MICROGLIAL GLUTAMATE TRANSPORTERS

Regulation of Expression and Possible Physiological Functions

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To my parents,

for endless support and encouragement

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ABSTRACT

Microglia are considered as the immunocompetent cells of the central nervous system (CNS). Being the first line of defence, they have prominent roles in monitoring the homeostasis and the extracellular milieu and can rapidly and specifically react to any disturbances such as brain trauma, ischemia, neurodegenerative diseases, or infections. Microglia are normally adapted to a resting state, but due to alterations in the homeostasis they become activated. During pathological conditions it has been shown that microglia are able to express Na^+-dependent high affinity glutamate transporters which are important for the uptake of the neurotransmitter glutamate. However, the mechanisms underlying the expression and the physiological role of it are not fully understood. In this thesis, it was found that the microglial glutamate transporter expression is connected to microglial activation and inflammatory events. The bacterial endotoxin lipopolysaccharide (LPS) was used to induce an environment mimicking neuroinflammation, a condition that occurs during almost any pathological condition in the CNS. It was found that LPS was able to increase the expression of the microglial glutamate transporter GLT-1 in a model system of essentially pure rat microglia. This effect was most likely mediated by the cytokine tumour necrosis factor-α (TNF-α), since the cytokine was able to mimic the effect of LPS by itself and the fact that antibodies against the TNF-α abolished the expression. Additionally, the LPS-induced increase in microglial GLT-1 expression could be inhibited by decreasing the release of TNF-α with the anti-inflammatory glucocorticoid corticosterone. The anaphylatoxin C5a, a component of the complement system, was also found to be able to induce microglial GLT-1 expression but in a different manner than LPS. The increased GLT-1 expression led to increased glutamate uptake from the extracellular space, which may be important to limit the excitotoxic effect of glutamate during pathological conditions. Furthermore, the increased glutamate uptake was directly coupled to an increased synthesis of the antioxidant glutathione. The glutamate partly fuelled the intracellular pool of glutamate in order to allow uptake of cystine, an amino acid that is one of the building blocks of the antioxidant glutathione, and was partly directly incorporated into glutathione. As a major antioxidant, glutathione was able to provide microglia with a self defence against reactive oxygen species. Furthermore, the increased glutathione levels provided microglia with better resistance to infections with herpes simplex virus due to the antiviral properties of the antioxidant. In response to herpes simplex virus infections, microglia are able to release TNF-α and up-regulate their GLT-1 expression in order to provide means for an increased glutathione synthesis and thus an increased viral resistance. In summary, the results show how microglial GLT-1 can be modulated and that increased resistance against oxidative stress and viral infections are two possible physiological functions of the increased microglial glutamate uptake.

Keywords: *anaphylatoxin, central nervous system, herpes simplex virus, glutamate transport, glutathione, GLT-1, lipopolysaccharide, microglia, neuroinflammation, oxidative stress, protection, tumour necrosis factor-α*

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Det centrala nervsystemets immunförsvar utgörs till stor del av en celltyp som kallas för mikroglia. Dessa celler bildar nervsystemets första försvarslinje mot invaderande patogener genom att ständigt övervaka balansen i nervsystemets miljö för att snabbt och specifikt kunna svara på störningar som uppstår vid exempelvis stroke, hjärnskador, neurodegenerativa sjukdomar och infektioner. Mikroglian förekommer normalt sett i ett vilande tillstånd, men kan snabbt övergå till att bli aktiverade celler. Det har visats att aktiverade mikroglia kan börja uttrycka Na^+-beroende glutamattransportörer vid patologiska situationer, vilket är viktigt för upptag av glutamat, ett av nervsystemets viktigaste signalämnen. I den friska hjärnan tags glutamat främst upp av andra celltyper som astrocyter för att förhindra att glutamat överaktiverar och dödar nervceller vilket annars kan ske vid olika sjukdomstillstånd i hjärnan då astrocyternas glutamatupptagsförmåga är minskad. Mekanismerna för regleringen av de mikrogliala glutamattransportörerna och dess fysiologiska betydelse har tidigare inte varit kända.

I den här avhandlingen presenteras nya forskningsrön som visar att uttrycket av de mikrogliala glutamattransportörerna är sammankopplat med inflammatoriska förlopp i hjärnan. För att skapa och efterlikna ett inflammatoriskt tillstånd, vilket förekommer vid nästan alla patologiska tillstånd i nervsystemet, användes lipopolysackarid (LPS), ett ämne som förekommer i cellväggen hos vissa typer av bakterier. I ett modellsystem där mikroglia från råtta odlades i frånvaro av andra hjärnceller visade det sig att LPS kunde öka uttrycket av glutamattransportören GLT-1 på mikroglia. Effekten förmedlades troligen av en inflammatorisk signalsubstans som kallas för tumörnekrosfaktor-α (TNF-α) eftersom TNF-α själv kunde öka mängden av GLT-1 på mikroglia. Dessutom visade det sig att antikroppar mot TNF-α, eller blockad av TNF-α frisättning med läkemedel eller antiinflammatoriska hormon som kortikosteron, förhindrade ökningen av GLT-1. Anafylatoxinet C5a, som är en del av immunförsvaret, visade sig också kunna öka mängden mikroglialt GLT-1 men genom en annorlunda mekanism än LPS. Anmärkningsvärt nog verkar vissa ämnen som nedreglerar glutamattransportörer på andra typer av hjärnceller uppreglera GLT-1 på mikroglia.

En ökad mängd GLT-1, och därmed ett ökat upptag av glutamat, för mikroglia kan ha stor betydelse vid olika sjukdomstillstånd i det centrala nervsystemet. Förutom att mikroglian delvis kan förhindra glutamat från att överaktivera och döda nervceller visar den här avhandlingen för första gången att det ökade mikrogliala glutamatupptaget även leder till ett bättre skydd mot oxidativ stress, ett tillstånd där syremolekyler ger upphov till skada, samt högre resistans mot virusinfektioner. Detta sker genom att det ökade glutamatupptaget leder till ökade produktionsförutsättningar och koncentrationer av antioxidanten glutathion. Forskningsrönen visar att mikroglia på ett unikt sätt kan svara på herpesvirusinfektioner och starta ett självförsvar genom att frisätta TNF-α, börja uttrycka GLT-1 och öka nivåerna av glutathion. Detta gör dem mer motståndskraftiga mot herpesvirus än vad astrocyter och nervceller är.

Sammanfattningsvis så visar avhandlingen hur de mikrogliala glutamattransportörerna kan regleras både positivt och negativt. Dessutom visas oxidativt skydd och motståndskraft mot virus som två möjliga fysiologiska betydelser av mikroglialt GLT-1. En ökad förståelse för regleringen av de mikrogliala glutamattransportörerna skulle på sikt kunna leda till en bättre förståelse för mekanismer bakom sjukdomsförlopp där neuroinflammation är en betydande komponent, som exempelvis stroke och hjärnskador, samt bättre behandling vid patologiska tillstånd som virusinfektioner i det centrala nervsystemet.

PAPERS INCLUDED IN THE THESIS

This thesis is based on the following original papers, which will be referred to by their Roman numerals in the text:

Paper I

Lipopolysaccharide increases microglial GLT-1 expression and glutamate uptake capacity *in vitro* by a mechanism dependent on TNF-α.

Mikael Persson, Mona Brantefjord, Elisabeth Hansson, and Lars Rönnbäck

GLIA (2005) 2:111-120

Paper II

Corticosterone inhibits expression of the microglial glutamate transporter GLT-1 *in vitro*.

Jenny Jacobsson[*], Mikael Persson[*], Elisabeth Hansson, and Lars Rönnbäck

Neuroscience (2006) 139:475-483

Paper III

Microglial glutamate uptake is coupled to glutathione synthesis and glutamate release.

Mikael Persson, Mats Sandberg, Elisabeth Hansson, and Lars Rönnbäck

European Journal of Neuroscience (2006) 24:1063-1070

Paper IV

The complement-derived anaphylatoxin C5a increases microglial GLT-1 expression and glutamate uptake in a TNF-α-independent manner

Mikael Persson, Marcela Pekna, Elisabeth Hansson, and Lars Rönnbäck

Manuscript (2007)

Paper V

Microglial GLT-1 is up-regulated in response to herpes simplex virus infection to provide an antiviral defence via glutathione

Mikael Persson, Mona Brantefjord, Jan-Åke Liljeqvist, Tomas Bergström, Elisabeth Hansson, and Lars Rönnbäck

Manuscript (2007)

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ABBREVIATIONS

ALS: amyotrophic lateral sclerosis

AMPA: α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid

ATP: adenosine-tri-phosphate

BBB: blood brain barrier

BSA: bovine serum albumin

CNS: central nervous system

DNA: deoxyribonucleic acid

EAAT: excitatory amino acid transporter

EAAC1: excitatory amino acid carrier 1

EDTA: ethylene diamine tetraacetic acid

ELISA: enzyme linked immunoabsorbent assay

FITC: fluorescein isothiocyanate

GFAP: glial fibrillary acidic protein

GLAST: glutamate and aspartate transporter

GLT-1: glutamate transporter 1

GPx: glutathione peroxidase

GR: glucocorticoid receptor

GRed: glutathione reductase

GS: glutamine synthetase

GSH: glutathione

GSSG: glutathione disulfide

HIV: human immunodeficiency virus

HPLC: high pressure liquid chromatography

HRP: horse radish peroxidase

HSV: herpes simplex virus

IFNγ: interferone γ

IP_3: inositol-triphosphate

LPS: lipopolysaccharide

MAC: membrane attacking complex

MEA: mercaptoethanol ethylene diamine tetraacetic acid azide

MEM: minimum essential medium

MHC: major histocompatability complex

MR: mineralocorticoid receptor

MRP: multi drug resistance protein

MTT: 3-4-5-dimethylthiazole-2-yl-2,5-diphenyl-tetrazolium bromide

NFκB: nuclear factor κB

NGF: nerve growth factor

NMDA: N-methyl-D-aspartate

OPA: ortho-phtaldialdehyde

PCR: polymerase chain reaction

ROS: reactive oxygen species

SIV: simian immunodeficiency virus

SLC1: solute carrier 1

TACE: Tumour necrosis factor-α converting enzyme

TLR: Toll-like receptor

TNF-α: tumour necrosis factor-α

TNFR, Tumour necrosis factor-α receptor

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INTRODUCTION

The central nervous system

The central nervous system (CNS) is composed of the brain and the spinal cord, which are both divided into grey and white matter (Kandel et al, 2005). The grey matter refers to areas with cell bodies, while white matter refers to areas dominated by myelinated axons. The CNS is the main information processing organ in the body and it is built up from numerous cell types organized into highly complex networks. Neurons are classically considered as the main information processing units while glial cells, being more numerous than the neurons, have been considered as supportive cells. However, the glial cells, which consist of astrocytes, microglia, oligodendrocytes and ependymal cells (Kettenmann and Ransom, 1995), have several specific functions of their own. Each cell type can express a specific set of receptors and channels which gives the cell type its unique functions. The cells are dependent on each other to function properly and the anatomical and physiological properties of the glial cells allow them to interact in complex ways.

Microglia

Microglia are considered as the immunocompetent cells of the CNS, forming the interface between the brain and the immune system. First discovered by Ramón y Cajal in 1913 as part of the "third element" besides neurons and neuroglia, it was not until 1932 that microglia was noted as a cell type of its own by del Rio-Hortega. Although discovered nearly a century ago, the origin of microglia in the CNS is not yet a clear issue. There are evidence supporting three views of origin (reviewed by Kaur et al, 2001). Microglia may be of mesodermal origin, that is, they are cells that invade the CNS during embryonic development through distinct entry points called "fountains of microglia" before settling down as normal microglia. Another view is that the microglia are true CNS cells, being of neuroectoderm lineage along with neurons and astrocytes, probably deriving from a common stem cell. The third school of thought is that circulating monocytes enter the developing brain and later transform into resident microglia.

In the mature brain, microglia make up 5-20% of the glial population and they are more numerous in grey matter than white matter (Lawson et al, 1990). They are present throughout the CNS and form a network of cells with the capacity of immune surveillance and control, and can be described as sensors for pathological events (Kreutzberg, 1996). They survey the CNS homeostasis by expressing ion channels, neurotransmitter and pathogen receptors which

make them able to sense neuronal activity, pH shifts and other physiological changes (reviewed by Farber and Kettenmann, 2005). Microglia are extraordinarily sensitive to changes in their microenvironment and can rapidly be prepared to deal with infections, physical injuries, and physiological changes by becoming activated (Barron, 1995). Normally, microglia are adapted to a resting state, called ramified microglia. However, "resting" is a somewhat misleading terminology since it has been shown that the fine microglial branches are constantly moving and sensing the environment, making it an active process (Raivich, 2005). Upon activation, the microglia transform to an amoeboid morphology and up-regulate their phenotype by expressing cell surface markers, cytokines, chemokines, reactive oxygen species (ROS) and membrane proteins such as receptors and channels (Gebicke-Haerter et al, 1996; Streit et al, 1999). They are also able to proliferate and migrate to the injured area and to remove invading or dying cells by phagocytosis, making them the resident macrophages of the brain (Streit et al, 1999). Several factors for microglial activation have been identified including bacterial cell wall components such as lipopolysaccharide (LPS), cytokines, plaque related molecules such as the β-amyloid peptide and prion proteins, serum factors and several other stimuli (for review see, Nakamura, 2002).

Due to the microglial characteristics and the fact that the blood brain barrier (BBB) shields the CNS from access by blood-born immune cells and various pathogens (Abbott et al, 2006), making it an immunoprivileged organ, microglia are thought of as the immunocompetent cells of the CNS (Aloisi, 2001; Gehrmann et al, 1995). As the first line defence cells, microglia are able to participate in the regulation of both non specific inflammation and adaptive immune responses (Aloisi, 2001). Activated microglia can express major histocompatability complex (MHC) I and II (Gehrmann et al, 1995), making them antigen presenting cells and able to control T cell responses (for review, see Aloisi, 2001). Furthermore, they express surface receptors for immune system components such as immunoglobulins, complement, and apoptotic cell markers (Farber and Kettenmann, 2005; Raivich, 2005; Streit et al, 1999).

