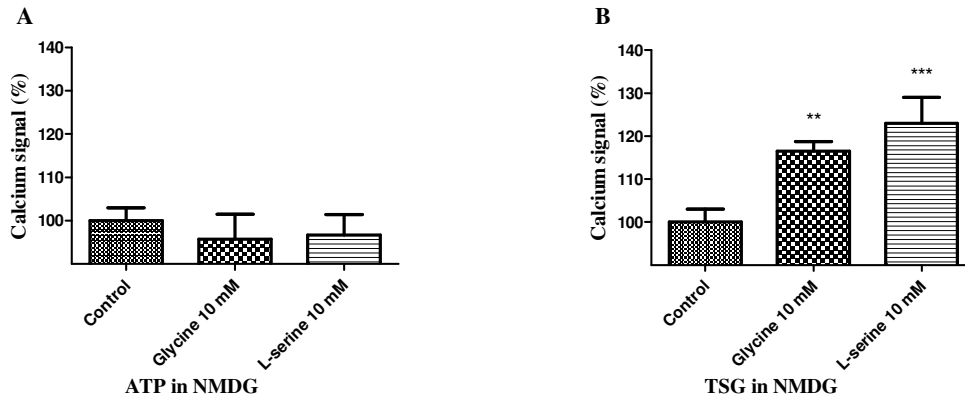


Figure 17. Representation of Ca^{2+} signal modulation by glycine and L-serine in Na^+ -free conditions. Conditions were compared using ANOVA with the Dunnett post hoc test ($N \geq 3$). Na^+ was replaced by NMDG. **A:** Modulation of ATP-induced Ca^{2+} signals by glycine and L-serine disappears in Na^+ -free conditions. **B:** Modulation of TSG-induced Ca^{2+} signals by glycine and L-serine is still significantly present in Na^+ -free conditions (glycine 10 mM: p-value < 0,01; L-serine 10 mM: p-value < 0,001; $N = 3$). TSG: thapsigargin, ATP: adenosine triphosphate, NMDG: N-methyl-D-glucamine

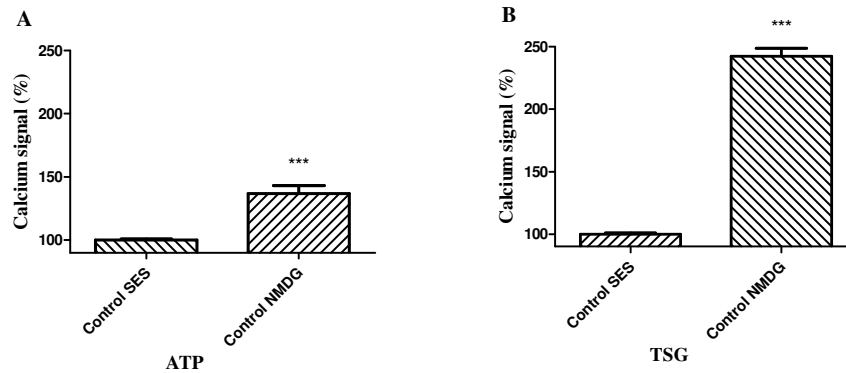


Figure 18. Representation of Ca^{2+} signals evoked in the NMDG control condition normalized to the SES control condition. Conditions were compared using the student's t test ($N \geq 3$). **A:** ATP-induced Ca^{2+} signals are significantly enhanced in the NMDG control condition compared to the SES control condition (p-value < 0,001). **B:** TSG-induced Ca^{2+} signals are significantly enhanced in the NMDG control condition compared to the SES control condition (p-value < 0,001). TSG: thapsigargin, ATP: adenosine triphosphate, NMDG: N-methyl-D-glucamine

2.4 TSG-induced Ca^{2+} signals are amino acid specific

To determine the amino acid selectivity of the Ca^{2+} signal modulation, other amino acids besides glycine and L-serine were investigated. Valine and leucine were selected, both substrates for different transporters and without known immune regulatory effects have. They were tested together with mannitol to investigate a potential contribution of osmolarity changes in the glycine effect. Results show a significant enhancement in the 10 mM valine conditions (TSG: Fig 19A; ATP, LPS: Table 2; p-value < 0,001), similar to 10 mM glycine and L-serine. The conditions with 10 mM leucine and mannitol were not able to enhance microglial Ca^{2+} signals compared the control. The observed enhancement under glycine conditions compared to control, remained when 10 mM glycine was compared to 10 mM leucine and mannitol (p-value < 0,001; Fig. 19B).

