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Neuron-glia interactions in the CNS

Weering, Hilmar Rienke Jan van

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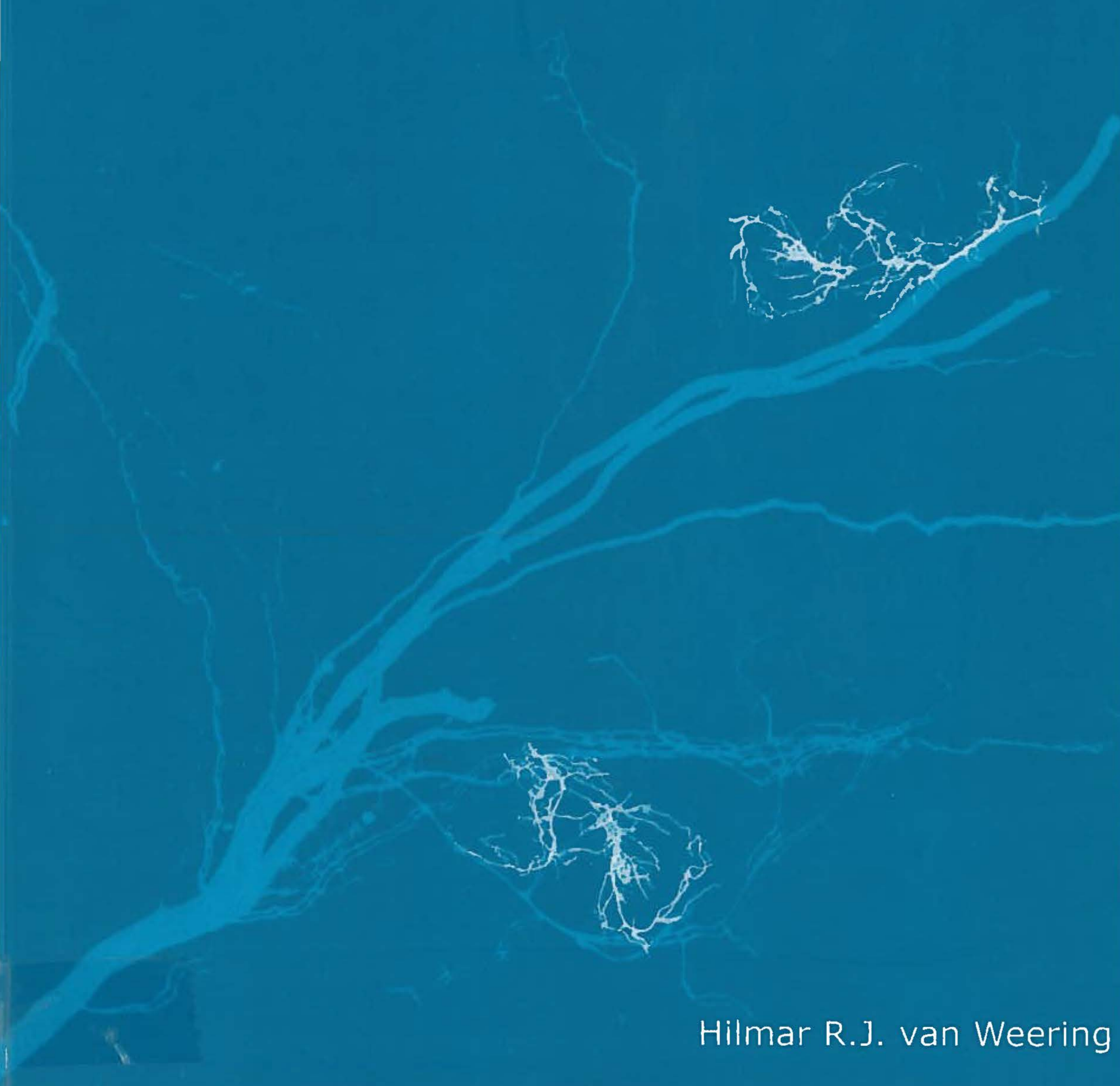
Neuron-Glia interactions in the CNS

Implication of CXCR3-signaling in excitotoxicity-induced neurodegeneration



Hilmar R.J. van Weering

NEURON-GLIA INTERACTIONS IN THE CNS

Implication of CXCR3-signaling in excitotoxicity-induced
neurodegeneration

The studies described in this thesis were carried out at the Department of Neurosciences, Section Medical Physiology, University Medical Center Groningen (UMCG), University of Groningen (RUG), The Netherlands. This work was supported by the University of Groningen (RUG) and the graduate school of Behavioral and Cognitive Neurosciences (BCN).