Microglia are not only involved in monitoring the CNS environment and responding to disturbances, but also in promoting both neurodegeneration and neuroregeneration. Deleterious effects and a pathogenic role for activated microglia have been reported to be involved in many disease states including Alzheimer's disease (Combs et al, 1999; Combs et al, 2001; Kalaria, 1999; Noda et al, 1999), human immunodeficiency virus (HIV) infection (Giulian et al, 1996; Vallat-Decouvelaere et al, 2003), prion diseases (Eitzen et al, 1998; Giese et al, 1998; Peyrin et al, 1999) and multiple sclerosis (Juedes and Ruddle, 2001; Smith,

1999) just to name a few (for review, see Aldskogius, 2001b). However, microglia are also involved in tissue repair. Proper activation of microglia results in removal of dead or dying cells which separates the disease process from the adjacent functional tissue to promote neuroregeneration (Aldskogius, 2001a; Graeber et al, 1993; Lazarov-Spiegler et al, 1996; Prewitt et al, 1997). Additionally, microglia are capable of producing and releasing neurotrophins and growth factors such as brain derived neurotrophic factor and nerve growth factor (NGF; Nakajima et al, 2001a). In fact, microglial derived neurotrophins and phagocytosis seem to be involved in synaptic damage and recovery (Bruce-Keller, 1999). Ironically, microglia have also been shown to participate in β-amyloid degradation during Alzheimer's disease (Mentlein et al, 1998) and to be a source of growth factors supporting remyelination during demyelinating disorders (Hinks and Franklin, 1999). This highlights that microglial activation can potentially be both beneficial and harmful. The fundamental, but yet unknown question, is whether the microglia causing the disease or simply there as a consequence of it.

Glutamate and glutamate receptors

The amino acid L-glutamate is considered as the main excitatory neurotransmitter in the mammalian nervous system (Fonnum, 1984). It is involved in many aspects of normal brain function, including cognition and memory (Nakanishi et al, 1998). Glutamate exists in all cells, but is not used as a signal transmitter by most cells. It is mostly neurons that use glutamate as a neurotransmitter, but also astrocytes to some extent (Nedergaard, 1994; Parpura et al, 1994). Glutamate, released mainly from the presynaptic terminal in neurons where it is stored in synaptic vesicles, can activate different subtypes of glutamate receptors located on postsynaptic membranes and cells in the vicinity (Danbolt, 2001). Several glutamate receptors have been cloned and characterized (Steinhauser and Gallo, 1996). Based on their pharmacology and electrophysiological properties, they have been divided into ionotropic and metabotropic receptors. The ionotropic receptors are further subdivided into α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainate, and N-methyl-D-aspartate (NMDA) receptor subtypes (Dingledine et al, 1999). They are composed of several subunits and when activated, they are permeable to Na^+ and K^+ except for the NMDA receptor which is additionally permeable to Ca^{2+} (Hansson et al, 2000). The metabotropic receptors contain seven transmembrane domains, are G-protein coupled, and can be further divided into three subtypes based on their sequence homology, agonist sensitivity, and signal transduction mechanisms. Group I receptors are coupled to the inositol-triphosphate

(IP_3)/Ca^{2+} system, while group II and III receptors inhibit adenylate cyclase (Hansson et al, 2000; Nakanishi et al, 1998). Glutamate receptors have been identified on neurons (Nakanishi et al, 1998), astrocytes (Hansson et al, 2000), microglia (Noda et al, 2000), and other cell types.

Taken together, it is clear that glutamate can transduce signals to cells in complex and multitude ways depending on the expression pattern and localisation of receptors on the postsynaptic cell. However, although glutamate is essential, it can also potentially be toxic. The brain contains large amounts of glutamate, about 5-15 mmol/kg wet weight, but only a small fraction is present extracellularly (Schousboe, 1981). There is a several thousand fold concentration gradient of glutamate across the plasma membrane of cells, with glutamate concentrations of only 2-4 μM in the extracellular fluid and approximately 10 μM in the cerebrospinal fluid (Benveniste et al, 1984; Danbolt, 2001), while there is 1-10 mM cytosolic glutamate (Erecinska and Silver, 1990). This is in order to avoid over-activation of glutamate receptors which otherwise can lead to cell death, a process termed excitotoxicity by Olney and co-workers (1969). Excitotoxicity is considered a major mechanism in many human disease states such as cerebral ischemia, nervous system trauma, epilepsy, and neurodegenerative disorders (Dodd, 2002; O'Shea, 2002), though the mechanisms behind it remains to be fully explored. Although the molecular basis for the excitotoxicity is still uncertain, it is believed that it is due to a Ca^{2+} overload that ultimately triggers intracellular Ca^{2+}-dependent signalling cascades that eventually lead to neuronal cell death (Sattler and Tymianski, 2000). Since it is glutamate, or glutamate analogues, that make the glutamate receptors overactive, it is of vital importance to keep the extracellular glutamate concentrations low.

Glutamate transporters

The extracellular concentration of glutamate must be kept below the activating threshold to avoid over-activation of glutamate receptors which could potentially cause excitotoxicity. Studies by Frandsen and Schousboe (1990) show that, providing that glutamate uptake is blocked, as little as 1 μM glutamate is sufficient to kill 50% of neurons in culture.

Unlike most neurotransmitters, glutamate does not appear to have any degrading enzyme in the extracellular space. Consequently, the glutamate must be rapidly removed by glutamate transporters that can transport it across the plasma membrane and against the steep concentration gradient. In addition to preventing excitotoxicity, rapid clearance of glutamate will also ensure a high signal to noise ratio in the glutamate signalling (Hansson et al, 2000; Riedel, 1996; Rönnbäck and Hansson, 2004). In the normal brain, astrocytes are thought of as

the main glutamate scavengers (Schousboe, 1981). In fact, neuronal vulnerability to glutamate is hundred fold greater in astrocyte-poor cultures than in cultures abundant with astrocytes (Rosenberg et al, 1992). Glutamate taken up by astrocytes is converted to glutamine by glutamine synthetase (GS) which can be transported back to neurons since it does not have any known synaptical activity (reviewed by Sonnewald et al, 1997, and Hertz et al, 1999).

Not much is known about how the glutamate transporters are expressed and regulated in microglial cells, and, consequently, most data referred to will be on astroglial cells and the glial specific transporters. A schematic figure of how glutamate is utilized and transported in the CNS can be seen in Figure 1.

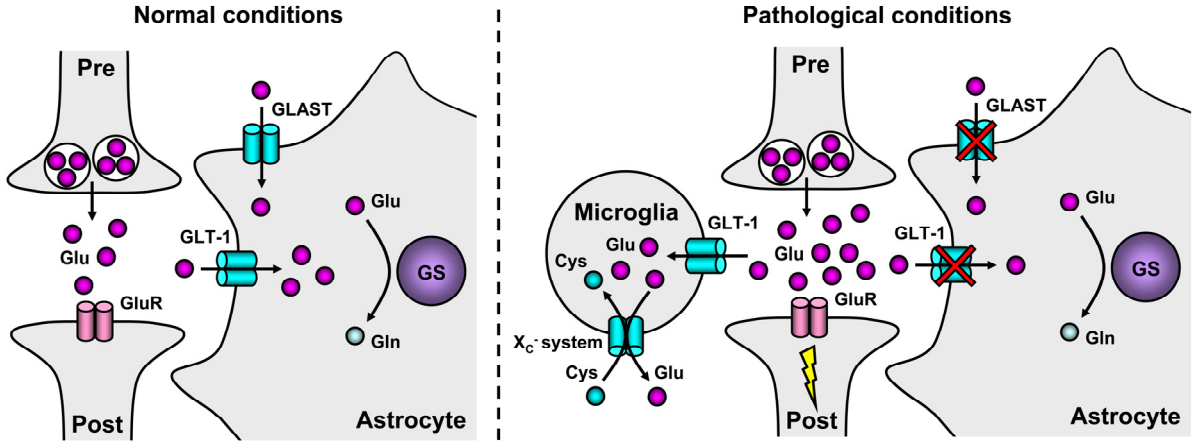

Figure 1. *Schematic drawing of the synaptic cleft and surrounding cells. Glutamate (Glu) is stored in synaptic vesicles in presynaptic nerve terminals (Pre) and can be released into the synaptic cleft. Glutamate can activate glutamate receptors (GluRs) on postsynaptic cells such as postsynaptic neurons (Post) and transmit signals. During physiological conditions (left), the glutamate is sequestered from the synaptic cleft mainly by the astroglial glutamate transporters GLT-1 and GLAST to prevent excitotoxicity due to prolonged activation of glutamate receptors. Astrocytes metabolize glutamate into glutamine (Gln) using glutamine synthetase (GS). Glutamine can then be safely transported back to neurons. During pathological conditions (right), the astroglial glutamate transporters are down-regulated and microglial transporters are induced. Consequently, there will be an accumulation of glutamate in the extracellular space which may lead to excitotoxicity due to excessive activation of glutamate receptors. Microglia may transport glutamate from the extracellular space and exchange it for cystine (Cys) using the X_C^- system.*

High affinity Na^+-dependent glutamate transport

To date, not counting various splice variants, five high affinity Na^+-dependent glutamate transporters in the CNS, called excitatory amino acid transporters (EAATs), have been cloned: EAAT1 (Storck et al, 1992), EAAT2 (Pines et al, 1992), EAAT3 (Kanai and Hediger,

1992), EAAT4 (Fairman et al, 1995), and EAAT5 (Arriza et al, 1997). EAAT1 and EAAT2, the human homologues to the murine glutamate and aspartate transporter (GLAST) and glutamate transporter 1 (GLT-1), respectively, are predominantly expressed by astrocytes in the normal brain, while EAAT3, also known as excitatory amino acid carrier 1 (EAAC1), is expressed by neurons (for review, see Gegelashvili and Schousboe, 1998). EAAT4 is expressed by Purkinje neurons in the cerebellum (Yamada et al, 1996) and EAAT5 is expressed by neurons in the retina (Arriza et al, 1997). See Table 1 for an overview of the transporters. Furthermore, astroglial GLAST and GLT-1 have different expression patterns. GLAST is the major transporter for glutamate uptake during development while expression of GLT-1 increases with the maturation of the CNS (Guillet et al, 2002). This is also reflected in the *in vitro* situation where the presence of neurons is needed for GLT-1 expression (Swanson et al, 1997).

Although messenger RNA for both GLAST and GLT-1 has been found in microglia (Kondo et al, 1995), they do not express any EAATs under physiological conditions *in vivo*. However, during pathological situations, in response to harmful conditions or stress, microglia have been shown to be able to express EAATs both *in vivo* and *in vitro*. It has been shown that activated microglia surrounding motoneurons express GLT-1 in a facial nerve axotomy paradigm (Lopez-Redondo et al, 2000). Furthermore, microglia can express glutamate transporters after controlled cortical impact (van Landeghem et al, 2001) and in connection with infectious diseases and neurodegenerative diseases such as prion diseases (Chretien et al, 2004; Gras et al, 2006). However, there are indications that the expression of microglial EAATs is species specific. Murine microglia have only been shown to express GLT-1 (Nakajima et al, 2001b). Microglia from macaque monkeys infected with simian immunodeficiency virus (SIV) express GLT-1 (Chretien et al, 2002) while microglia from patients infected with HIV express GLAST (Vallat-Decouvelaere et al, 2003).

The glutamate transporters have high affinity for glutamate and are dependent on Na^+ and energy in form of adenosine-tri-phosphate (ATP) to function. The transport system, named X_{AG}^-, couples the co-transport of three Na^+ and one H^+ to the counter transport of one K^+ with no dependency on Cl^- when transporting one glutamate (Levy et al, 1998; Zerangue and Kavanaugh, 1996). Since the electrogenic transport occurs against a steep concentration gradient, it is an energy consuming process, requiring more than one ATP for every glutamate. This has been estimated to account for a large fraction of the brain's total ATP turnover (Sibson et al, 1998). In the case of ATP depletion, which occurs during severe

ischemia, there will be less glutamate uptake and transport reversal may even occur, giving rise to excitotoxicity (Longuemare and Swanson, 1995; Seki et al, 1999; Zeevalk et al, 1998).

There is 50-60% structural homology and 90% sequence homology between the EAATs and they belong to the gene family solute carrier 1 (SLC1; Gegelashvili and Schousboe, 1997; Kanai and Hediger, 2004). A general membrane topology has been predicted. The EAATs have eight transmembrane domains and a re-entrant loop between domain seven and eight that provides transport specificity (Kanai and Hediger, 2004).

Pharmacological, knockout, and antisense studies have shown some of the physiological functions of the different transporters. Homozygous mice lacking GLT-1 show spontaneous lethal seizures and an increased susceptibility to acute cortical injury (Tanaka et al, 1997b). Similar results were presented by Rao and co-workers (2001). Additionally, it has been shown that lack of GLT-1 augments brain edema after transient ischemia (Namura et al, 2002). Rothstein and co-workers (1996), using chronic antisense against the transporters, showed that loss of GLAST and GLT-1 produced elevated extracellular glutamate levels, neurodegeneration due to excitotoxicity, and progressive paralysis, while loss of EAAC1 did not elevate the glutamate levels but produced mild neurotoxicity that led to epilepsy. GLAST knockout mice show increased susceptibility to injury in the cerebellum and consequently fail when challenged with complex motor tasks, consistent with cerebellar abnormality (Watase et al, 1998).

Alterations of EAAT expression, or EAAT function, have been implied in several disease states, including Alzheimer's disease, cerebral ischemia, epilepsy, traumatic brain injury, and amyotrophic lateral sclerosis (ALS; for reviews, see Danbolt, 2001, and O'Shea, 2002). The regulation and expression patterns of the EAATs are highly complex and the focus of many studies. Numerous substances have been found that down-regulate the transporters, but only a few that up-regulate them. For instance, dibuturylic cyclic adenosine monophosphate (Schlag et al, 1998), epidermal growth factor (Suzuki et al, 2001), pituitary adenylate cyclase activating peptide (Figiel and Engele, 2000), and β-lactams (Rothstein et al, 2005) have been used to increase astroglial GLT-1 expression. The relative lack of substances able to increase the EAAT expression has made it exceedingly difficult to counteract the neurodegenerative diseases with excitotoxic characteristics. Instead, research focus has been on factors that down-regulate astroglial EAAT expression or glutamate uptake like acidosis (Swanson et al, 1995), hypoxia (Swanson, 1992), endothelin-1 (Leonova et al, 2001), cytokines (Fine et al, 1996; Liao and Chen, 2001), and oxidative stress (Pogun et al, 1994; Trotti et al, 1996; Volterra et al, 1994). Interestingly, in conditions where the astroglial

glutamate transporters are down-regulated, such as after controlled cortical impact, their microglial counterparts are being induced or up-regulated.