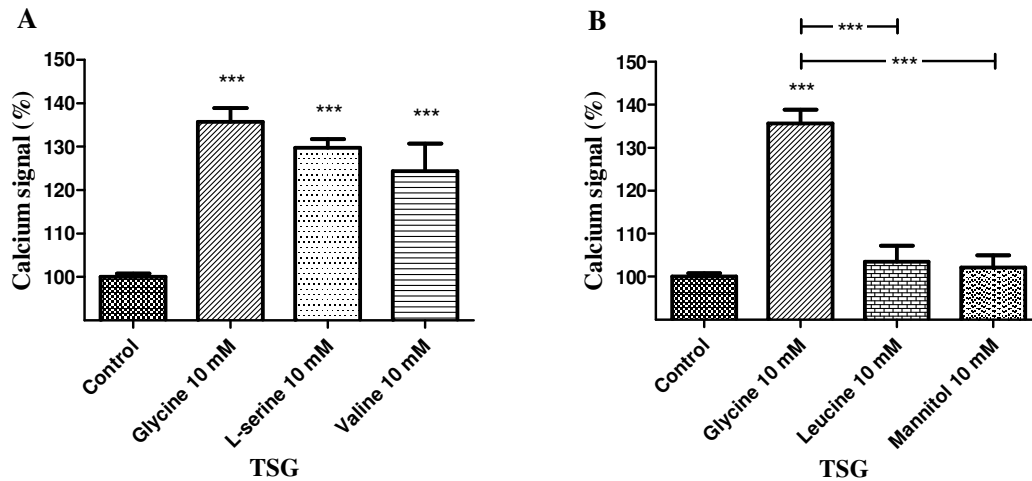


Figure 19. TSG-induced Ca^{2+} signals show amino acid specific modulation. Conditions were compared using ANOVA with the Tukey post hoc test ($N \geq 3$). **A:** The condition containing 10 mM valine was able to significantly enhance microglial Ca^{2+} signals compared to the control condition (p -value $< 0,001$). There was no difference between 10 mM valine and 10 mM glycine or L-serine. **B:** The conditions containing 10 mM Leucine and mannitol showed no significant enhancement of microglial Ca^{2+} signals compared to control. The observed enhancement of 10 mM glycine compared to control remained when glycine was compared to leucine and mannitol (p -value $< 0,001$).

2.5 Verification in primary cultured microglia

To confirm the results seen with the BV-2 murine cell line, a verification was done on PMCs. The first results show that ATP-induced Ca^{2+} signals are significantly enhanced by 1 mM L-serine (p -value $< 0,05$; Fig. 20) in the acute setting. These results were similar to the results obtained for the 1 mM glycine and L-serine conditions in the BV-2 cells (Fig. 13A). In the chronic setting, no enhancement could be observed under 1 mM conditions of glycine and L-serine.

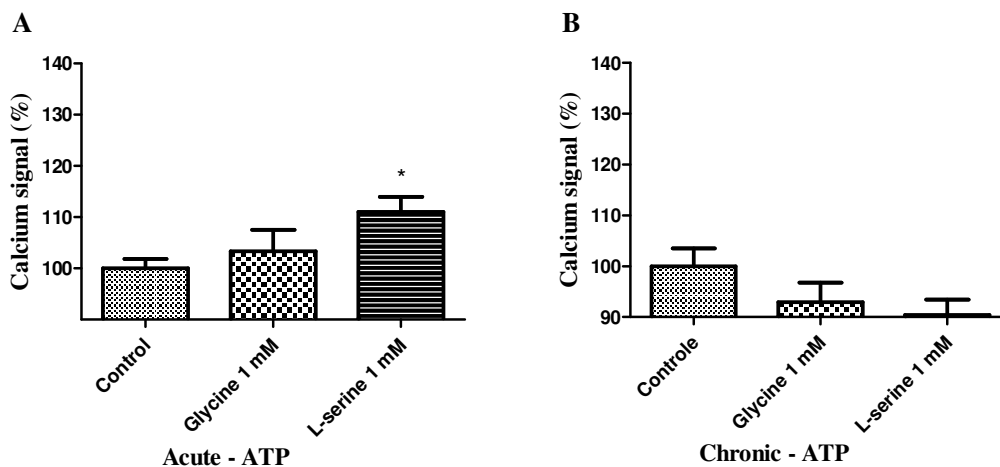


Figure 20. Modulation of ATP-induced Ca^{2+} signals by glycine and L-serine in PMCs. Conditions were compared to control by ANOVA with the Dunnet post hoc test ($N \geq 3$). **A:** In acute conditions 1 mM of L-serine was able to enhance the Ca^{2+} signals compared to control. (p -value $< 0,05$). **B:** In chronic conditions, no significant enhancement of Ca^{2+} signals was present. ATP: adenosine triphosphate

Table 2. Summary of normalized Ca²⁺ signals of BV-2 cells evoked by ATP, TSG and LPS in all acute conditions. Conditions are represented by means with 95% confidence interval (CI) and the p-value of each CI compared to 100% using the t-test. Gly = glycine, L-ser = L-serine, AIB = α -amino-isobutyric acid, Stry = strychnine, NMDG: N-methyl-D-glucamine