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Stellingen behorende bij het proefschrift

Neuron-Glia interactions in the CNS; implication of CXCR3-signaling in excitotoxicity-induced neurodegeneration

Hilmar R.J. van Weering

1. De rol van microglia bij - door excitotoxiciteit geïnduceerde - neuronale celdood is primair van een neuroprotectieve aard. Echter, het klassieke 'geactiveerde' microglia fenotype is niet noodzakelijk voor de bijdrage van deze cellen in de bescherming van neuronen in dit paradigma (*dit proefschrift*).

2. Gezien de beperkingen van het gebruik van geïsoleerde microglia als model voor het bestuderen van microglia functies *in vitro*, zou het gebruik van het "organotypische slice culture model", waarin de fysiologische neuron- en/of glia interacties bewaard blijven, de voorkeur moeten hebben (*dit proefschrift*).

3. De communicatie tussen neuronen en/of glia cellen via chemokine-signaaltransductie, waaronder CXCL10/CXCR3 en CCL21/CXCR3, beïnvloedt in sterke mate het overleven van neuronen onder excitotoxische omstandigheden. Echter, het verschil in zowel het expressiepatroon als het effect van bovengenoemde liganden suggereert dat deze twee vormen van communicatie via verschillende signaalsystemen verlopen (*dit proefschrift*).

4. Het begrip "kamertemperatuur" in experimentele protocollen dient voorzichtig gehanteerd te worden, gezien deze gevoelsmatig vele graden lager ligt bij mannen dan bij vrouwen.

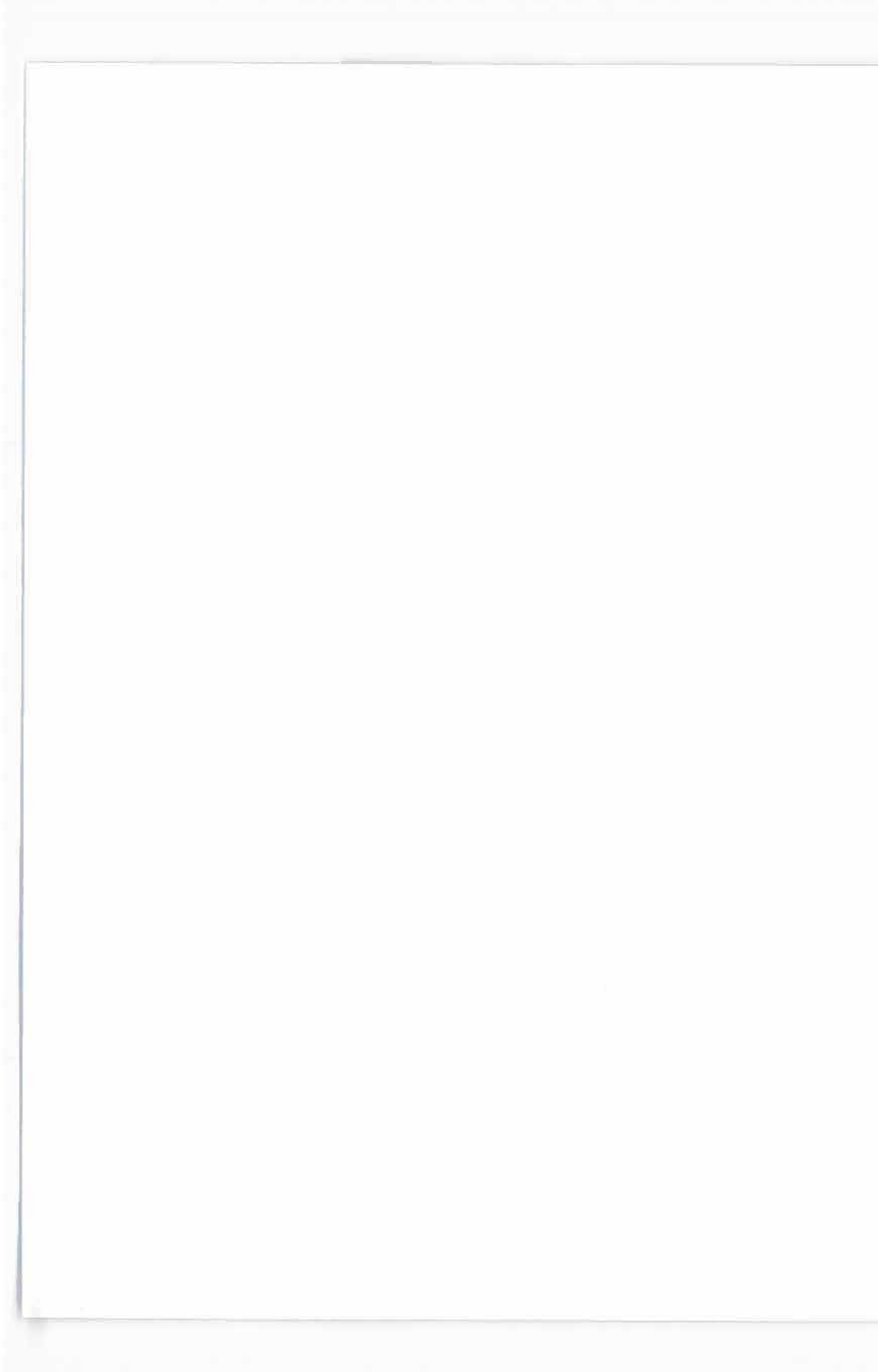
5. De observatie dat CCL21 effecten induceert in CXCR3-deficiente astrocyten suggereert de aanwezigheid van een additionele (nog onbekende) receptor voor dit ligand in het brein (*dit proefschrift*).