Protein name	Human gene name	Tissue distribution and cellular expression
EAAT1 GLAST	SLC1A3	Brain (astrocytes and reactive microglia), heart, skeletal muscle, placenta
EAAT2 GLT-1	SLC1A2	Brain (astrocytes and reactive microglia), liver
EAAT3 EAAC1	SLC1A1	Brain (neurons), intestine, kidney, liver, heart
EAAT4	SLC1A6	Cerebellum (Purkinje cells)
EAAT5	SLC1A7	Retina

Table 1. *Overview of some of the members of the SLC1 family of Na^+-dependent high affinity glutamate transporters. Adapted from Kanai and Hediger (2004).*

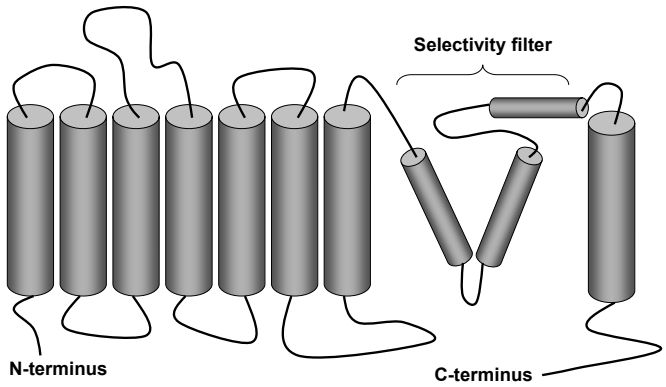

Figure 2. *Schematic drawing of the proposed structure of a Na^+-dependent high affinity glutamate transporter. The transporters are predicted to have eight α-helical transmembrane domains that span the plasma membrane of the cells. A re-entrant loop is thought to provide substrate specificity and forms the translocation pore through which glutamate can be transported. Both the N-terminus and the C-terminus are predicted to reside intracellularly. The figure is adapted from Kanai and Hediger (2004).*

Na^+-independent glutamate transport

Na^+-independent glutamate transport systems are typically Cl^--dependent glutamate/cystine antiporters, which exchange internal glutamate for cystine, the oxidized form of cysteine, across the plasma membrane (Bannai, 1986). This transport system has been termed the X_C^- system. Such a system have been cloned from macrophages (Sato et al, 1999). The X_{AG}^- and the X_C^- system have similar affinity for glutamate, but the X_C^- system has lower transport velocity, suggesting that it may have a limited role in the physiological brain for glutamate transport. Instead, it has been proposed that the antiporters have a primary role in cystine uptake and in maintenance of glutathione levels in astrocytes (Cho and Bannai, 1990) and in microglia (Rimaniol et al, 2001). Using internal glutamate as a driving force, it is no surprise

that the cystine uptake is inhibited by extracellular glutamate (Murphy et al, 1989). If the extracellular glutamate concentrations are sufficiently high, the X_C^- system may even release cystine which consequently leads to cell death due to oxidative stress (Cho and Bannai, 1990; Murphy et al, 1989; Murphy et al, 1990) since cystine is used for synthesis of the antioxidant glutathione (Dringen and Hirrlinger, 2003).

Neuroinflammation

The concept of inflammation in the CNS is termed neuroinflammation and is normally associated with glial, and especially microglial, activation. This can occur with local microglial activation and without the classical infiltration of T cells seen during normal inflammatory brain disease (Bradl and Hohlfeld, 2003). In fact, neuroinflammation is often defined as the presence of activated microglia, reactive astrocytes, and inflammatory mediators (Minghetti, 2005). Inflammation is a self-defensive reaction aimed to eliminate or neutralize injurious stimuli, and restoring tissue integrity (Minghetti, 2005).

Today, it is well known that the immune system and the CNS are interconnected, communicate bidirectionally, and interact in both health and disease (for reviews, see (Engblom et al, 2002, Engelhardt and Ransohoff, 2005, and Tracey, 2002). However this has not always been the case. The brain was once regarded as an organ devoid of an immune system, being largely separated from the rest of the organism by the BBB and lacking immune reactions and functions (for review, see Carson et al, 2006). The main reasons for this view is that the intact CNS practically lacks professional antigen presenting cells such as dendritic cells, macrophages, or B cells, and there is a very limited trafficking of T cells, leading to highly suppressed immunological reactions. Furthermore, there is very little expression of immune recognition molecules such as MHCs, and foreign tissue grafts survive longer periods without rejection in the CNS. There is also a high expression of molecules, such as FasL expressed by neurons, that are directly apoptotic for lymphocytes (Flugel et al, 2000). However, as previously mentioned, these statements are only partly true. The CNS should be regarded as an immuno privileged organ and not as an immuno isolated organ. The virtual absence of immune reactions is only true for the normal CNS. During pathological situations, the BBB can be penetrated by activated T cells in a process that is dependent on adhesion molecules, chemokines, and cytokines (for review, see Engelhardt and Ransohoff, 2005). There is also anatomical locations where the BBB is more open, such as in the plexus chorideus, which allows for lymph drainage of the CNS (Engelhardt and Ransohoff, 2005; Rebenko-Moll et al, 2006). MHCs can be expressed by cells such as neurons (Darnell, 1998),

astrocytes (Dong and Benveniste, 2001), and microglia (Kreutzberg, 1996). Most importantly, there is an induction of immune reactions, such as release of cytokines and complement activation, in response to pathological events (Aloisi, 2001; Gebicke-Haerter et al, 1996; van Beek et al, 2003). It is also important to note that the CNS immune privilege refers only to adaptive immune responses since innate immune responses are readily initiated within the CNS (Carson et al, 2006).

Neuroinflammation has mainly been connected to microglia, but other CNS cells are involved as well. Activated microglia are able to secrete proinflammatory cytokines and chemokines in addition to more non specific inflammatory mediatiors such as ROS and nitric oxide (Streit et al, 1999), and are thus major contributors to neuroinflammation. In comparison to microglia, astrocytes have a delayed response but can become reactive, express MHC class II, and form glial scars to isolate a damaged area (for review, see Dong and Benveniste, 2001, Fawcett and Asher, 1999, and Pekny and Nilsson, 2005). Neurons have classically been considered as passive bystanders, only regulating immune reactions, but are now known to be able to express MHC class I and produce several cytokines (for review, see Piehl and Lidman, 2001).

Neuroinflammation occurs during almost any pathological event in the CNS such as brain trauma, ischemia, infections, and chronic neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Creutzfeldt-Jacob's disease (Bradl and Hohlfeld, 2003; Minghetti, 2005; Piehl and Lidman, 2001). In the laboratory environment, a neuroinflammatory state is often induced using LPS as a model substance both *in vitro* and *in vivo*. LPS is a cell wall component of Gramm-negative bacteria and a very potent inducer of inflammation (Nakamura, 2002). Regardless of which stimuli it is that initiates it, neuroinflammation can be both beneficial and detrimental (for review, see Minghetti, 2005). Like the classical peripheral inflammation, neuroinflammation acts as a two-edged sword and must be tightly regulated. Both deficient and excessive responses will result in pathological conditions. A delicate balance must be reached and this is likely to be dependent on a crosstalk between different cells, such as microglia and neurons, that modulates the inflammatory reactions (Polazzi and Contestabile, 2002). Neuroinflammation is clearly a very complex situation which is regulated by cellular crosstalk and pro- and anti-inflammatory signals.

Tumour necrosis factor-α

Tumour necrosis factor-α (TNF-α) is a member of the cytokine family, molecules that are thought of as important messengers in the cross-talk between different cells. Tumour necrosis factors are primarily produced as transmembrane proteins arranged in stable homotrimers (Tang et al, 1996). They are cleaved by the metalloproteinase TNF-α converting enzyme (TACE) to yield the soluble form of TNF-α (Black et al, 1997; Moss et al, 1997). TNF-α is considered as a pro-inflammatory cytokine, meaning that it is usually produced and released during inflammatory events (Vitkovic et al, 2000). TNF-α production in the CNS has been attributed to neurons, astrocytes, and microglia (Cheng et al, 1994; Lee et al, 1993). To exert their biological functions, members of the TNF family have to interact with their cognate membrane receptors, comprising of the TNF receptor (TNFR) super family (Locksley et al, 2001). There are two main receptors, TNFR1 and TNFR2, that bind both membrane bound and soluble TNF-α (Wajant et al, 2003). TNFR1 is expressed constitutively by most tissue, while TNFR2 is highly regulated and expressed mostly by cells connected to the immune system. TNFRs have been detected in microglia both *in vitro* (Dopp et al, 1997) and *in vivo* (Sippy et al, 1995).

TNF-α can also be expressed constitutively in small amounts in the normal brain, giving it a possible role as a neuromodulator (Vitkovic et al, 2000). As an example, TNF-α has been proposed to affect sleep in the normal adult brain (Krueger et al, 1998). Although TNF-α is mostly known for its capability to destroy tumour cells by necrosis, thereby its name, it is not clear whether TNF-α is harmful or beneficial when expressed in the brain. It has been found that TNF-α is toxic during ischemia (Barone et al, 1997) but also that it is involved in ischemic preconditioning (Marchetti et al, 2004; Romera et al, 2004) which protects against neuronal death. These results highlight the complexity of action by TNF-α.

The complement system

The complement system provides the innate immune system with a mechanism to protect against pathogenic organisms (Frank and Fries, 1991). It was first discovered more than a century ago in 1895 by Jules Bordet as a component in plasma that was complementing antibodies in the removal of pathogens (Laurell, 1990). Today, it is known that the functions of the complement system include the recognition and killing of foreign cells, while preserving normal endogenous cells, by controlling inflammatory mediators and lytic complexes (for review, see van Beek et al, 2003). Although most prominent in plasma, the

complement proteins also exist in the CNS, being synthesized by astrocytes, microglia, and neurons (Barnum, 1995; Eikelenboom et al, 1991). Complement have also been implicated to be, whether beneficial or harmful, part of many neurological pathologies including Alzheimer's disease, Parkinson's disease, Huntington's disease, prion diseases, and other neurodegenerative diseases (for review, see Bonifati and Kishore, 2007).

The complement system can be activated in three different ways: either by the classical, alternate, or the lectin pathway (for review, see Bonifati and Kishore, 2007). All three pathways share the common activating molecule C3, but are initiated in different ways (see Figure 3). The classical pathway is initiated by binding of C1q to IgG- or IgM-containing immune complexes, the C-reactive protein, as well as some virus membranes and LPS. The alternate pathway is antibody independent and activated by low level spontaneous breakdown of C3 and activated plasma factor B, while the lectin pathway is initiated by the binding of microbial saccharides to mannose binding lectins. The pathways lead, through different activation cascades, to activation of C3 convertase that cleaves C3 to generate C3a and C3b. At 1 mg/ml, C3 is the most abundant complement protein in human plasma and increase rapidly upon inflammation (Kushner et al, 1972). The C3b will bind to structures near its activation site, promoting opsonisation and clearance of pathogens. C3b can also interact with C3 convertase itself and form C5 convertase. This convertase will promote the cleavage of the complement protein C5 which results in the formation of C5a and C5b. C5b will be loosely attached to C5 convertase and promote downstream activation of the complement cascade. The cascade will ultimately end in the terminal pathway, generating the membrane attacking complex that can lyse cells directly by forming a pore through the target membranes.

The C3a and C5a are very interesting molecules called anaphylatoxins. They are small polypetides and exert their effect at the pico molar to nano molar range on their specific receptors C3aR and C5aR, respectively, expressed on astrocytes, neurons, and microglia (Ember et al, 1998; Gasque et al, 2000). The anaphylatoxins have very diverse effects in the CNS. C3a have been shown to promote chemotaxis (Ember et al, 1998), induce release of NGF from human microglia (Heese et al, 1998), suppress the production of proinflammatory cytokines elicited by LPS (Takabayashi et al, 1996), protect neurons against NMDA toxicity (van Beek et al, 2001), as well as being important for ischemia-induced neurogenesis (Rahpeymai et al, 2006). C5a, on the other hand, has been shown to be of a more proinflammatory character (Guo and Ward, 2005). C5a is an important chemoattractant and stimulates cells to release cytokines, chemokines, complement components, and also up-regulate adhesion molecules (Ember et al, 1998). Like C3a, C5a has been shown to protect

against excitotoxicity both *in vitro* and *in vivo* (Osaka et al, 1999) and mice genetically deficit in C5, and thus C5a, are more susceptible to excitotoxic lesions in the hippocampus (Pasinetti et al, 1996). Indeed, the anaphylatoxin receptors have been shown to be up-regulated in response to cerebral ischemia (Barnum et al, 2002; van Beek et al, 2000) and brain trauma (Stahel et al, 1997b; Stahel et al, 2000), suggesting that they may play an important role during these pathological conditions.