Celltype	Stimulus	Acute condition	Mean (%)	95% CI	p-value	N
BV-2	ATP	Glycine 1 mM	101,9	96,23 - 107,5	n.s.	4
		L-serine 1 mM	110,7	106,5 - 114,9	< 0,0001	4
		Glycine 10 mM	117	111,4 - 122,7	< 0,0001	9
		L-serine 10 mM	114,5	111,9 - 117,2	< 0,0001	7
		Strychnine 1 μ M	99,21	90,74 - 107,4	n.s.	3
		Gly 10 mM + Stry 1 μ M	118	110,9 - 125,1	< 0,0001	3
		Valine 10 mM	114,4	108,2 - 120,6	< 0,0001	4
		NMDG Glycine 10 mM	95,76	83,20 - 108,3	n.s.	3
		NMDG L-serine 10 mM	96,7	86,4 - 107	n.s.	3
	LPS	Glycine 10 mM	127,4	122,3 - 132,4	< 0,0001	6
		L-serine 10 mM	130,9	118,6 - 143,1	0,0004	3
		AIB 10 mM	112,8	105,8 - 119,9	0,0031	3
		Gly 10 mM + AIB 10 mM	116,8	112 - 121,5	< 0,0001	3
		Valine 10 mM	129,4	120,7 - 138,1	< 0,0001	3
	TSG	Glycine 1 mM	111	103,8 - 118,3	< 0,0001	3
		L-serine 1 mM	121,6	112,2 - 131	< 0,0001	3
		Glycine 10 mM	135,7	129,4 - 142,1	0,0086	15
		L-serine 10 mM	129,8	125,8 - 133,7	< 0,0001	13
		AIB 1 mM	113,4	101,8 - 125	0,0285	3
		AIB 3 mM	110,6	97,12 - 124,1	n.s.	3
		AIB 10 mM	108,5	101,7 - 115,2	0,0163	8
		Gly 10 mM + AIB 10 mM	115,2	107 - 123,3	0,0014	5
		L-ser 10 mM + AIB 1 mM	128,5	114,6 - 142,5	0,0015	3
		L-ser 10 mM + AIB 3 mM	111,4	101,4 - 121,3	0,03	3
		L-ser 10 mM + AIB 10 mM	102,9	96,69 - 113,1	n.s.	3
		Valine 10 mM	124,4	110,4 - 138,3	0,0027	4
		Leucine 10 mM	103,5	94,64 - 112,3	n.s.	3
		Mannitol 10 mM	102,2	95,56 - 108,7	n.s.	3
NMDG Glycine 10 mM	116,5	111,7 - 121,2	< 0,0001	4		
NMDG L-serine 10 mM	123	110,4 - 135,7	0,0015	4		
PMC	ATP	Glycine 1 mM	103,4	94,32 - 112,4	n.s.	4
		L-serine 1 mM	111	104,5 - 117,5	0,0028	4

3. Glycine uptake in microglia is neutral amino acid transporter mediated.

Immunostaining experiments were performed to visualise a possible glycine transport into the cells. The conditions and their incubation times were set up similar to the Ca^{2+} measurements, in order to indicate a possible correlation. The images show a clear difference in glycine staining between the cells incubated in SES buffer and cells incubated in SES buffer containing 10 mM glycine. In the control condition there is a low level staining, while this is severely augmented in the glycine condition (Fig. 21 Top). When adding 10 mM AIB to these conditions, there is no visual change in the control condition (Fig. 21 Left). In the glycine condition however, a decrease in staining can be observed when AIB is added (Fig. 21 Right). Though there is a decrease, the staining does not appear to decrease to the level of the AIB-control condition (Fig. 21 Bottom). The images were also quantified and results confirm the visual analysis. Significant differences were found between all conditions (p-value < 0,001), except between control and AIB-control (Fig. 22). Glycine staining is augmented 436% \pm 13% in glycine conditions compared to control, AIB decreases this augmentation to 217 \pm 10% (Table 3).