6. Voor een "immune-privileged" orgaanstelsel bevat het CZS verdacht veel componenten van het afweersysteem.

7. Het huidige beleid van het openbaar vervoer in Noord-Nederland doet de slogan "Er gaat niets boven Groningen" eer aan.

8. Alhoewel af en toe "de zinnen verzetten" een promotietraject vele malen draaglijker maakt, kan dit in letterlijke zin desastreuze gevolgen hebben voor de uiteindelijke lay-out van een proefschrift.

9. Mochten Pasen en Pinksteren ooit op één dag vallen, dan zou deze feestdag "Sint Juttemis" moeten heten.



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NEURON-GLIA INTERACTIONS IN THE CNS

Implication of CXCR3-signaling in excitotoxicity-induced neurodegeneration

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ABBREVIATIONS

AD	Alzheimer's disease
AIDS	acquired immune deficiency syndrome
ALS	amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionate
APC	antigen-presenting cell
ATP	adenosine-5'-triphosphate
BBB	blood-brain barrier
cAMP	cyclic adenosine monophosphate
CA	cornu ammonis
CNS	central nervous system
DG	dentate gyrus
EAE	experimental autoimmune encephalitis
GAG	glycosaminoglycan
GFAP	glial fibrillary acidic protein
GPCR	G-protein coupled receptor
HAD	HIV-associated dementia
HIV	human immunodeficiency virus
IP3	inositol triphosphate
KA	kainate
Lip-CL	liposome-encapsulated clodronate
NMDA	*N*-methyl-D-aspartic acid
MAPK	mitogen-activated protein kinase
MCAO	middle cerebral artery occlusion
mGLUR	metabotropic glutamate receptors
MHC	major histocompatibility complex
MS	multiple sclerosis
NO	nitric oxide
OHSC	organotypic hippocampal slice culture
PI	propidium iodide
PI3K	phosphatidylinositol-3-kinase
PLC	phospholipase C
PLT	paucity of lymph node T cells
PTX	pertussis toxin
RT-PCR	real time polymerase chain reaction
SLC	secondary lymphoid-tissue chemokine

CHAPTER 1

General Introduction and Thesis Outline

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and in Molecular Neurobiology (2007), 36(2):137-51