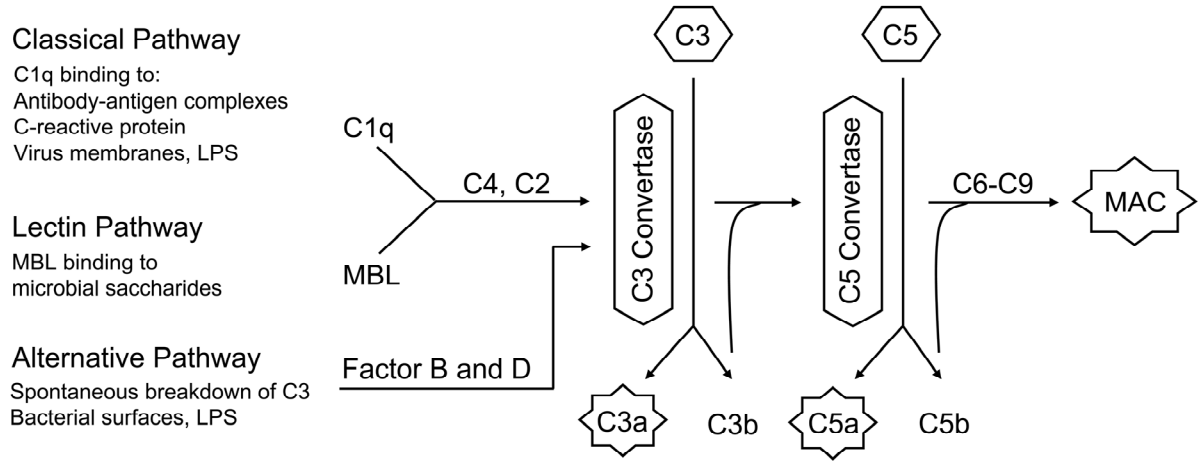

Figure 3. *The complement activation pathways. Complement can be activated by either the classical, lectin, or the alternative pathways. The classical pathway is initiated by binding of C1q to antibody-antigen complexes, the C-reactive protein, virus membranes, or LPS. The lectin pathways is initiated by the binding of mannose binding lectins to microbial saccharides. The classical and the lectin pathway converge at the activation of C4 and C2 which in turn activates C3 convertase to cleave C3 to the anaphylatoxin C3a and the C3b peptide. The C3 convertase can also be activated by the alternative pathway by spontaneous breakdown of C3 together with factor B and D. The C3b peptide can react with C3 convertase to form the C5 convertase that cleaves C5 to the anaphylatoxin C5a and the C5b peptide. C5b can initiate the terminal pathway through C6-C9 to form the membrane attacking complex (MAC) that will promote cell lysis.*

Glucocorticoids

Glucocorticoids are a family of endogenous steroid hormones with great anti-inflammatory effects. They exert their function by interacting with glucocorticoid receptors (GRs) or mineralocorticoid receptors (MR) that reside in an inactive form in the cytoplasm of cells (McKay and Cidlowski, 1999). Glucocorticoids are small lipophilic molecules and can easily translocate across the cell membrane to activate GRs to induce both transcriptional activation and repression (Smoak and Cidlowski, 2004). Both GRs and MRs have been identified in microglia (Tanaka et al, 1997a). Part of the anti-inflammatory effects of glucocorticoids come from the ability of the activated GR to form protein-protein interactions with transcription

factors such as NFκB and AP-1 (Almawi and Melemedjian, 2002; McKay and Cidlowski, 1999). Such interactions have been shown to repress production of pro-inflammatory cytokines such as TNF-α (Crinelli et al, 2000), interferone gamma (IFNγ) and several interleukins (Almawi and Melemedjian, 2002; Kunicka et al, 1993). Indeed, glucocorticoids have been shown to be key players in the feedback loop that is elicited by pro-inflammatory cytokines during an injury in order to keep the immunological response within certain limits (Glezer and Rivest, 2004). Prolonged and exaggerated inflammation, or too high glucocorticoid levels, could otherwise be detrimental for the CNS. In fact, glucocorticoids, or GR agonists like dexamethasone, have been widely used as immunosuppressants.

Glutathione and oxidative stress

Glutathione (GSH) is a tripeptide (γ-glutamylcysteinylglycine) and is synthesized from cysteine, glutamate, and glycine. It is synthesized by the consecutive actions of two enzymes in an ATP consuming process (for review, see Dringen, 2000). First, the dipeptide γ-glutamylcysteine is formed from glutamate and cysteine by γ-glutamylcysteine synthetase. The dipeptide is then further synthesized to GSH with the addition of glycine in a reaction catalyzed by glutathione synthetase. The GSH synthesis is balanced by a feedback inhibition of γ-glutamylcysteine synthetase by the end product GSH (Richman and Meister, 1975).

Being the most abundant thiol in mammalian cells, with concentrations up to 12 mM (Cooper, 1997), GSH is a major antioxidant and protects cells against oxidative stress by detoxifying ROS. The imbalance between the production of ROS, or other free radicals, and the inability of cells to defend against them is termed oxidative stress (Gilgun-Sherki et al, 2002). Oxidative stress can thus occur when there is an increase in ROS or other free radicals (Simonian and Coyle, 1996). Compared to other organs, the brain appears to be especially endangered when it comes to generation and detoxification of ROS. The human brain comprise about 2% of the body weight but uses 20% of the total oxygen consumption (Clarke and Sokoloff, 1999). Free radicals are constantly generated by the mitochondria when it uses oxygen to supply the energy needs of the CNS. Several mechanisms are active in the formation of ROS, including some enzyme activities, i.e. activity of monoamino oxidase or tyrosine hydroxylase, and even metabolism of glutamate (for review, see Gilgun-Sherki et al, 2002). Oxidative stress can produce functional and detrimental alterations in lipids, proteins, and deoxyribonucleic acid (DNA) to name a few things, and have been implied in most

pathological states in the CNS, including neurodegenerative diseases and acute CNS injuries (Gilgun-Sherki et al, 2002; Simonian and Coyle, 1996).

Several cellular defence systems, including superoxide dismutase, catalase, and antioxidants such as GSH, exists in the brain (Simonian and Coyle, 1996). GSH can react directly in a non-enzymatic way with radicals, or it can act as an electron donor in the reduction of peroxides by glutathione peroxide (GPx; Chance et al, 1979). The product of the reaction is glutathione disulfide (GSSG), the oxidized form of GSH. GSSG can be recycled to GSH by the enzyme glutathione reductase (GRed; Dringen et al, 2000). Additionally, GSH is also antiviral (Garaci et al, 1992; Palamara et al, 1995; Palamara et al, 1996b), exerting its effect by a mechanism that it connected to the redox state of the cells (Ciriolo et al, 1997; Palamara et al, 1996a).

Microglia posses a prominent glutathione system with a glutathione content significantly higher than in neurons or astrocytes (Hirrlinger et al, 2000). Furthermore, microglia have been shown to express the highest immunoreactivity for GPx (Lindenau et al, 1998), and high levels of GRed in microglia have been reported (Gutterer et al, 1999).

Herpes simplex virus infections in the CNS

Herpes viridae is a large family of viruses of which herpes simplex virus 1 and herpes simplex virus 2 (HSV-1 and HSV-2) are the most serious human pathogens (Whitley and Roizman, 2001). They are double stranded DNA viruses and the virus particles are composed of at least 84 different polypeptides and the virus genome, encapsulated by a membrane envelope called a capsid (Homa and Brown, 1997). The viral genome encodes for viral glycoproteins that are necessary for viral attachment and penetration as well as polypetides with many diverse functions such as viral host shut-off proteins which enables the virus to take over the invaded cell and allow viral replication (Matis and Kudelova, 2001; Mossman et al, 2001; Whitley and Roizman, 2001).

HSV infections are very common. It is estimated that up to 90% of the population in the USA are seropositive for either HSV-1 or HSV-2 (Morrison, 2002; Whitley and Roizman, 2001). The subtypes of HSV have different, although clinically somewhat overlapping, pathogenesis. HSV-1 leads normally to orolabial herpes vesicles and blistering, and HSV-2 often causes genital herpes vesicles and blistering (Whitley and Roizman, 2001). Recurrent HSV is very common. HSV may be dormant without causing any effects, but may be rapidly activated due to stress, fever, or tissue damage to name a few things (Whitley and Roizman, 2001). Although HSV infections are usually comparatively benign, HSV can enter the CNS

and have devastating effects. HSV can gain access and enter the CNS by lytic infection of epithelial or mucosal surfaces which allows the virus to enter axons of sensory neurons in which the virus can be axonally transported to the neuronal nuclei in either the dorsal root ganglia or the trigeminal ganglion and spread to neighbouring cells (for review, see Frampton, Jr. et al, 2005). The effect of the virus in the CNS is then determined by its neuroinvasiveness, neurotoxicity, and latency. HSV-1 typically causes encephalitis while meningitis are most commonly caused by HSV-2 (for review, see Schmutzhard, 2001, and Tyler, 2004). Herpes simplex encephalitis is relatively infrequent in the population occurring in one case for every 250.000 people in the USA (Whitley and Roizman, 2001) and 2-3 per 1 million people in Sweden (Sköldenberg et al, 1984). Herpes simplex encephalitis develops when HSV-1 infects brain tissue in a lytic/necrotic manner (Studahl et al, 2000). It has a very high mortality without treatment, and even with treatment, severe neurological complications such as seizures, paresis, and cognitive deficits are common (Tyler, 2004). Neonatal herpes simplex encephalitis can be caused by either HSV-1 or HSV-2, causing severe neurological disorders and mental retardation (Kimberlin, 2004). HSV-2 mediated meningitis, called Mollaret's meningitis when it is recurrent, often lead to transient neurological abnormalities such as seizures, cranial nerve paresis, fever, and pathological reflexes (Tyler, 2004). Viral mediated meningitis, including HSV-2 mediated meningitis, have an estimated incidence of 5-15 cases per 100.000 per year in the UK (Chadwick, 2005).

Microglia are interesting cells when it comes to viral infections. They have been shown to be able to recognize HSV by Toll-like receptors (Aravalli et al, 2005; Finberg et al, 2005), and subsequently initiate an immunological response by secreting cytokines (Lokensgard et al, 2001; Lokensgard et al, 2002). In fact, neuroinflammation with circulating cytokines are one of the hallmarks of HSV infections in the brain (Sköldenberg, 1996). It has been theorized that glial cells, with microglia as key players, can orchestrate a defence against HSV in the CNS by evoking an inflammatory and immunological response (Lokensgard et al, 2002).

AIMS

The general aim of the thesis was to study the regulation of expression and physiological functions of microglial glutamate transporters using an *in vitro* culture system of essentially pure microglia, and an *in vitro* system of primary neuron enriched cultures from the cerebral cortex.

The specific aims of the thesis were to:

I. Examine the expression of glutamate transporters on microglia in culture.

II. Identify possible factors responsible for the activation dependent expression of glutamate transporters.

III. Examine the anti-inflammatory effect of glucocorticoids on microglial glutamate transporters.

IV. Examine to role of the complement anaphylatoxins in microglial glutamate transporter expression.

V. Elucidate the physiological function of the microglial glutamate uptake.

METHODS

Microglial cultures (I, II, III, IV, V)

Microglial cultures consisting of >90% microglia (Figure 4A) were obtained from primary astroglial enriched cerebral cortex cultures (Figure 4B), as described in Paper I.

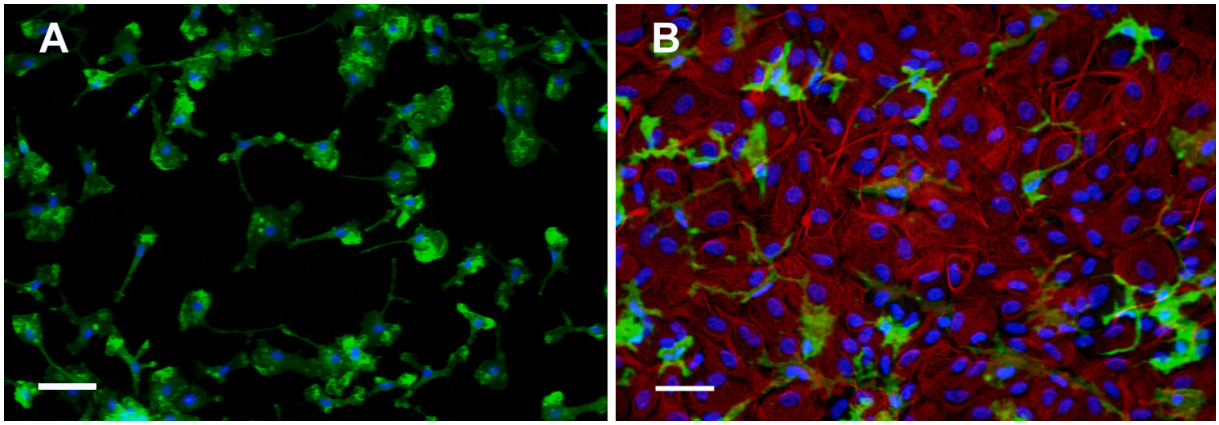

Figure 4. *Images of immunocytochemically stained cultures. Microglia are visualized with antibodies against OX42 (green) and astrocytes are visualized with antibodies directed against glial fibrillary acidic protein (GFAP; red). The nuclei are visualized with Hoechst's 33258 (blue). Essentially pure microglial cultures (A) can be obtained from primary astroglial enriched cerebral cortex cultures (B). In the latter cultures, microglia are evenly distributed on top of and inside a monolayer of confluent astrocytes. Scale bar = 50 µm.*

Comments on microglial cultures

Microglia in culture are most often of secondary culture character. They can be isolated from primary cultures enriched in astroglia by using one of two common methods, either by mild trypsinization or by shaking the astroglial enriched cultures. Both methods take advantage of the different attachment properties of microglia and astroglia. In the mild trypsinization method by Saura and co-workers (2003), confluent astroglial cultures are gently subjected to trypsinization in presence of ethylene diamine tetraacetic acid (EDTA) and Ca^{2+}, which results in detachment of an intact layer of cells comprising of mostly astrocytes. This leaves an undisturbed population of microglial cells since they adhere more firmly to the cell culture dish. Throughout this thesis, the classical method of shaking primary astroglial cultures, described simultaneously by Giulian and Baker (1986) and Frei and co-workers (1986), has been used. This method has the advantage that the astroglial cultures can be used continuously as long as the culture medium is replenished after cultivation, although the method gives a lower yield of microglia than the trypsinization method. When culturing in T75 culture flasks, the cultures will be usable every second day after they have reached

confluence, due to proliferation of microglia in these cultures. The primary astroglial enriched cultures, from which the microglia are obtained, have previously been extensively studied according to parameters such as brain regions and seeding time and their effect on amino acid transport, receptor activities, GFAP content, and protein synthesis (Hansson et al, 1984; Hansson et al, 1985b; Hansson, 1985; Hansson, 1986). Throughout this thesis, primary astroglial enriched cultures derived from 2 day old inbred Sprague-Dawley rats have been used to minimize biological variance.