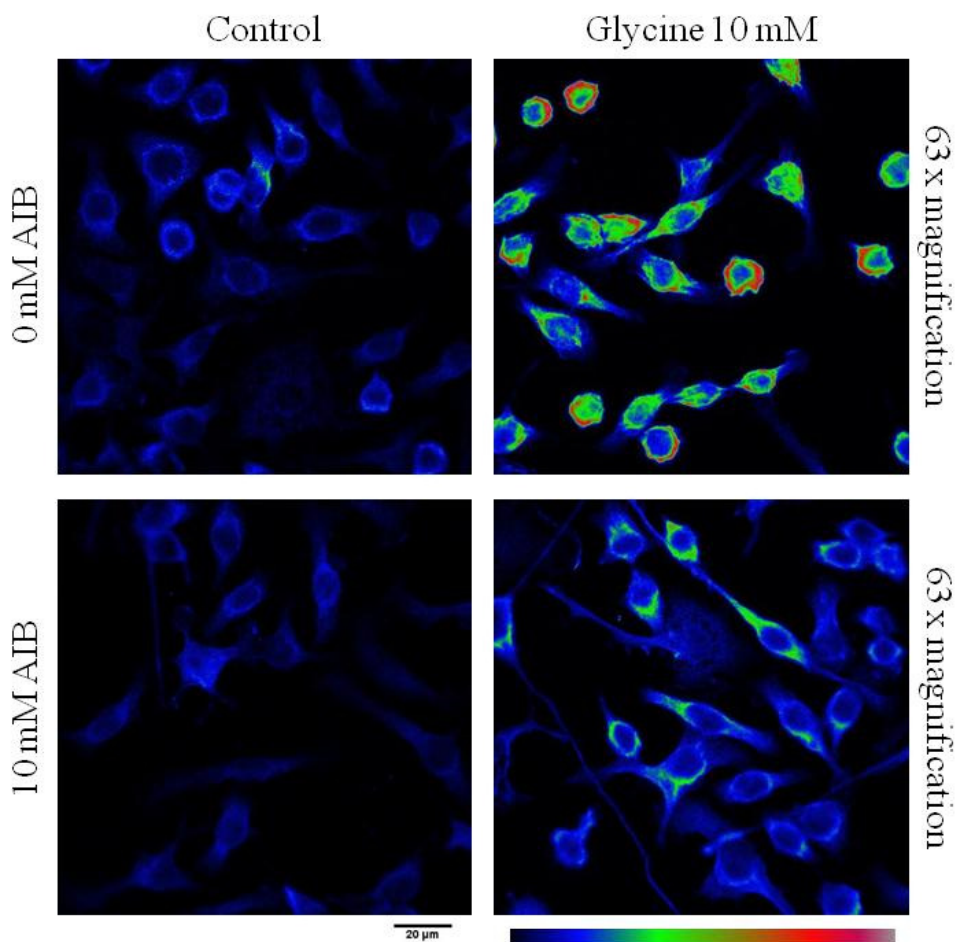


Figure 21. Confocal microscopy images of BV-2 cells labeled for glycine. Cells incubated in 10 mM glycine show increased staining compared to control. When AIB is added there is no change in staining of the control condition. In glycine conditions there is a visual decrease in glycine staining when AIB is added. However, staining in glycine + AIB conditions still appears to be augmented compared to control + AIB and control conditions. AIB = α -amino-isobutyric acid

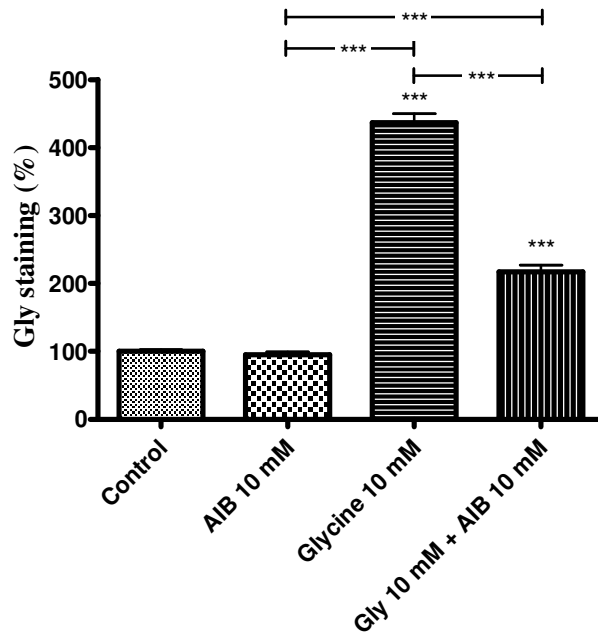


Figure 22. Quantification of the glycine immunostainings. The mean of the individual cell fluorescence was determined for all conditions and they were compared using ANOVA with the Tukey post hoc test. Cells incubated in 10 mM glycine show significantly enhanced staining compared to control (p-value < 0,001). When AIB is added there is no change in staining of the control condition. In glycine conditions there is a significant decrease in glycine staining when AIB is added (p-value < 0,001). However, staining in glycine + AIB conditions is still significantly augmented compared to control + AIB and control conditions (p-value < 0,001). Results are expressed as mean \pm S.E.M. of 3-5 independent experiments, with at least 6 cells quantified for every individual experiment. AIB = α -amino-isobutyric acid

Table 3. Overview of the average staining \pm S.E.M. normalized to the control condition (100%).

Gly = glycine, AIB = α -amino-isobutyric acid

Control	100% \pm 3%
AIB 10 mM	95% \pm 4%
Glycine 10 mM	436% \pm 13%
Gly 10 mM + AIB 10 mM	217% \pm 10%

4. Glycine increases TNF- α production

Primary cultured microglia were plated out into medium containing a control and four different conditions: glycine 1 mM, strychnine 1 μ M, glycine 1 mM + Strychnine 1 μ M and L-serine 1 mM. The concentrations normalized to the stimulated control are presented together with normalized cell viability results. This was done in order to determine a possible correlation between changes in TNF- α production and altered cell viability. At six hours stimulation, the results show a significant increase in TNF- α production in all conditions compared to control (p-value < 0,05). The matching results for cell viability show no significant differences in cell viability of the conditions compared to control. At 10 hours stimulation, only glycine 1 mM, strychnine 1 μ M and glycine 1 mM + Strychnine 1 μ M show significant increase of TNF- α production compared to control (p-value < 0,001). Matching cell viability assay shows no significant differences in cell viability compared to control (Fig. 23).