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1.1 INTRODUCTION

The primary units of the central nervous system (CNS) are the neurons, which are highly specialized in the processing and transmission of electrochemical signals from the brain to the periphery and vice versa. Neurons are post-mitotic (non-dividing) cells and are highly vulnerable to changes or disturbances in their environment. Subsequently, their proper functioning and survival depends on the support of *glia cells*, the most abundant cell population within the CNS. Three glia cell-types have been identified: oligodendrocytes, astrocytes and microglia. Each of these glia cell-types has distinct functions, ranging from metabolic and trophic support (CNS homeostasis) to neuronal signal propagation, providing a stable environment for neurons to function properly under physiological conditions (Fig. 1.1).

The CNS as an "immune-privileged" organ

Due to its limited regenerative capacity the CNS is particularly sensitive to injury. Any type of inflammatory- or immune response within the CNS is a potential threat to neuronal survival if this response is not tightly controlled. In the early 1950s, it was already appreciated that the CNS responds differentially to immune-related threats when compared to the periphery (Barker and Billingham, 1977; Billingham and Boswell, 1953; Medawar, 1948). Early transplantation experiments with allogeneous (non-self) skin grafts directly in the brain or retina revealed that an immune response in the CNS would only occur when a similar graft was simultaneously transplanted into the skin (Medawar, 1948). Normally, foreign tissue grafts are directly recognized and attacked ("rejected") by the peripheral immune system. Based on the unusual tolerance to foreign transplants, it was concluded that the CNS could only submit to, but not induce, an immune response (Medawar, 1948). For a long time, this immune-tolerant or "immunologically-privileged" state (Barker and Billingham, 1977; Billingham and Boswell, 1953) of the CNS was considered to be a passive state, contributed to an apparent lack of lymphatic vessels in the CNS and the existence of the blood-brain barrier (BBB), an anatomic barrier between neural tissue and the blood-stream which limits/prevents the entry of potentially harmful blood-derived factors (e.g. antigens, immune cells and pathogens), while still allowing exchange of metabolic factors essential for CNS homeostasis (Abbott, 2002; Becher et al., 2006; Carson et al., 2006; Galea et al., 2007). Therefore, an immune response within