Most importantly, it should be remembered that all microglia in culture are in a partially activated state and not reminiscent of microglia *in vivo* (for review, see Streit et al, 1999). This is probably due to a lack of cell to cell contacts and factors normally produced in the intact CNS. The microglia are separated from their normal tightly regulated cellular and electrochemical environment. This has effects on the expression of transporters and receptors. It should also be kept in mind that the cells are subject to stress and could therefore react in a way different from their normal response. Several strategies to induce normal behaviour of microglia have been attempted, such as use of astrocyte conditioned media or monolayers of fixed astrocytes (Tanaka and Maeda, 1996), but none of these have been completely successful. In Paper I, serum free MEM was used to keep the microglia in a functionally resting state (Tanaka et al, 1998). However, despite the partially activated state of the cells, microglia in culture is a powerful model system to study basal functions of microglia due to the simplicity and pure cellular composition of the culture. One important aspect of cell culturing is the use of antibiotics. The use of β-lactams such as penicillin have recently been shown to increase GLT-1 expression in astrocytes (Rothstein et al, 2005), although it is not known if they regulate microglial GLT-1 as well. Either way, all microglial cultures in the studies have been treated with the same amounts of antibiotics. To verify data obtained *in vitro*, one should use more *in vivo* like systems such as mixed primary cultures, brain slices, and ultimately, experimental animals.

Primary neuron enriched cultures (V)

Primary neuron enriched cultures (Figure 5) were obtained from the cerebral cortices of rats, as described in Paper V. This model system has been extensively characterized to show that it contains both glutamatergic and GABAergic neurons, differentiated astrocytes, and semi-ramified microglia, as well as functional receptors for several neurotransmitters and a prominent glutamate uptake through EAATs (Björklund et al, 2007).

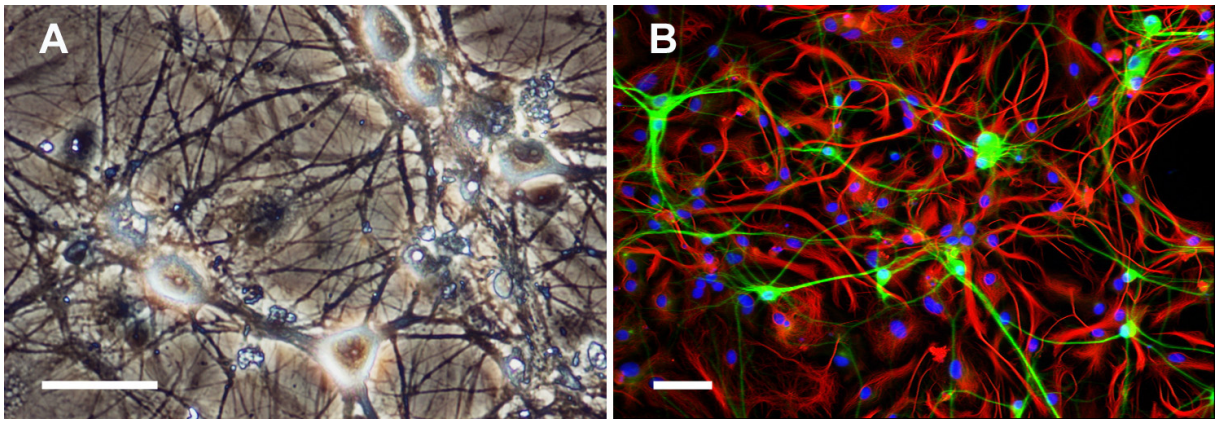

Figure 5. *Images of primary neuron enriched cultures after 20 days of cultivation. (A) Neurons growing on a confluent monolayer of astrocytes are visualized by light microscopy. (B) The cellular composition of the cultures is visualized by immunocytochemistry. Neurons are visualized with antibodies against the microtubuli associated protein 2 (green) and astrocytes are visualized with antibodies directed against GFAP (red). Scale bar = 50 µm*

Comments on primary neuron enriched cultures

These cultures represent a higher model system than the microglial cultures, containing a mixture of astrocytes, neurons, microglia, as well as other cell types such as oligodendroblasts and ependymal cells (Björklund et al, 2007). This allows for closer resemblance to the complexity of the *in vivo* situation, allowing cell to cell contacts, effects of soluble factors released by different cell populations, and more complex signalling in general. However, it is not certain that the same cell populations that exist *in vivo* are represented in the model system. It could be that some populations or phenotypes are selected/destroyed during cell plating. Of course, general cell culturing conditions, i.e. the use of different media or other factors, greatly influence the cell populations. As an example, the mitosis inhibitor cytosine-1-β-D-arabinofurosemide is used at day six in order to suppress the glial cell growth of dividing cells to prevent them from quenching the neurons. Likewise, insulin and a higher glucose concentration are needed to stimulate insulin receptors on the neurons. It has been proposed that serum, which is used in the majority of cell culture systems, can influence the phenotypes of neurons in the cultures since the serum contains high levels of glutamate which are sufficient to kill sensitive neurons, leaving a population of more glutamate tolerant neurons (Ye and Sontheimer, 1998). No serum was used in our culture system except for the first day. Clearly, the primary neuron enriched cultures are manipulated from the start by adding, substituting, or preventing factors that are present in the cells unique neurochemical milieu which is only seen *in vivo*. Therefore, the model system is not a true reflection of an

organism, and, just like with the microglial cultures, results obtained in the cultures should be verified in brain slices and ultimately in experimental animals. Still, the primary neuron enriched cultures remain a good model system for finding and investigating basal mechanisms that would be virtually impossible to analyse *in vivo* without prior basic knowledge of them.

Morphology (I, II, IV)

Morphological examination of cells was conducted before and after treatment with different stimuli using a Nikon phase contrast microscope coupled to a digital camera.

Comments on morphology

Morphological examination of microglia *in vitro* can be utilized to examine effects of different treatments. The cells are very sensitive to changes in their extracellular milieu and will react rapidly to any disturbances. The reactions are stimuli-directed, that is the cells will react in different ways to different stimuli, and they are most often reversed when the stimuli are removed. However, it should be kept in mind that the microglia are not in their natural environment and not in a ramified state. Therefore, it is uncertain if the cells will react in the same manner *in vivo* when they are influenced by the CNS specific environment.

Protein measurements (I, II, III, IV, V)

Protein concentrations were determined with the Bio-Rad DC protein assay according to the manufacturer's instructions. Bovine serum albumin (BSA), diluted in the same buffer as the samples, was used as a standard over the range of 0-2 mg/ml.

Comments on protein measurements

The concentration of protein was determined by a colorimetric assay similar to the Lowry assay (Lowry et al, 1951). The proteins react with copper in an alkaline solution and subsequently with Folin reagent to give a characteristic blue colour with a maximal absorbance at 750 nm. It is important to dilute the standard in the same buffer as the samples and to avoid contaminating peptides, salts, or extraction chemicals.

Immunocytochemistry (I, II, V)

Immunocytochemistry was used to show the presence and distribution of specific proteins in the cells. The cells were grown on cover slips and fixed with formaldehyde. Unspecific binding was blocked with BSA, and the cells were permeabilized with saponin. Primary

antibodies were used to bind the antigens, and fluorescein isothiocyanate (FITC) or Texas red conjugated secondary antibodies were subsequently used to visualize the primary antibodies. Hoechst's 33258 were used to visualize nuclear DNA. The cover slips were mounted using fluorescent mounting medium and viewed with a Nikon Optiphot-2 microscope.

Comments on immunocytochemistry

Immunocytochemistry is an important tool for characterization of cell type distribution, morphology and visualisation of sub-cellular structures. It is based on the specific binding of antibodies to an antigen. Therefore the quality of the binding, and thus the specificity of the antibody, is of utmost importance. Poor specificity will lead to false positives and/or false negatives. Precautions should be taken to avoid unspecific binding. BSA was used throughout the experiments to block unspecific binding and controls without primary antibody, or exchange of the antibody with normal serum at the same total protein concentration was performed in order to verify the results.

Gel electrophoresis and Western blot (I, II, IV, V)

Gel electrophoresis and Western blot was used to determine increases or decreases of specific proteins in the cell cultures. The cells, controls or treated, were lysed in RIPA lysis buffer and the protein concentration was measured prior to gel electrophoresis. An equal amount of protein was introduced into each lane of the gel. Electrophoresis was conducted at 200V for 50 minutes according the manufacturer's instructions. The separated proteins were then transferred to a nitrocellulose membrane by Western blot at 30V for 60 minutes. Unspecific binding of antibodies were blocked with 5% skim milk and the membrane was probed with primary antibodies. These antibodies were then detected with HRP-conjugated secondary antibodies and visualized using enhanced chemiluminiscence. The membranes in Paper I were exposed to light sensitive film and the membranes in Papers II, IV, and V were detected using a Fuji LAS-3000 system. The resulting bands from the chemiluminiscence reaction were then quantified by densitometry using ImageJ (NIH software).

Comments on gel electrophoresis and Western blot

The problem with antibody specificity also applies to Western blot. However, Western blot has some advantages over the immunocytochemical technique since the proteins have been separated according to size. This will help in identifying the correct antigen. The biggest

advantage is that samples of equal protein loading can be quantified and measured by densitometry in an objective manner.

It should be mentioned that the Covalab GLT-1 antibody gives an unspecific band at 40 kDa (not shown in the thesis) in addition to the specific band at 64 kDa. However, the unspecific band can be blocked by incubating with normal serum.

Enzyme linked immunoabsorbent assay (I, II, IV, V)

Enzyme linked immunoabsorbent assay (ELISA) was performed to determine the extracellular levels of TNF-α and IL-6 using commercial kits. These kits use a sandwich ELISA technique where capture antibodies are coated on a microtitre plate. Supernatants or standards of known concentrations of recombinant cytokines are added, and the plates are incubated with biotinylated detection antibodies. These antibodies are then bound by streptavidin-HRP which reacts with a chromogen to produce a blue colour. The absorbance is determined at 450 nm. Since the intensity of the colour is proportional to the concentration of cytokine, the unknown samples can be determined.

The GLT-1 ELISA in Paper I uses the same principles but it is not a sandwich technique. Instead, samples of equal protein concentrations are coated directly into the wells of a microtitre plate. Serially diluted control peptide was used as a positive control and as a standard. The antigen was detected with primary antibodies and subsequently with HRP-conjugated secondary antibodies that can react upon the chromogen. Since the standard consists of peptides and not recombinant protein of known concentrations, the GLT-1 ELISA is only semi-quantitative. Samples can be compared against each other but the concentration of the protein is not known.

Comments on enzyme linked immunoabsorbent assay

ELISA is a very useful technique when it comes to capturing soluble compounds. This makes it particularly useful when analysing proteins that are released into the extracellular medium, like cytokines. ELISA can also be used on cell lysates, such as in the GLT-1 ELISA, with high sensitivity. However, from the results in Paper I, it is clear that the GLT-1 ELISA is not as sensitive as Western blot analysis of GLT-1. It would probably have been a better approach to use a biotinylated secondary antibody followed by streptavidin-HRP instead of using a directly HRP-conjugated antibody. This would have amplified the signal from the ELISA and probably produced lower intra-variability and higher sensitivity.

Glutamate uptake assay (I, II, III, IV)

Glutamate uptake assays were performed essentially as described by Hansson and co-workers (1985a) with some modifications. A specific amount of ^{3}H-glutamate was added to various concentrations of un-labelled glutamate. The cells were then incubated in the uptake medium and intracellular and extracellular ^{3}H-glutamate was measured using liquid scintillation counting. During liquid scintillation counting, radioactivity is transformed into fluorescence and can be detected and quantified. In Paper I, increasing concentrations of un-labelled glutamate was used in the uptake medium to produce uptake curves for the different treatments. These curves can be used to calculate the transport velocity V_{max} and affinity K_m. In Papers II, III, and IV, single point uptake was used.

Comments on glutamate uptake assay

Glutamate uptake assays are important to conduct in order to investigate the functional capacity of the transporters. The glutamate uptake consists of two parts, active transport and passive diffusion. A concentration of 100 µM of un-labelled glutamate was used in the single point uptake, since the active uptake is relatively large compared to the diffusion at this concentration. It is important to perform the uptake acutely, an incubation time of 4 minutes was used throughout this thesis, since the system can reach equilibrium otherwise, especially if there is a release in addition to the uptake. It is possible to use radioactively labelled glutamate analogues or substrates, such as D-aspartate which is not metabolized, that are transported by the glutamate transporters. Care should be taken when selecting glutamate uptake inhibitors. Some glutamate uptake inhibitors, such as L-trans-2,4-pyrrolidine dicarboxylate, are transportable and should be applied in excess of glutamate, while others, like dihydrokainate, are non-transportable and not as dependent on the glutamate concentration.

Glutathione analysis (III, IV)

The glutathione contents were determined using a commercial kit or by high pressure liquid chromatography (HPLC) with ortho-phtaldialdehyde (OPA) precolumn derivatization with fluorescence detection, as described elsewhere. The commercial kit uses a carefully optimized enzymatical method, using glutathione reductase, for quantification of glutathione (Tietze, 1969). The sulfhydryl group of GSH reacts with,5'-dithiobis-2-nitrobenzoic acid and produces yellow coloured 5-thio-2-nitrobenzoic acid which is directly proportional to the GSH concentrations and can be measured at 405 nm.

Comments on glutathione analysis

The glutathione contents of the samples can be analysed in two ways. Either kinetically or by end point measurement. The less complicated method of endpoint measurement was used since both methods were tested and gave similar results. When measuring glutathione contents enzymatically, it is important to keep total protein concentrations below 1 mg/ml to avoid signals from contaminating thiols.