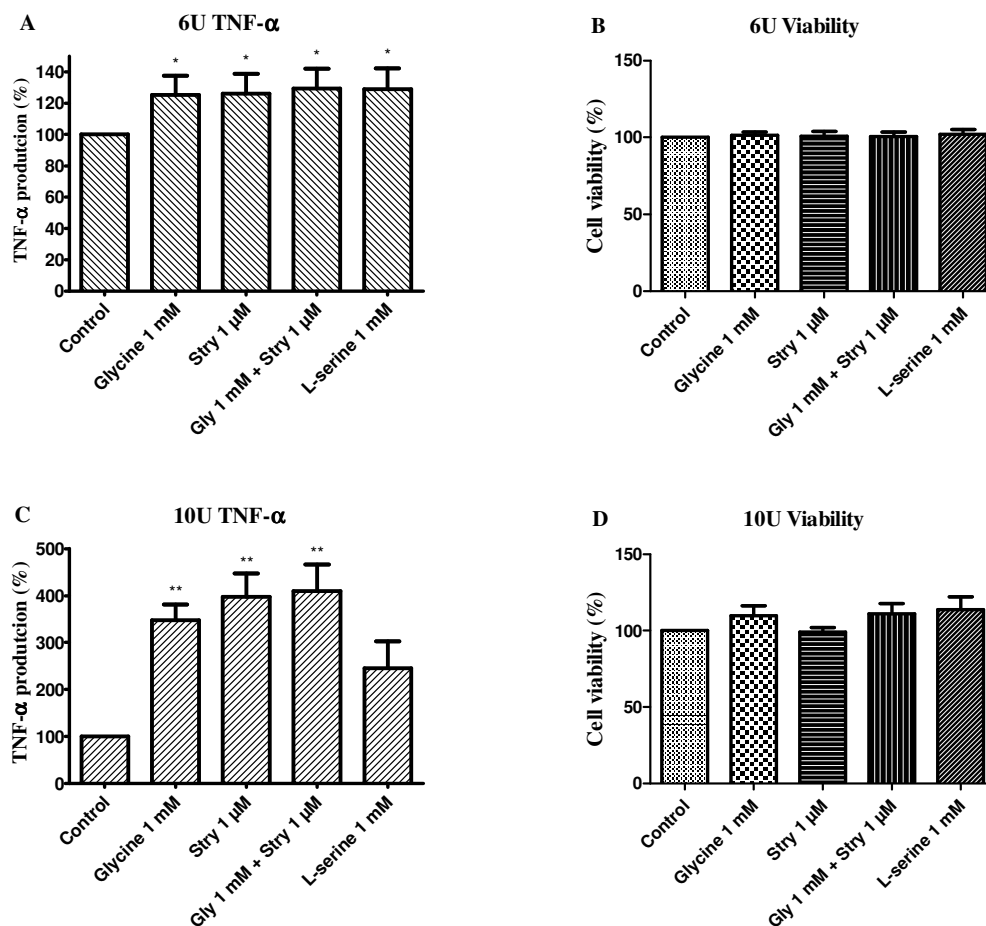


Figure 23. Results from the TNF- α assay showing TNF- α production and cell viability under the different conditions, normalized to control. Results were compared to their respective control using ANOVA with the Dunnett post hoc test. **A.** Six hours after LPS stimulation TNF- α production is significantly increased in all conditions compared to control (p-value < 0,05). **C.** Ten hours after LPS stimulation TNF- α production is significantly increased in glycine 1 mM, Strychnine 1 μ M and Glycine 1 mM + strychnine 1 μ M conditions (p-value < 0,001). **B, D.** Results show no significant differences in cell viability between the conditions at 6 and 10 hours stimulation with LPS. Gly = Glycine, Stry = strychnine, LPS = lipopolysaccharide

Discussion

The Fluo-3 based Ca^{2+} measurement protocol developed in this study has, like any other technique, its inherent strengths and weaknesses. The fact that the fluorescence signal was a cumulative signal from an entire population of cells, made this technique less prone to intercellular variation. However, this also makes it impossible to detect any differences in response within a population, making the technique unsuitable for mixed cell type populations, but ideal for cell lines and purified cultures. Another limitation of the technique was the lack of absolute Ca^{2+} quantification, due to the non-ratiometric nature of the Fluo-3 Ca^{2+} tracer. As demonstrated, a quantification is possible, but would be less reliable because the fluorescence signal is dependent on the cell number during the different steps of the quantification. This cell number can be altered by washing steps between the different measurements required for quantification. Merritt et al. (55) also reported that ionomycin, used in quantifications for evoking the maximum Ca^{2+} signal, was not capable of doing this when Fluo-3 was applied. They demonstrated that digitonin, a lipid solubilizing compound, was still able to further increase the maximum Ca^{2+} signal after ionomycin application. To avoid any issues with a non-normalized quantification method, raw fluorescence ratio's from the Ca^{2+} signal measurement itself were applied. To this end the raw fluorescence signal was normalized to the mean of the baseline fluorescence, which created a cell number independent trace. The normalized maximum of the Ca^{2+} signal provided a stable measurement parameter. In retrospect, the selected Fluo-3 AM based protocol allowed for an elaborate study of microglial Ca^{2+} signals at a time resolution of 1 s/measurement (well mode). This time resolution could not have been achieved had Fura-2 AM been applied. This proves the technical and scientific value of the developed protocol and shows the potential of plate reader technology for Ca^{2+} measurements.

Applying this new protocol we clearly showed an enhancement of the induced Ca^{2+} signals by glycine and L-serine (10 mM) and this modulation was dose dependent. At 1 mM concentrations, L-serine but not glycine significantly enhanced Ca^{2+} signals indicating a more potent role for L-serine. Fifteen minutes of pre-incubation (acute) with glycine and L-serine also appeared to be more potent for enhancing Ca^{2+} signals, compared to 24 hours of pre-incubation (chronic). In the chronic experiments, the conditions were applied in the medium which was washed off when the cells were loaded. A decrease of the modulation in this protocol could imply the required presence of glycine for maximum Ca^{2+} flux enhancement. The demonstration of the acute nature for glycine enhancement of microglial Ca^{2+} signals contradicts a metabolic mechanism underlying glycine effects. Because glycine is an important metabolic building block of the cell, for example in glutathione synthesis, altered amino acid availability could also influence chronic cellular processes such as protein synthesis and gene transcription.