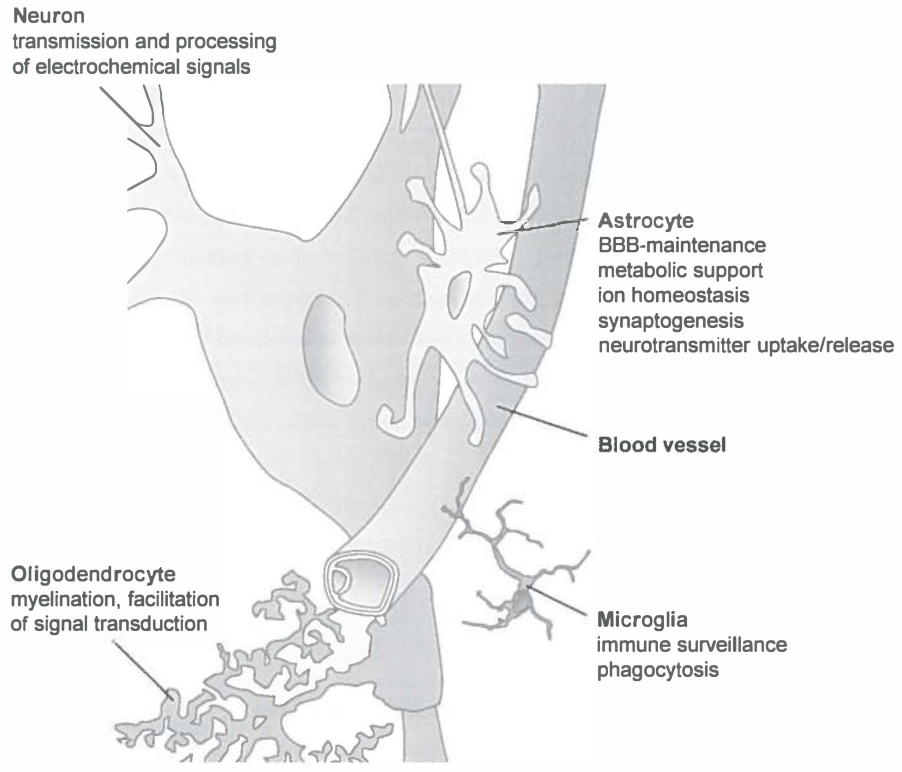


Figure 1.1 Glia cells in the healthy CNS. Oligodendrocytes support and enhance neuronal transmission by forming myelin sheaths around neuronal axons, allowing fast propagation of signals along the neuronal fibre. Microglia continuously monitor their environment for irregularities and are considered as the endogenous immune cells of the CNS. Astrocytes are essential for brain homeostasis and neuronal function as they provide metabolic- and trophic support to neurons, regulate extracellular ion- and water levels, and are actively involved in neurotransmitter uptake and release from neuronal synaptic clefts. Moreover, astrocytes provide structural support to the BBB as they make close contact with the cerebro-microvasculature and regulate tight junction formation between vascular endothelial cells.

the CNS was thought only to occur in case of BBB-disruption caused by severe inflammation. Although the concept of immune-privilege still holds true today, its definition has changed radically in recent years. First of all, it is now recognized that antigens from the CNS are drained to the cervical lymph nodes and that lymphocytes (peripheral immune cells) readily monitor and infiltrate the CNS, irrespective of an intact BBB (Cserr and Knopf, 1992; Hickey, 2001). Furthermore, the immune-privileged state of the CNS is not a passive, but an active process, in which CNS-residing cells actively

maintain an immune-suppressive environment (Galea et al., 2007). Moreover, being immune-privileged does not imply that immune responses cannot be facilitated within the CNS. On the contrary, several immune-regulatory defence mechanisms exist within the CNS parenchyma, which can initiate and regulate a direct response (innate immune response) against "foreign" disturbances, including neuronal injury and infiltration of microbial pathogens or blood-derived components (Dong and Benveniste, 2001; Farina et al., 2007; Hanisch, 2002; Streit, 2002). In addition, it is becoming clear that CNS-residing cells may also be critically involved in the regulation of adaptive (antigen-specific) immune responses by effective presentation of captured antigens and secretion of (anti-) inflammatory factors to infiltrating lymphocytes (Aloisi et al., 2000; Becher et al., 2000; Tian et al., 2009). The view of glia cells being mere homeostatic, supportive cells has changed radically in recent years and it is now generally accepted that both microglia and astrocytes (and also neurons) are prominent players in the initiation and regulation of both innate and adaptive immune responses.

MICROGLIA

Microglia: sentinels and immune-effector cells of the CNS

Microglia comprise approximately 10-20% of the total glia population and are considered the primary endogenous immune-effector cells of the CNS (Aloisi, 2001; Hanisch, 2002; Hanisch and Kettenmann, 2007; Kreutzberg, 1996; Streit, 2002; van Rossum and Hanisch, 2004). Unlike other glia cell-types, microglia are derived from the haematopoietic (monocytic) lineage and share similarities in function and phenotype with peripheral monocytes, such as macrophages and dendritic cells (Cuadros and Navascues, 2001; Soulet and Rivest, 2008). Early during development, microglia precursors infiltrate and populate the CNS, where they adapt a typical ramified morphology, characterized by a small cell body with elongated secondary- and tertiary processes (Cuadros and Navascues, 2001; Soulet and Rivest, 2008).

Little is known about the function of microglia under physiological conditions, although recent findings indicate that ramified microglia directly monitor the functional state of neurons (Davalos et al., 2005; Nimmerjahn et al., 2005; Wake et al., 2009). Indeed, it has been shown *in vivo* that ramified microglia continuously scan their environment by dynamic extension and retraction of their dendritic processes (Davalos et al., 2005; Nimmerjahn et al., 2005). Moreover, recent findings suggest that ramified microglia

directly monitor the functional state of neuronal synapses by brief and direct, one-hour interval contacts (Wake et al., 2009). In response to ischemia, the frequency and duration of these contacts were markedly increased, often followed by breakdown of the presynaptic terminal (Wake et al., 2009). These results suggest that, next to their monitoring function, microglia actively contribute to the increased turn-over of neuronal synapses in response to injury.