High pressure liquid chromatography (III)

HPLC was performed to separate the amino acid contents of the microglial cells using OPA precolumn derivatization with fluorescence detection as described earlier (Lindroth and Mopper, 1979; Sandberg et al, 1986) and in Paper III. MEA solution consisting of 20 mM β-mercaptoethanol, 1 mM EDTA, and 5 mM NaN_3 was added to samples and standards of known concentrations to keep glutathione in its reduced form and prevent bacterial growth. The samples were then derivatized with OPA solution in an autosampler before injection. The amino acids were separated by reverse phase HPLC on a Nucleosil C_{18} column with a mobile phase consisting of NaH_2PO_4 and methanol in a gradient from 25 to 90% methanol. A flow rate of 1 ml/min was used, and 1.5 minute fractions were collected for subsequent analysis by liquid scintillation counting. Detection was carried out by excitation at 333 nm and emission over 418 nm. Peaks were identified and quantified using standards of known concentrations.

Comments on high pressure liquid chromatography

The method with OPA-derivatization can be used to analyse traditional amino acids, sulphur containing amino acids, glutathione and metabolites. It allows rapid derivatization that is selective and sensitive. Potential draw backs with the method may include formation of unstable derivates and the fact that the derivatization reaction only occurs with a 1°-amine. The derivatization reaction can be seen in Figure 6.

The MEA solution was prepared fresh each day in order to keep its reducing capability, and the OPA solution was prepared weekly to avoid precipitations in the solution. This is important for the derivatization step. Poor derivatization could lead to poor resolution and underestimations in the fluorescence detection since underivatized amino acids will not have the same retention time in the column and will not be detected. HPLC with OPA derivatization is a sensitive method able to detect subpicomole amounts of amino acids (Lindroth and Mopper, 1979), and coupled to liquid scintillation counting it is possible to

analyse relatively small amounts of radioactivity in different amino acid fractions. This makes it possible to study how the glutamate taken up by the cells is utilized.

In the metabolic labelling experiments, steps were taken to ensure the specificity and recovery of the ^{3}H-labelled contents after HPLC separation. Pure ^{3}H-glutamate was mixed with external standards, derivatized with OPA, and separated with the HPLC. Analysis of the glutamate fraction revealed that there was approximately 85% recovery of the ^{3}H-glutamate with no significant radioactivity in the other fractions. No quenching effects were discovered when ^{3}H-glutamate was added to HPLC separated fractions and analysed with liquid scintillation counting.

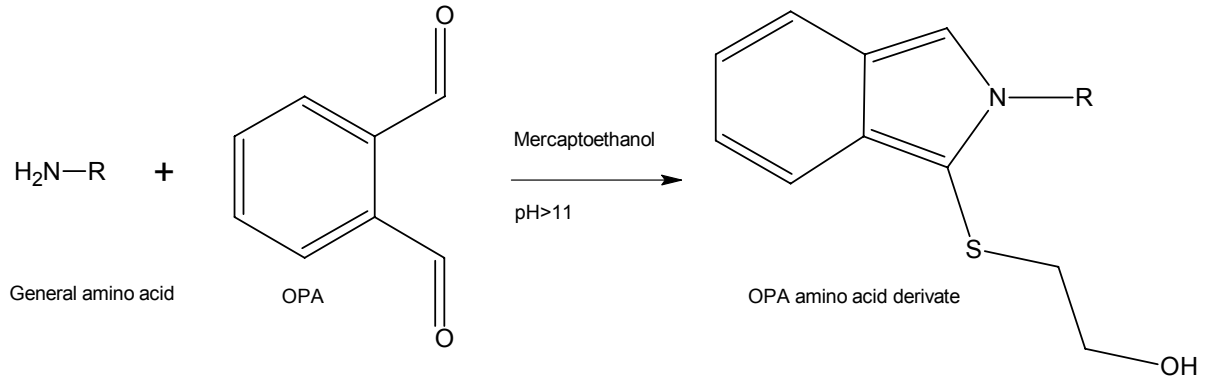

Figure 6. *The OPA-derivatization reaction. Non-fluorescent OPA reacts with the 1°-amine of the general amino acid H_2N-R together with mercaptoethanol under basic conditions (pH>11). The resulting reaction yields the fluorescent OPA amino acid derivate.*

Release of glutamate and/or glutamate metabolites (III, IV)

Release of glutamate and/or glutamate metabolites measurements were performed in relation to the radio-labelling experiments in Paper III. The cells were incubated with ^{3}H-glutamate for 60 minutes and then washed three times with buffer. Released radio-labelled compounds were then collected during five minutes before the cells were washed and subsequently lysed for protein concentration determination and HPLC separation and analysis.

Comments on release of glutamate and/or metabolites

Since the release experiment only measures radio-labelled contents, it does not give a quantitative value of the release, but only an indication that there is a release. The release of radioactive compounds will of course be subject to the concentration of radioactive compounds in the cells, which in turn is dependent on how much is taken up in the first place. However, the effect of different treatments can be compared relative to each other. The major

drawback of this experiment is that it is not possible to know if it is ^{3}H-glutmate *per se* that is released or some glutamate metabolite. One way to analyse if it is glutamate or glutamate metabolites that are released is to use glutamate release inhibitors. Such inhibitors should only inhibit the glutamate release and not the release of metabolites, providing that they are specific.

Cell viability assay (III, V)

Cell viability assays were conducted in order to investigate proliferative effects and to ascertain that microglial cytokine release were not due to cell death. The viability of the cells were evaluated using the 3-4-5-dimethylthiazole-2-yl-2,5-diphenyl-tetrazolium bromide (MTT) assay. The cellular medium was aspirated and an MTT solution was added to the cells. MTT is taken up by endocytosis and is then reduced to formazan in the endosomal/lysosomal compartment (Molinari et al, 2005). The non-soluble formazan crystals, that have a characteristic blue-purple colour, are then exocytosed to the cellular membrane. The formazan crystals can then be dissolved in dimethylsulfoxide and measured at 592 nm. Since only living cells endocytose the MTT, the amount of formed formazan crystals give a direct measurement of the amount of living cells.

Comments on cell viability assay

The MTT test is an often used method, most likely due to its simplicity. However, it is not a very sensitive method and is only able to detect comparably large effects on both proliferation and cell death. Furthermore, it only gives a semi-quantitative estimation of the number of living cells. Fluorescence-based methods, such as bromodioxyuridine incorporation for proliferation, or propidium iodide or terminal deoxynucleotidyl transferase dUTP nick end labelling for cell death, often provide better sensitivity though it should be noted that they often give information from a fixed field in the microscope instead of the total cell population as the MTT test does. Additionally, they can be performed in a fully quantitative manner. Since the MTT test only utilizes the cells for the test, it is possible to combine it with a lactate dehydrogenase assay on the supernatant in order to increase the specificity and diversity. In Paper III and V, it was judged sufficient to use the MTT test alone. It should be noted that phenol red must be excluded from the medium since it will otherwise interfere with the colorimetric assay.

Infection of CNS cells with HSV (V)

The viral infections were performed as described in Paper V. In order to avoid effects that are due to strain differences, two HSV-1 and two HSV-2 strains were used. The HSV are used at 1 PFU/cell, calculated at plating densities, for all cell cultures in Paper V. The virus were allowed to absorb for 60 minutes before the cell cultures were carefully washed two times to remove unabsorbed virus.

Comments infection of CNS cells with HSV

HSV infections can be studied both in experimental animals and *in vitro*. CNS cells obtained from rat pups were used in Paper V. Although CNS cells from rats have been reported to have a higher resistance to viral infections than cells from other animal models, such as mice, it is only partially true. The resistance displayed by rats is only obtained after 4-6 weeks, namely in relatively mature rats (Bergström et al, 1991). Younger rats display no such resistance (Bergström et al, 1991), and since our cells are derived from rat pups, no older than 2 days, the resistance should not be a factor that affects the obtained results. Furthermore, such cultures have been used to study viral replication in astrocytes where even differences in brain regions could be identified (Bergström et al, 1994).

Calculations of infected cells (V)

A method for semi-automatic counting of the number of HSV infected cells were used to estimate the difference between treatments. The method uses immunocytochemistry to identify the viral glycoproteins gC1 or gG-2, for HSV I or II, respectively, and Hoechst's 33258 to visualize all cell nuclei. Random fields are selected in the fluorescence microscope in a blinded manner and then examined using Image J software. Using cut off values for soma size in pixels and bins, it is possible to automatically count the total number of cell nuclei and the number of infected cells by separating each field into images of cell nuclei and immunoreactivity. Cut off values were selected and then employed for all images. After automatic calculation, each image were manually corrected to adjust for cells that were situated in too close proximity to each other to be calculated as separate cells, using the cell nuclei for guidance.

Comments on calculations of infected cells

It should be noted that the method only shows the amount of cells that express gC1 or gG-2. This is an indirect estimation of the number of infected cells. A cell can be infected without

expressing the viral glycoprotein. An alternative method that can be used is quantitative polymerase chain reaction (PCR) where you can count the actual viral genomes and compare them to the number of cells. This will yield a more accurate number of infected cells or, more accurately, viral DNA replication. Nonetheless, the method used in Paper V provides a suitably accurate estimation, at least when there is large differences between treatments. It should also be noted that the immunofluorescence method gives a very conservative estimation of the percentage of infected cells, since many cells that were immunoreactive were excluded due to the set thresholds. It should also be mentioned that expression of viral antigens is not directly comparable to viral replication. Cells like microglia can be subjected to viral infections and express early antigen markers, such as for ICP4, without allowing viral replication (Lokensgard et al, 2001). Low viral replicate titers, despite presence of early antigens, strongly indicate that a step post infection is responsible for possible resistance.

Quantitative polymerase chain reaction (V)

Taqman PCR was used to measure HSV DNA copies in order to determine the viral replication. The cell cultures were incubated with HSV-1 or HSV-2 for 60 minutes to allow viral absorbtion. Viral replication was then allowed to occur for 24 hours. The cells were harvested by a cell scraper and the DNA was extracted using a MagnaPure LC automatic robot using a MagnaPure LC kit. The extracted DNA was then amplified using HSV-1 or HSV-2 specific primers together with their respective probes. The probes are labelled with fluorescent substances such as carboxyfluorescein or 6-carboxy-4',5'-dichloro-2',7'-dimethoxy-fluorescein and are used to give a real time value of the number of primer specific DNA sequences that can easily be monitored.

Comments on quantitative polymerase chain reaction (V)

In contrast to common PCR, which is a semi-quantitative method, Taqman PCR is a real time PCR method that gives a quantitative value. PCR has been extensively used to analyze HSV samples. The Taqman PCR is a very accurate method provided that the primers and probes are constructed in such a way that they are specific with high fidelity to their intended DNA targets. The method used in Paper V has been reported to have an accuracy of 99.5% with a correct identification of type for HSV samples (Namvar et al, 2005). Since it is a very sensitive method due to the amplification steps, it is very important to avoid contamination of the samples since even relatively small contaminations can have large consequences. It is also important that all samples are handled in the same way. Ideally, the samples should be

handled and analyzed at the same time, which was the case in Paper V, in order to avoid discrepancies due to different handling conditions such as freeze-thawing and PCR sample preparations. The validity of the results is of course dependent on the quality of the prepared DNA samples. In Paper V, the DNA was extracted using an automated process in a robotic work station, thereby ensuring that all samples were handled equally and that cross-contamination was limited.

RESULTS AND DISCUSSION

I. Lipopolysaccharide increases microglial GLT-1 expression and glutamate uptake capacity *in vitro* by a mechanism dependent on TNF-α

Microglia are able to express Na^+-dependent high affinity glutamate transporters *in vivo* during pathological conditions such as controlled cortical impact (van Landeghem et al, 2001), facial nerve axotomy (Lopez-Redondo et al, 2000), viral infections (Chretien et al, 2002; Porcheray et al, 2006a; Vallat-Decouvelaere et al, 2003), and prion diseases (Chretien et al, 2004). These are situations where the astroglial glutamate transporters are down-regulated, which could contribute to neurodegeneration (O'Shea, 2002). Microglia have also been shown to express glutamate transporters *in vitro* and have a capacity for glutamate uptake (Nakajima et al, 2001b; Rimaniol et al, 2000). However, not much is known about how the glutamate transporters are regulated.

With the knowledge that microglia are activated in a number of CNS diseases, it is reasonable to think that glutamate transporter expression is activation dependent and possibly linked to inflammatory events. Examining the effects of several different stimuli known to activate microglia and to be associated with a down-regulated astroglial glutamate transport, it was found that LPS, the only stimulus in the study to induce release of cytokines, increased expression of GLT-1. The fact that LPS induces/up-regulates GLT-1 in microglia has now been confirmed in a study by O'Shea and co-workers (2006) using microglia in culture and for human monocyte derived macrophages by Porcheray and co-workers (2006a). In our study, it was also found that the pro-inflammatory cytokine TNF-α was able to mimic the LPS induced GLT-1 increase and that the TNF-α synthesis inhibitor thalidomide (Sampaio et al, 1991), or neutralization with TNF-α antibodies, was able to inhibit the increase in GLT-1 expression. This suggests that microglial GLT-1 expression could be coupled to inflammatory events and that TNF-α can act as an inducer of the expression. However, it is not known if TNF-α *per se* induces the expression or if it is some other factor that in turn is induced by the cytokine. One such factor could be interferon-γ (IFN-γ) which has been shown to increase microglial glutamate uptake (Shaked et al, 2005). One possibility to find out about the importance of TNF-α would be to use cultures from TNF-α knock out animals. Nonetheless, it is plausible that pathological conditions that lead to release of TNF-α can also lead to an induced expression of microglial glutamate transporters.

The increase in transporter expression leads to increased functional glutamate uptake. However, the glutamate uptake capacity of microglia is only about 10% of that measured for

astrocytes (Persson et al, 2005; Shaked et al, 2005). This could suggest different physiological functions for astroglial and microglial glutamate transporters although it should be mentioned that microglia does indeed clear added glutamate from medium *in vitro* (Rimaniol et al, 2000). Interestingly, TNF-α has been shown to inhibit glutamate uptake in astrocytes (Fine et al, 1996). This could suggest a reciprocal control of glutamate transporters between astrocytes and microglia. Indeed, microglia have been proposed to serve as a backup system for diminished astroglial glutamate transport (Gras et al, 2006; Rimaniol et al, 2000).