The millimolar dose response, observed for glycine enhancement of Ca^{2+} signals in microglia, conflicts with glycine and L-serine effects on NO production that are already present at micromolar concentrations. The stimulation for NO production was also applied after 24 hours pre-incubation with glycine, an incubation time at which enhancement of Ca^{2+} signals by glycine was no longer present. This could indicate that both effects are unrelated, but this was contradicted by a similarity in the effect of glycine on both parameters. Both elevation of NO production and enhancement of Ca^{2+} signals showed a similar glycine effect size ($\pm 35\%$) and a more potent role for L-serine. Even more striking, is that they also both seem to suggest a GlyR-independent mechanism underlying the effect. This is argued by the fact that L-serine, which is not a GlyR agonist in these concentrations, evoked a similar effect and that both the effects of glycine and L-serine were strychnine-insensitive. A possible explanation for the concentration discrepancy could be confined in the difference of effect level. Elevation of NO production is present on a chronic level (days), while elevation of the studied Ca^{2+} signals is measured on an acute level (minutes).

GlyR-independent modulation of Ca^{2+} signaling can also be correlated with the patch clamp experiments measuring the glycine-evoked currents. Glycine evoked currents, which were present at equimolar range, were also strychnine-insensitive. Schilling et al. (36) also showed that glycine-evoked currents were not Cl^- - but Na^+ -dependent and could be blocked by applying the SAT substrate MeAIB. Similar results were obtained by the Ca^{2+} measurements, which showed a dose-dependent decrease in L-serine enhancement of Ca^{2+} signals when AIB was applied. Glycine enhancement of Ca^{2+} signals was also inhibited by AIB, but this inhibition was not as complete as observed for L-serine. L-serine and glycine may have responded to AIB in different ways, because of a difference in transporter affinity and thus competition with AIB. The fact that 10 mM AIB did not completely inhibit the glycine effect was also seen in the immunostainings visualizing glycine uptake. These glycine immunostainings show a highly significant presence of intracellular glycine compared to control, when cells are exposed to glycine (10 mM) for 30 minutes ($436 \pm 13\%$). This is mainly an argument for glycine uptake in microglia. The fact that this uptake could also be inhibited with AIB, provides a strong correlation between glycine enhancement of Ca^{2+} signals and glycine uptake by microglia. As mentioned before applying AIB to the glycine condition could not completely block the glycine uptake compared to control ($217 \pm 10\%$). Since there was no significant difference between AIB-control and AIB-glycine conditions in the Ca^{2+} measurements, it is likely that this residual uptake is not sufficient for evoking an enhancement of Ca^{2+} signals by glycine. Only SATs have glycine and AIB as substrates, implicating this transporter system in glycine enhancement of microglial Ca^{2+} signals (59). The System A transporter family consists of three subtypes (SAT 1-3), which all have a stoichiometry of 1:1 (amino acid : Na^+). Possible physiological roles for these transporters have been described in the GABA/Glutamate – Glutamine cycle in the brain, in ammonia detoxification and gluconeogenesis in the liver, in the renal response to acidosis and even in neoplasia. (60).

Na⁺-free experiments were attempted in an effort to learn more about a possible role for Na⁺-coupled transporters. When Na⁺ was replaced by NMDG an overall decrease in Ca²⁺ signal enhancement by glycine and L-serine was observed. For ATP-induced Ca²⁺ signals, this decrease was a full inhibition, while glycine enhancement of TSG-induced Ca²⁺ signals was decreased by approximately 20%. The residual enhancement of TSG-induced Ca²⁺ signals makes it difficult to conclude that Na⁺-dependent neutral amino acid transport is responsible for enhancing microglial Ca²⁺ signals. The relevance of these experiments is further compromised by reports of physiological effects inherent to NMDG, when used as Na⁺-replacement (61, 62). These reports imply that NMDG increases resting intracellular Ca²⁺, possibly through inhibition or reversal of the Na⁺-Ca²⁺ exchanger. The results also showed an increase in Ca²⁺ signal under the control NMDG condition compared to the Na⁺-containing control condition. It is possible that NMDG caused the intracellular Ca²⁺ stores of the microglia to be loaded with extra Ca²⁺, which created a larger Ca²⁺ signal upon store emptying by ATP and TSG. This side effect could influence the enhancement of Ca²⁺ signals by glycine and L-serine.