Microglia play a key role in the early innate immune response and are considered as the first line of defence against potential threats to normal CNS homeostasis (Kreutzberg, 1996; Streit, 2002; van Rossum and Hanisch, 2004). Accordingly, microglia express a battery of surface receptors which can detect even the smallest changes in their environment. These receptors include several types of pattern recognition receptors, such as mannose- and Toll-like receptors (which recognize bacterial, viral and fungal components) and complement receptors (which recognize various pathogens and serum components) (Aloisi, 2001). Upon any type of disturbance, ranging from impaired neuronal activity to full-blown pathological conditions, microglia become rapidly activated and change their phenotype from a "surveillance" state to a "reactive" profile. This phenotypic shift involves drastic changes in morphology and function. Upon activation, microglia retract their processes and transform into amoeboid, macrophage-like cells with phagocytic properties. Subsequently, these activated microglia migrate to the site of injury where they remove pathogens and cellular debris by phagocytosis and excrete (anti-) inflammatory and/or neurotrophic factors, all in order to limit neuronal damage and to facilitate recovery (Hanisch, 2002; Kreutzberg, 1996; Streit, 2002; van Rossum and Hanisch, 2004).

Furthermore, activated microglia may also participate in the regulation of adaptive immune responses as professional antigen-presenting cells (APCs) (Aloisi, 2001). Upon activation, microglia rapidly express major histocompatibility complex (MHC) class II receptors and various adhesion- and co-stimulatory molecules, which are required for proper antigen presentation to CD4+ T-cells (Aloisi et al., 2000; Aloisi, 2001; Becher et al., 2000). Indeed, several *in vitro* studies have shown that microglia are able to take up, process and present antigens to T-cells, leading to T-cell proliferation and/or T-cell effector functions (Aloisi et al., 2000; Becher et al., 2000). Even though the antigen-presenting capacity of microglia might be less pronounced than that of their peripheral counterparts due to the immune-tolerant environment of the CNS, the contribution of activated microglia in the re-stimulation of infiltrating T-cells in the CNS might be

significant (Aloisi et al., 2000; Aloisi, 2001; Becher et al., 2000; van Rossum and Hanisch, 2004).

Activation of microglia: “ON”- and “OFF”-signals

The course and outcome of microglia activation depends on the type of injury and signals involved. Accordingly, microglia responses are highly variable and adaptive as these cells are capable of integrating multifarious inputs and respond accordingly (Hanisch, 2002; Hanisch and Kettenmann, 2007; Schwartz et al., 2006). Two types of signal principles which control microglia activity can be distinguished here: so-called “ON”- and “OFF”-signals (Biber et al., 2007; Hanisch and Kettenmann, 2007) (Fig. 1.2). ON-signals trigger microglia activation and include factors which are normally not present in the CNS (microbial pathogens, blood-derived components, complement factors, protein aggregates), and endogenous factors which are presented in case of injury (cell debris, inflammatory mediators such as cytokines, chemokines, purines, etc.) (Aloisi, 2001; Hanisch, 2002; Hanisch and Kettenmann, 2007). Conversely, microglia activation in the healthy and injured CNS can be downregulated or suppressed through molecular interactions and/or soluble factors expressed by endogenous cells, in particular neurons and astrocytes (Biber et al., 2007; Galea et al., 2007). Disappearance of these OFF-signals, for example caused by decreased neuronal activity (= decrease in local neurotransmitter levels) or disruption of molecular interactions, results in activation of microglia. An example is the cell-cell interaction between neuron and microglia through CD200 and CD200L, respectively, which keeps microglia in the naïve brain in an immune-restive state (Hoek et al., 2000; Wright et al., 2000).Transgenic animals that lack CD200 expression display spontaneous microglia activation in the CNS and have worsened disease outcome and microglia activity in a model for multiple sclerosis (MS) as well as in other disease models (Broderick et al., 2002; Deckert et al., 2006; Hoek et al., 2000).