The role of TNF-α in pathological conditions has been debated. TNF-α has classically been considered as deleterious since it has the ability to induce inflammation and cell death, but it also has several other functions that are non-deleterious (for review see Hehlgans and Pfeffer, 2005). In fact, TNF-α has been shown to be neuroprotective in some paradigms (for reviews, see Hallenbeck, 2002, and Shohami et al, 1999). This seems to be true for several cytokines since they can act as neuromodulators of the CNS (Vitkovic et al, 2000) and even protect against excitotoxic insults (Carlson et al, 1999). The concept of an immunological protection against glutamate excitotoxicity, coined protective autoimmunity, has been proposed (Schwartz et al, 2003).

It has been shown that TNF-α has deleterious effects during and after ischemia (Nawashiro et al, 1997a), but that pre-treatment with the cytokine is protective against the insult (Marchetti et al, 2004; Nawashiro et al, 1997b; Romera et al, 2004). It has also been shown that pre-treatment with a single dose of LPS reduces ischemic damage after middle cerebral artery occlusion with TNF-α as the likely mediator (Tasaki et al, 1997). Furthermore, Marchetti and co-workers (2004) have shown that signalling through TNFR1 aggravates neuronal loss while signalling through TNFR2 has the adverse effect. Taken together, these studies show that the timing, context, and concentration of TNF-α is important for determining the effects of the cytokine. It is not known whether the neuroprotective abilities of cytokines, especially TNF-α, is mediated at least in part by microglial glutamate transporters, but it makes an interesting hypothesis and field of research.

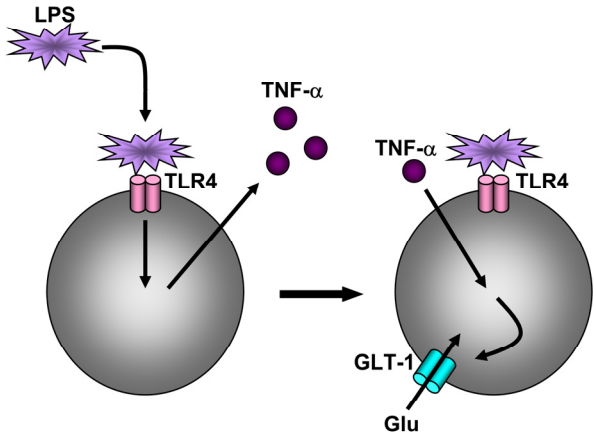

Figure 7. *A schematic figure presenting the results in Paper I. The inflammatory stimulus LPS is detected by microglia via the Toll-like receptor 4 (TLR4), causing a prominent release of the cytokine TNF-α into the extracellular space. TNF-α can then activate the microglia in an autocrine manner and induce/increase the microglial expression of the glutamate transporter GLT-1, making the cells able to transport glutamate from the extracellular space.*

II. Corticosterone inhibits expression of the microglial glutamate transporter GLT-1 *in vitro*

The expression of microglial high affinity glutamate transporters seems to be regulated by inflammatory events. Incubation with LPS has been shown to induce expression of microglial GLT-1 as shown in Paper I and II, and this expression seems to be connected to the proinflammatory cytokine TNF-α. Therefore, it is believable that anti-inflammatory molecules that are known to affect microglia and microglial production and/or release of TNF-α, might negatively regulate the expression. One such potential molecule is corticosterone, the species specific glucocorticoid of rats.

Our study showed that corticosterone, at physiological stress response related levels, was indeed able to inhibit the microglial expression of GLT-1. Corticosterone was found to inhibit LPS induced synthesis/release of TNF-α. The effect of corticosterone on microglial GLT-1 is probably due to the inhibition of TNF-α since this cytokine has previously been linked to glutamate transporter expression in both microglia (Persson et al, 2005) and in blood-born monocytes (Rimaniol et al, 2000). The inhibitory effect of corticosterone could also be seen for otherwise unstimulated microglia. The low basal levels of TNF-α that are responsible for the basal microglial GLT-1 expression seen *in vitro* are likely to be inhibited by corticosterone and subsequently, the basal microglial GLT-1 expression is lowered. It should be noted that dexamethasone, a synthetic glucocorticoid analogue, have been shown to

be a potent inducer of EAAT1 in human monocyte derived macrophages (Gras et al, 2006; Porcheray et al, 2006a). The difference between murine microglia and human monocyte derived macrophages may respond to differences in TNF-α sensitivity since the macrophages do not secrete detectable TNF-α when differentiated (Gras et al, 2006).

The results in Paper II further highlight the connectivity between neuroinflammatory mediators, such as TNF-α, and microglial glutamate transporter expression. Anti-inflammatory substances like corticosterone are indeed able to inhibit microglial GLT-1 expression by inhibiting the release of TNF-α. They do not affect the mechanism for GLT-1 expression *per se*, since exogenously added TNF-α could induce microglial GLT-1 while the TNF-α synthesis/release was inhibited by corticosterone.

Taken together, the results raise some interesting questions regarding the mechanisms behind microglial glutamate transporter expression. Microglia are capable of *de novo* expression of glutamate transporters after controlled cortical impact while their astroglial counterparts are down-regulated (van Landeghem et al, 2001). During traumatic brain injury there is a release of TNF-α (Stover et al, 2000) and glucocorticoids (McCullers et al, 2002). There will also be an accumulation of extracellular glutamate (McCullers et al, 2002), which in part can be due to inhibition of astroglial glutamate uptake. In fact, TNF-α has been shown to inhibit astroglial glutamate uptake (Fine et al, 1996). Corticosterone has been shown to inhibit glutamate uptake in hippocampal cultures (Brooke and Sapolsky, 2003) by a mechanism that has been suggested to impair astroglial glutamate uptake during neurological crisis (Virgin, Jr. et al, 1991) although it increases astroglial GLT-1 expression and glutamate uptake in cortical cultures (Zschocke et al, 2005). On the other hand, the present studies (Paper I and II) have shown that TNF-α and corticosterone have differential effects on microglial GLT-1 expression and glutamate uptake when compared to astrocytes derived from cortex. This further highlights that microglial and astroglial GLT-1 may be regulated reciprocally although differences may occur in different brain regions.

The timescale of TNF-α and glucocorticoid presence may be of importance for the microglial glutamate transporter expression. Glucocorticoid levels are elevated early and until six hours after the insult (McCullers et al, 2002), while TNF-α has its peak 8-48 hours after the insult (Stover et al, 2000), indicating that the microglial transporters may be induced with some delay. In van Landeghem and co-workers' (2001) controlled cortical impact model, they detected microglial glutamate transport expression as early as 4 hours after the impact which reached stable levels after 48 hours. This at least allows for a time window where the

microglia are affected by both TNF-α and corticosterone. Therefore, it is plausible that the microglial GLT-1 expression is regulated during pathological events by a balance of inflammatory and anti-inflammatory substances.

Certainly, more research is needed to understand the expression pattern of microglial glutamate transporters during patophysiology and the physiological meaning of the expression.

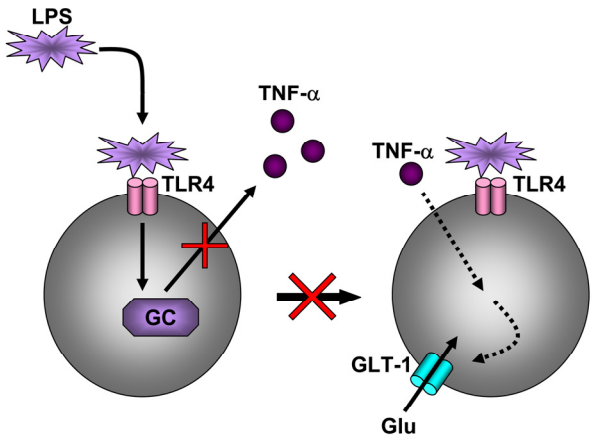

Figure 8. *A schematic figure presenting the results in Paper II. The inflammatory stimulus LPS is detected by microglia via the Toll-like receptor 4 (TLR4) which normally causes a prominent release of TNF-α into the extracellular space. However, antinflammatory glucocorticoids (GCs), like corticosterone, inhibit the synthesis and thereby the release of TNF-α. Consequently, there is an inhibition of the TNF-α-dependent expression of GLT-1 in microglia. As depicted by the dotted arrows, the microglial GLT-1 expression can be rescued by exogenously added TNF-α.*

III. Microglial glutamate uptake is coupled to glutathione synthesis and glutamate release

The physiological function of glutamate uptake through microglial glutamate transporters has been questioned. At least three major hypotheses exist. The first two theories concern the actual transport of glutamate in microglia. According to one theory, the microglial glutamate uptake serves as a back up system for the diminished astroglial glutamate uptake (Gras et al, 2006; Nakajima et al, 2001b; Schwartz et al, 2003), but this has been debated since the microglial glutamate uptake capacity is only 10% of that measured for astrocytes (Persson et al, 2005; Shaked et al, 2005). Another plausible theory is that the glutamate uptake is used for glutamate release (Noda et al, 1999). The third theory, presented by Rimaniol and co-workers (2001), proposes that the glutamate uptake is used for cystine uptake and for a direct incorporation into glutathione. During virus infection, microglia have been shown to express GS (Chretien et al, 2002; Vallat-Decouvelaere et al, 2003), like astrocytes do, which could

make them capable to metabolize glutamate into glutamine in order to aid neurons (Gras et al, 2006; Hertz et al, 1999; Sonnewald et al, 1997).

Using LPS and TNF-α as molecular tools to modulate microglial GLT-1 expression together with radioactively labelled glutamate, this study has investigated how the transported glutamate is metabolized. It was found that both TNF-α and LPS, even when TNF-α synthesis was inhibited by thalidomide, increased microglial glutathione levels. This shows that although TNF-α can induce GSH synthesis by itself, LPS induced GSH synthesis is TNF-α-independent. With the metabolic labelling, it was shown that most of the ^{3}H-glutamate ended up in the large intracellular pool of glutamate although there was seemingly only an increase in labelled ^{3}H-glutamate after TNF-α treatment. One might expect an increase after LPS treatment as well, but this stimulus also led to the largest release of labelled compounds in our model system which likely explains the result. Interestingly, there was ^{3}H-labelled contents in the glutathione fraction, with more labelling after LPS and TNF-α treatment. This is in line with the model proposed by Rimaniol and co-workers (2001) where microglia use the glutamate uptake through EAATs to fuel the X_C^- system for cystine uptake and for direct incorporation of glutamate into glutathione (see schematic figure in Paper III).

Glutathione is released from brain cells, reported mainly for astrocytes (Sagara et al, 1996), through multi drug resistance protein (MRP) 1 and 2 (Leier et al, 1996; Paulusma et al, 1999; Rebbeor et al, 2002). MRP1 has been reported to be expressed functionally in microglia (Dallas et al, 2003). This leads to the conclusion that microglia may use the GSH system, and subsequently the EAAT system, to protect, at least themselves but also possibly other cell types, from oxidative stress. In fact, in cell cultures, microglia have a more prominent GSH system than neurons or astrocytes (Hirrlinger et al, 2000), and stain intensively for both GSH and GR (Chatterjee et al, 1999; Gutterer et al, 1999). This prominent antioxidant system has been proposed to reflect the need of microglia to protect themselves from ROS that they can release upon activation (Hirrlinger et al, 2000). The question still remains whether the GSH system may protect other vulnerable cells like neurons as well. This would give the microglia some neuroprotective capabilities as previously suggested (Rimaniol et al, 2001; Vallat-Decouvelaere et al, 2003). In fact, microglial conditioned media has been shown to have neuroprotective effects (Watanabe et al, 2000).

It should be mentioned that the results apply to our model system. In other model systems, such as *in vivo* experiments, other physiological functions for microglial glutamate uptake may occur. For instance, no significant radio activity was found in the glutamine

fraction in our microglial cultures, although GS have been found in EAAT expressing microglia *in vivo* (Chretien et al, 2002). This highlights the need for more basic knowledge of microglial glutamate transporters and their function, both in simple and complex model systems.

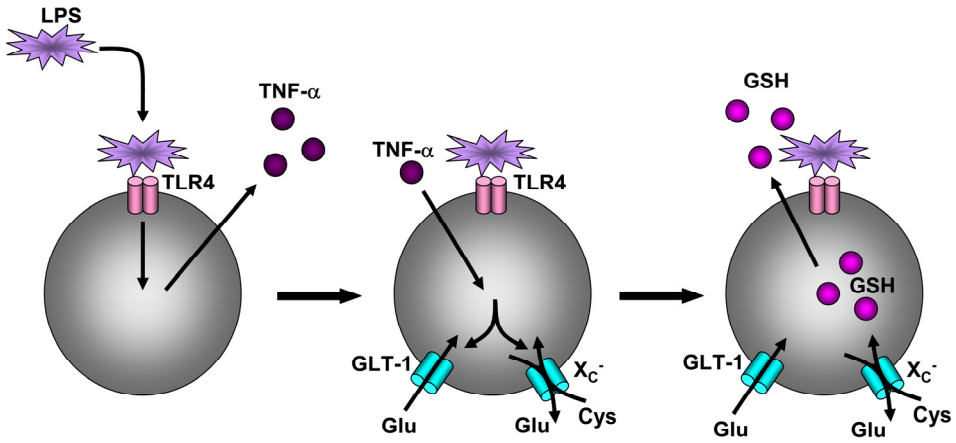

Figure 9. *A schematic figure presenting the results in Paper III. The inflammatory stimulus LPS is detected by microglia via the Toll-like receptor 4 (TLR4), causing a prominent release of the cytokine TNF-α into the extracellular space. TNF-α can then activate the microglia in an autocrine manner and induce/increase the microglial expression of the glutamate transporter GLT-1, making the cells able to transport glutamate from the extracellular space. The sequestered glutamate can then either be stored in the intracellular glutamate pool, used to fuel the X_C^- system for cystine uptake, or be used directly for synthesis of the antioxidant glutathione (GSH). Increased levels of glutathione provide the microglia with a self-defence against oxidative stress.*

IV. The complement-derived anaphylatoxin C5a increases microglial GLT-1 expression and glutamate uptake in a TNF-α-independent manner

The complement system is activated in several CNS diseases in which the microglial glutamate transporters are expressed such as brain trauma (Lopez-Redondo et al, 2000; van Beek et al, 2003; van Landeghem et al, 2001), viral infections (Chretien et al, 2002; Datta and Rappaport, 2006; Porcheray et al, 2006a; Porcheray et al, 2006b; Vallat-Decouvelaere et al, 2003), prion diseases (Bonifati and Kishore, 2007; Chretien et al, 2004), and during general neuroinflammatory conditions as seen during neurodegenerative diseases (Emmerling et al, 2000; McGeer et al, 1989; Persson et al, 2005; Rimaniol et al, 2000). Therefore, it seemed possible that complement activation may be a factor for microglial glutamate transporter expression. Especially the complement anaphylatoxins C3a and C5a were of interest since they have been shown to be connected to cytokine release, which is important for microglial GLT-1 expression (Jacobsson et al, 2006; Persson et al, 2005), and to be neuroprotective

against excitotoxicity both *in vivo* and *in vitro* (Osaka et al, 1999; Pasinetti et al, 1996; van Beek et al, 2001).