The fact that the mannitol condition was not able to enhance Ca²⁺ signals showed that the change in osmolarity, caused by adding 10 mM of a test product, does not influence the TSG-induced Ca²⁺ signals. Results also showed that the neutral amino acid valine, but not leucine, was able to enhance microglial Ca²⁺ signals to the same degree as glycine and L-serine. This indicates a clear amino acid specificity for enhancing TSG-induced Ca²⁺ signals. One amino acid transporter, which transports glycine, L-serine and valine but not leucine is SAT 1. This Na⁺-dependent neutral amino acid transporter is a System A transporter, which corresponds with a role for System A transporters shown by AIB. The fact that there is a neutral amino acid transporter selectivity that correlates with the selectivity exhibited in enhancing Ca²⁺ signals, makes these neutral amino acid transporters likely targets for directly enhancing Ca²⁺ signals. The electrogenic properties of the glycine transport, caused by Na⁺ cotransport, could have an influence on V_m and thereby directly influence certain Ca²⁺ fluxes. However, a role for AIB as a competitive agonist pleads against involvement Na⁺-dependent transport, seeing as Schilling et al. (36) also described an inward current upon application of MeAIB. It can however be argued that this current is visually smaller than the glycine-evoked current. This could be explained by the fact that MeAIB is specific to System A, but not an efficient model substrate (60). If current size is important, the smaller current evoked by MeAIB could correspond to a slightly significant enhancement of Ca²⁺ signals seen in AIB-control conditions and the larger current evoked by glycine could correspond with a highly significant enhancement of Ca²⁺ signals under glycine conditions. On the other hand, unless voltage gated channels or other membrane potential sensitive intermediaries are involved, a membrane depolarization due to a Na⁺ influx from glycine cotransport would also be expected to limit the driving force for Ca²⁺-entry instead of enhancing the induced Ca²⁺ signals.

If the electrogenic nature of the amino acid transport is not responsible for enhancing Ca^{2+} signals, it is also possible that the transported amino acids exert an unknown intracellular signaling effect. Leucine might for example be transported into microglia, but lack the intracellular signaling capabilities required for enhancing the studied Ca^{2+} signals. Concerning cellular signaling capabilities, one possible mechanism that may lead to enhanced Ca^{2+} signals has been described specifically for L-serine. Studies investigating anti-hypertensive effects of amino acids show L-serine was able to induce a fall in mean arterial pressure and this was mediated by opening of vascular Ca^{2+} -sensitive potassium channels (K_{Ca}) (63, 64). An increased conductance of K_{Ca} -channels present on microglia would lead to a hyperpolarization of the cell membrane and would increase the driving force for Ca^{2+} influx upon induction of Ca^{2+} signals. For now there is cumulative evidence for the involvement of amino acid transporters, but how they are exactly implicated in enhancing Ca^{2+} signals remains unknown.

Experiments using PMCs, instead of BV-2 cells, for Ca^{2+} measurements showed similar results for enhancement of ATP-induced Ca^{2+} signals by glycine and L-serine at 1 mM levels. This indicates that the cell line properties of the BV-2 cells do not confound the in vitro microglial responses measured. Enhanced Ca^{2+} signals at 1 mM glycine and L-serine levels in PMCs correspond with increased levels of TNF- α production shown at incubation with equimolar levels of glycine and L-serine. These results correlate with the increased immune response reported in other experiments (18, 19). TNF- α levels were also increased when glycine and strychnine were administered together. Unfortunately no conclusion could be drawn concerning the GlyR involvement, due to the unexpected finding that TNF- α levels were also increased in the strychnine control condition. Perhaps this issue could be avoided, not by excluding involvement of the GlyR, but by demonstrating the requirement of a different mechanism (e.g. SATs).

Conclusions and future perspectives

The development of a new and successful strategy for measuring intracellular Ca^{2+} was a major technical achievement. Not only microglial research, but all in vitro research concerning cellular activation, proliferation or signaling, gains a new approach for studying cellular processes involving Ca^{2+} . This study has proven the value of plate reader technology for Ca^{2+} measurements, but this does not mean there is no room for improvement. With the right equipment, a protocol can be set up which applies Fura-2 AM and allows for absolute quantification of Ca^{2+} concentrations.

The most important finding of the newly developed technique was that glycine and L-serine enhance microglial Ca^{2+} signals. Although it does not exclude all extracellular ligand-receptor interactions, the effect was proven to occur independently of the GlyR. On the other hand, applying a competitive transporter substrate for neutral amino acid transporters (AIB) did inhibit the effects of glycine and L-serine on Ca^{2+} signals. The fact that immunostainings confirmed an AIB-sensitive glycine uptake, advocates an important role for neutral amino acid transporters in enhancing Ca^{2+} signals. Transporter substrate selectivity for AIB, glycine and L-serine is exhibited by Na^+ -dependent SATs. The specificity for enhancing Ca^{2+} fluxes also correlated with specificity present in a System A transporter (SAT 1). Experiments in Na^+ -free conditions could not provide a uniform answer concerning the role of Na^+ -coupled transport in enhancing Ca^{2+} signals. For a full characterization of glycine transport present in microglia, specific glycine uptake experiments can be performed using tritium labeled glycine. This could be done parallel to a molecular transporter characterization, using RT-PCR and western blot.