In addition, CD200-expression has been observed in reactive astrocytes in primary MS lesions, suggesting also a role for astrocytes in the suppression of microglia activation under pathological conditions (Koning et al., 2009). Other neuron-microglia interactions, which have been shown to suppress microglia activation either *in vivo* or *in vitro*, are CX3CL1-CX3CR1 (Cardona et al., 2006), CD47-SIRP1α (Adams et al., 1998) and CD22-CD45 (Mott et al., 2004).

Alternatively, soluble factors expressed by neurons and astrocytes can inhibit or downregulate microglia function, in order to prevent or to minimize potential bystander damage. These inhibitory mediators of microglia activation include glucocorticoids, prostaglandins and anti-inflammatory cytokines such as transforming growth factor-β (TGF-β), interleukin (IL)-4, and IL-10 (discussed in Hanisch, 2002).

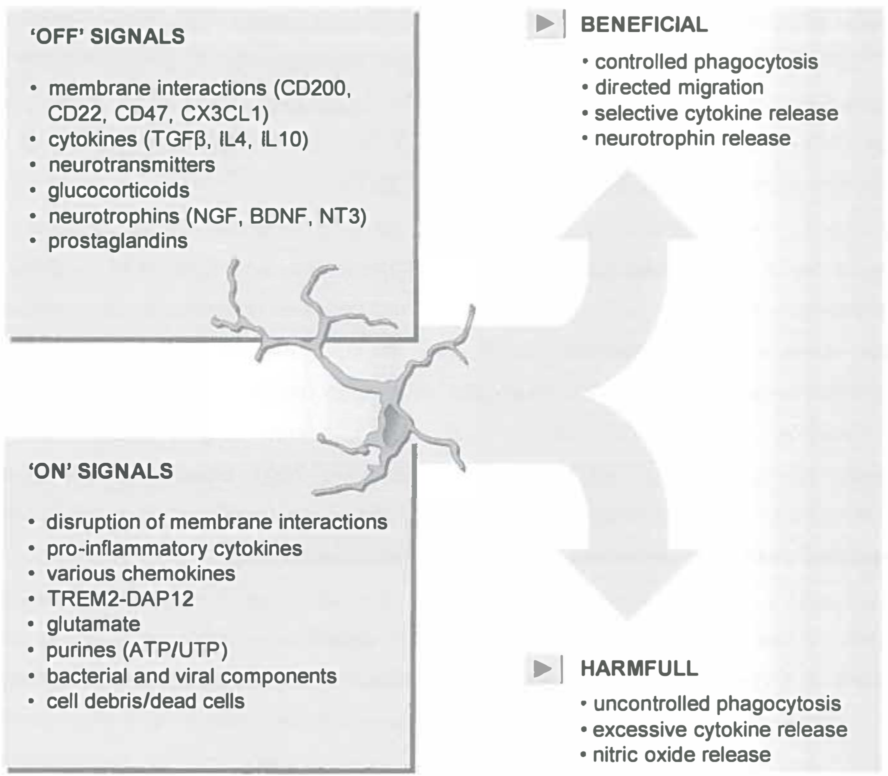


Figure 1.2 "ON" and "OFF" signals controlling microglia activity. ON-signals lead to microglia activation, while OFF-signals suppress or downregulate microglia activation. ON- and OFF-signals are defined by mode of action, not by outcome. Both signaling types can contribute to the beneficial or detrimental effects of microglia activation. Adapted from (Biber et al., 2007; Galea et al., 2007).

Neuroprotective versus neurotoxic effects of microglia activity

Taken together, microglia activation in the CNS is dictated by both ON- and OFF-signals (Fig. 1.2). Both types of signals individually or simultaneously shape microglia function. The outcome however can have neuroprotective as well as neurotoxic effects. In general, microglia activation is considered as a beneficial process, aimed at the protection of neurons under pathological conditions (Kreutzberg, 1996; Streit, 2002). Indeed, upon minor insults (e.g. small blood vessel rupture), microglia direct their dendritic processes within minutes to the site of injury and shield it in order to prevent leakage and to promote repair (Davalos et al., 2005; Nimmerjahn et al., 2005). Moreover, the neuroprotective action of microglia has been demonstrated in animal models for ischemia (Imai et al., 2007; Kitamura et al., 2004; Lalancette-Hebert et al., 2007), nitric oxide (NO)- and AMPA-induced excitotoxicity (Turrin and Rivest, 2006; Xapelli et al., 2008) and Alzheimer's disease (El Khoury et al., 2007).