It was found that C5a, but not C3a, increased microglial expression of GLT-1. However, the increase in GLT-1 expression was not as great as the one promoted by LPS. Furthermore, C5a did not induce GLT-1 by the same mechanism as LPS since C5a did not show any dependency on TNF-α. In fact, C5a did not induce any release of TNF-α at all despite being considered as a proinflammatory factor.

The increase in microglial GLT-1 induced by C5a was also accompanied by an increased glutamate uptake capacity. This could have some interesting implications if the mechanism holds true for the *in vivo* situation as well. Microglial glutamate uptake have been shown to protect neurons against glutamate-induced excitotoxicity (Rimaniol et al, 2000), produce glutamine as an energy source for neurons (Gras et al, 2006), and increase the levels of GSH (Persson et al, 2006; Rimaniol et al, 2001). Therefore, it is plausible that the increased microglial glutamate uptake, provided by C5a stimulation, may lead to neuroprotection, or at least self protection, for the microglia. The microglial glutamate uptake is about 10% of that measured for astrocytes (Persson et al, 2005; Shaked et al, 2005), but may still have significant effects and serve as a backup system for the diminished astroglial glutamate uptake in an insulted area during pathological events (O'Shea, 2002), such as an ischemia or brain trauma, since there is a massive infiltration in that area by microglia and macrophages (Streit et al, 1999). Indeed, microglial glutamate transporters have been shown to be expressed in the insulted area during brain trauma (Lopez-Redondo et al, 2000; van Landeghem et al, 2001). Of course, such hypothesis may also hold true for microglial glutamate uptake induced by TNF-α. It should be mentioned that there is a high level of interconnectivity between TNF-α and the complement anaphylatoxins, highlighting the complexity of microglial GLT-1 expression. For instance, LPS is a powerful activator of the complement system and generates C5a (Gasque et al, 2000). Additionally, it has been shown that TNF-α up-regulates C5aR (Stahel et al, 1997a), making the effect of C5a more pronounced. This can be seen during bacterial meningitis (Stahel and Barnum, 1997) which is a pathological state mimicked by our model system with LPS in Paper I-III.

Clearly, C5a can induce microglial glutamate transporters by a mechanism that differs from the one induced by LPS or TNF-α. In fact, C5a and TNF-α could have an additative or synergistic effect on the microglial glutamate transporter expression and glutamate uptake capacity, although such an effect has not been shown. There are also implications for an

increased glutamate uptake during pathological situations in the CNS, namely neuroprotection and/or self protection by the microglia, which deserves to be further investigated *in vivo*.

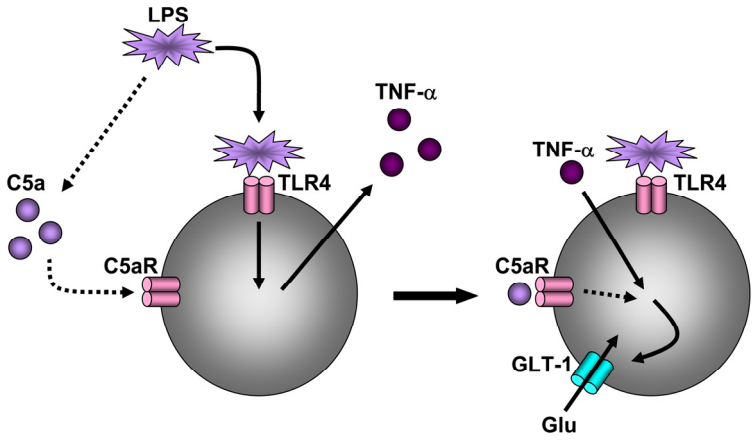

Figure 10. *A schematic figure presenting the results in Paper IV. It was found that the complement anaphylatoxin C5a could induce/increase the microglial GLT-1 expression, as indicated by the dotted arrows, in addition to the previously described pathway initiated by LPS, although to a lower extent. However, unlike the LPS initiated pathway, the C5a pathway is independent of TNF-α release and may represent another activation profile for microglial glutamate transporter expression. It may also be possible that C5a is part of the LPS-induced mechanism for microglial GLT-1 expression since complement, and thus C5a, is readily activated by LPS.*

V. Microglial GLT-1 is up-regulated in response to herpes simplex virus infection to provide an antiviral defence via glutathione

HSV infections in the CNS are relatively uncommon in the population since HSV normally have higher affinity and easier access to other non-neuronal tissue. Nonetheless, highly neurovirulent and neurotropic HSV strains exist (Broberg and Hukkanen, 2005; Frampton, Jr. et al, 2005; Kimberlin, 2004) and HSV infections normally have devastating effects, causing encephalitis or meningitis (Tyler, 2004). The research in paper I-III have mainly been focused on examining the effect of the inflammatory environment that occurs due to brain trauma, stroke or inflammation, or the inflammatory environment that is connected to neurodegeneration, with regards to microglial glutamate transporter expression and regulation. HSV infections in the CNS share several hallmarks with these conditions, including high levels of TNF-α (Lokensgard et al, 2002; Sköldenberg, 1996), and are therefore very suitable to mimic under experimental conditions and apply the knowledge gained in Paper I-III on in order to elucidate more of the physiological functions of the microglial glutamate transporters.

In our experimental paradigm, it was found that HSV indeed caused an inflammatory response by microglia. In line with the previous findings, increased levels of TNF-α led to increased expression of microglial GLT-1 and, presumably, increased microglial glutamate uptake. The up-regulation of microglial GLT-1 is remarkable since most proteins are down-regulated during infection due to viral host shut-off functions (Matis and Kudelova, 2001; Mossman et al, 2001). An induced/increased microglial GLT-1 expression can therefore be theorized to be part of an antiviral defence for microglia. Such a defence can be provided by GSH that is normally increased by increased glutamate uptake (Persson et al, 2006; Rimaniol et al, 2001) and have great antiviral properties (Palamara et al, 1995; Palamara et al, 1996a) by counteracting the oxidative state that is crucial for viral replication (Ciriolo et al, 1997). Indeed, our results show that the microglial GSH levels are neither decreased in response to HSV, nor increased as predicted by the increased levels TNF-α. This can be interpreted as if the microglial GLT-1 provides enough glutamate for the microglia to allow enough synthesis of GSH to counteract the effect of the virus, thus inhibiting viral entry and viral replication. This is reflected by the fact that microglia have increased resistance to HSV infections compared to neurons or astrocytes, as shown by us and others (Lokensgard et al, 2001), and the fact that microglia have the highest levels of GSH of the cells in the CNS (Hirrlinger et al, 2000). Additionally, it is known that cells with high GSH levels have higher resistance to viral infections than cells with lower GSH levels (Macchia et al, 1999). Although increased GSH production via glutamate uptake may not be the only factor for the increased resistance shown by microglia, it nonetheless shows a mechanism that can provide an antiviral defence. Interestingly, the antiviral mechanism provided by microglial GLT-1 and GSH up-regulation could be a general mechanism, holding true for several other types of viruses besides HSV. It has been shown that microglial glutamate transporters are involved in HIV infections of the CNS (Porcheray et al, 2006a; Vallat-Decouvelaere et al, 2003). HIV drives microglia towards a proinflammatory state (Porcheray et al, 2006b) and there is an induction of glutamate transporters in these cells (Porcheray et al, 2006a; Vallat-Decouvelaere et al, 2003). Furthermore, GSH has antiviral effects for HIV (Palamara et al, 1996b). Therefore, it seems plausible that the results obtained with HSV may hold true for HIV as well.

Greater knowledge about mechanisms such as the one presented in Paper V may in the long run be used to provide better clinical treatment for patients suffering from HSV infections in the nervous system. Of course, such mechanisms must be tested in higher model systems and ultimately in experimental animals. In fact, several studies have already successfully employed the end product GSH, either exogenously or in the form of GSH esters

that can cross the BBB, in order to counteract viral entry and replication (Garaci et al, 1992; Nucci et al, 2000; Vogel et al, 2005).

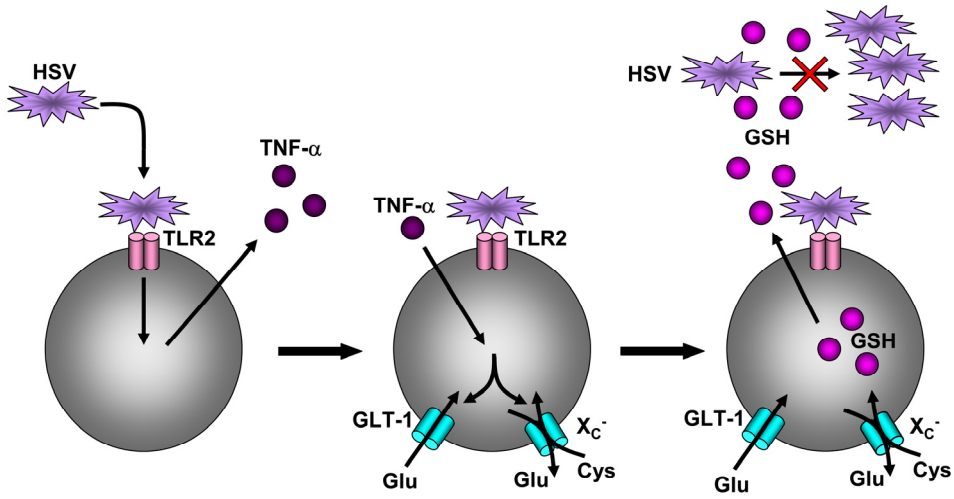

Figure 11. *A schematic figure presenting the results in Paper V. Herpes simplex virus (HSV) can be detected by microglia, possibly through the Toll-like receptor 2 (TLR2), and causes a prominent TNF-α release into the extracellular space. TNF-α can then activate the microglia in an autocrine manner and induce/increase the microglial expression of the glutamate transporter GLT-1, making the cells able to transport glutamate from the extracellular space. The sequestered glutamate can then either be stored in the intracellular glutamate pool, used to fuel the X_C^- system for cystine uptake, or be used directly for synthesis of the antioxidant glutathione (GSH). Increased levels of glutathione provide microglia with a viral defence since glutathione has prominent antiviral properties, making the microglia more resistant to herpes simplex virus infections than astrocytes or neurons.*

PERSPECTIVES

Knowledge about the regulation and physiological function of microglial glutamate transporters might give valuable insights into the mechanisms behind neurodegenerative diseases that are connected with excitotoxicity. Astrocytes, being responsible for most of the glutamate uptake in the CNS, have classically been, and are still, considered as key players during such pathological conditions. However, it is now becoming clear that other cell types are highly involved, especially the microglia. Important knowledge about the regulation and physiological function of microglial EAATs has emerged from this relatively new field of research.

The studies in this thesis highlight that there most likely is a connection between inflammatory events in the CNS, especially the pro-inflammatory cytokine TNF-α, and microglial EAAT expression. It shows that microglial glutamate transporters can be modulated in a relatively simple model system of pure microglia in culture. The results show the complexity of the regulation with TNF-α and C5a as two, possibly interacting, inducers of microglial GLT-1 and glucocorticoids as possible down-regulators. Most likely, the timing, strength, duration, and localization of these signals are crucial for determining the effect of the stimuli. It seems plausible that a balance in these regulating systems, and most importantly in the microglial activation, can have beneficial properties. One such mechanism is through increased synthesis of the antioxidant GSH which is highlighted in this thesis. The results show that the microglia, due to increased EAAT expression and thus increased glutamate uptake, can protect themselves from ROS and viral infections by increased synthesis of GSH. This is in contrast to the previously well known fact that microglial activation can lead to excitotoxicity due to release of microglial glutamate. An ideal situation would be to reach a level of microglial activation that harness all the beneficial properties of the activation with increased glutamate uptake and just enough glutamate release to provide increased GSH synthesis but prevent an exaggerated glutamate release. Such a balance is likely to be reached with the proper level of microglial activation.

The hope is that this research will give important insights into the regulation of microglial glutamate transporters, and their physiological functions, that can later be investigated and verified in more complex model systems such as mixed cultures, brain slices, and ultimately, in experimental animal. Successful modulation of microglial glutamate transporters, whether they have beneficial or harmful effects, may be important for treating, and understanding the mechanisms behind pathological conditions such as stroke, brain

trauma, infections and neurodegenerative diseases. In the future, it may be possible to limit excitotoxicity and oxidative damage during pathological conditions by harnessing the beneficial properties of microglial activation and glutamate transport using specific pharmacological treatment.

CONCLUSIONS

I. Microglia in culture were found to express the Na^+-dependent high affinity glutamate transporter GLT-1, but not GLAST.

II. The proinflammatory cytokine TNF-α were able to induce an increased GLT-1 expression in microglia, possibly associating microglial glutamate transporter expression with inflammatory events.

III. The antinflammatory effects of the glucocorticoid corticosterone inhibited microglial TNF-α release and inhibited both basal and LPS induced microglial GLT-1 expression.

IV. The complement anaphylatoxin C5a, but not C3a, was able to increase microglial GLT-1 without eliciting an inflammatory response in a manner that is different from the effect of LPS.

V. The glutamate taken up through GLT-1 was found to be utilized for direct incorporation into the antioxidant glutathione in addition to being stored in the intracellular glutamate pool. It was also found that there was a release of compounds, most likely of glutamate through the X_C^- system, from microglia. By increasing/maintaining the glutathione pool, microglia are able to provide self-defence against oxidative stress and viral infections.

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