In the future, more information on the exact mechanism behind the effect of amino acids on Ca^{2+} signals could also be gained by analyzing the different Ca^{2+} fluxes of a Ca^{2+} signal. Through these experiments, information concerning which specific Ca^{2+} fluxes are regulated can be revealed. Properties of the channels responsible for those Ca^{2+} fluxes could in turn provide clues to how glycine transport enhances Ca^{2+} signals. This especially holds true if the experiments can be set up to allow simultaneous measuring of Ca^{2+} and V_m or Na^+ , to test the electrogenic influence of glycine transport via neutral amino acid transporters. On the other hand if the underlying mechanism is mediated by intracellular amino acid signaling, measuring cyclic-AMP or protein kinases under glycine conditions may be a more suitable approach. Both possibilities will have to be explored to reveal how exactly glycine uptake can lead to enhanced Ca^{2+} signals (Fig. 24). The fact that these results appeared to require the acute presence of glycine, indicates that the mechanism is not a slow metabolic effect of altered amino acid availability effecting protein synthesis or gene expression. Future measurements with multiple time points fitted between 15 minutes and 24 hours can reveal at what time the enhancement of Ca^{2+} signals by amino acids is at its maximum level.

Research also has to be focused towards determining if the modulation of Ca^{2+} signals is also responsible or indicative of the modulation of immune function. To this end the relevance of findings concerning the mechanism of Ca^{2+} signal modulation should be tested further on cellular activation parameters modulated by glycine. $\text{TNF-}\alpha$ production has been revealed as one of those parameters, as glycine and L-serine significantly enhance $\text{TNF-}\alpha$ production. This confirms that the immune function of microglial cells is enhanced by glycine and that glycine effects on microglial cells are opposite to glycine effects on peripheral immune cells.

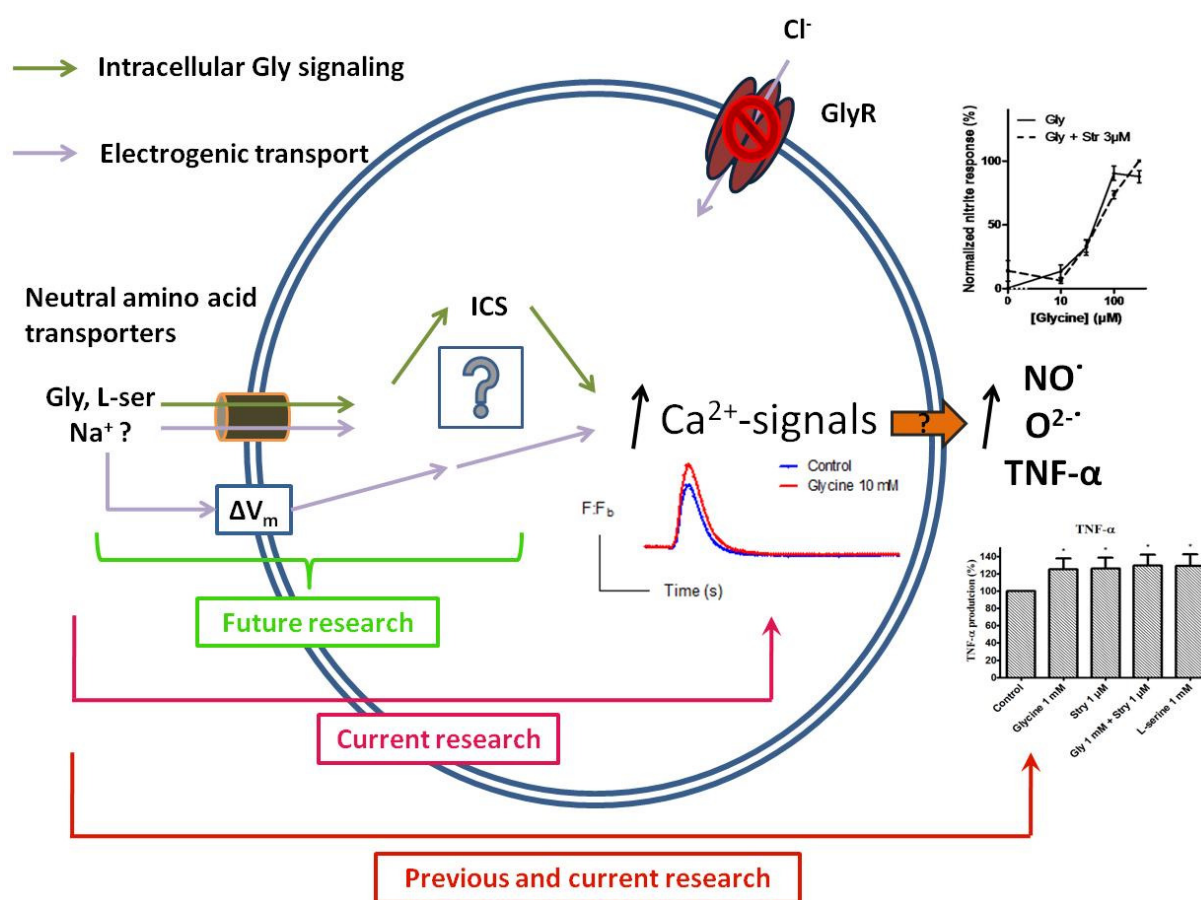


Figure 24: Schematic summary of the correlations between previous and current research and the aims for future research based on identifying the role of neutral amino acid transporters. Gly: glycine, GlyR: glycine receptor, L-ser: L-serine, V_m : membrane potential, ICS: intracellular signaling, $F:F_b$: Normalized fluorescence signal, NO^\bullet : Nitric Oxide, $\text{O}_2^{\bullet-}$: superoxide, $\text{TNF-}\alpha$: Tumor necrosis factor alpha, Str(y): Strychnine

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