Conversely, excessive/chronic activation can have detrimental effects on neuronal survival as it might lead to uncontrolled phagocytosis and excessive release of inflammatory mediators, such as TNFα, NO and reactive oxygen species (Hanisch, 2002; Hanisch and Kettenmann, 2007; van Rossum and Hanisch, 2004). Indeed, neurotoxic properties of activated microglia have been described in animal models for MS (Heppner et al., 2005; Huitinga et al., 1990), animal- and *in vitro* models for excitotoxicity (Hailer et al., 2005; Tikka and Koistinaho, 2001; Yrjanheikki et al., 1998) and Parkinson's disease (Mount et al., 2007), in which suppression of microglia function attenuated disease development and promoted neuronal survival.

In conclusion, microglia are critically involved in the pathology of various CNS disease models, in which their activity might prove either beneficial or detrimental for neuronal survival. It is therefore of importance to understand the molecular mechanism(s) behind the process of microglia activation in CNS disease development as it may provide opportunities to intervene therapeutically in order to either reduce/prevent neuronal injury or to promote neuronal survival and repair.

ASTROCYTES

Astrocytes in the healthy CNS

Astrocytes represent the most abundant glial cell population within the CNS and are essentially involved in brain homeostasis and neuronal function. In the healthy brain,

astrocytes have a variety of functions, including the regulation of the extracellular environment (extracellular ion buffering and water homeostasis), BBB-maintenance (e.g. tight junction formation and blood-flow regulation), metabolic support (production and release of glutamine after uptake of glutamate and release of glycogen and lactate) and release of trophic factors controlling neuronal maturation and synaptogenesis (Abbott, 2002; Seth and Koul, 2008; Wang and Bordey, 2008).

An exciting feature of astrocytes that has gained a lot of attention in recent years is their suggested involvement in the regulation and modulation of neurotransmission (Fig 1.3A) (Fellin, 2009; Perea and Araque, 2005; Wang and Bordey, 2008). Astrocytes are closely associated with neuronal synapses and express a wide range of neurotransmitter receptors (including receptors for glutamate, GABA, ATP, acetylcholine etc.) (Porter and McCarthy, 1997). Several lines of evidence suggest a bidirectional communication between neurons and astrocytes as astrocytes respond to presynaptic release of several neurotransmitters with intracellular calcium (Ca^{2+}) transients and subsequent Ca^{2+}-dependent release of gliotransmitters (such as glutamate, adenosine-5'-triphosphate (ATP), D-serine and TNFα) back to the neuron, thereby directly modulating neuronal excitability and/or synaptic transmission (Agulhon et al., 2008; Fellin, 2009; Perea and Araque, 2005; Perea and Araque, 2007; Volterra and Meldolesi, 2005). Moreover, spontaneous intracellular Ca^{2+}-oscillations and subsequent glutamate release from astrocytes in the absence of neuronal activity has been observed, suggesting that astrocytes might also generate and modulate neuronal activity autonomously (Parri et al., 2001).

In addition, astrocytic Ca^{2+} transients may be propagated to neighbouring astrocytes (so-called intercellular "Ca^{2+} waves") through gap junctions and/or via purinergic signaling, allowing long-distance astrocyte-astrocyte and astrocyte-neuron communication (Fig. 1.3B). Although this type of Ca^{2+}-signaling between astrocytes has been shown *in vitro* in primary brain cell cultures and in organotypic slices in response to receptor stimulation or mechanical/electrical stimulation (Scemes and Giaume, 2006), it remains to be elucidated whether Ca^{2+} waves are evoked under physiological conditions. Considering the fact that the induction of astrocytic Ca^{2+} waves requires powerful stimuli, this type of signaling may relate to neuropathological conditions. Indeed, intercellular Ca^{2+} waves in astrocytes have been associated with a process known as cortical spreading depression, which is characterized by a slowly moving wave of tissue depolarization evoked by brain trauma or ischemia (Nedergaard and Hansen, 1993; Peters et al., 2003).

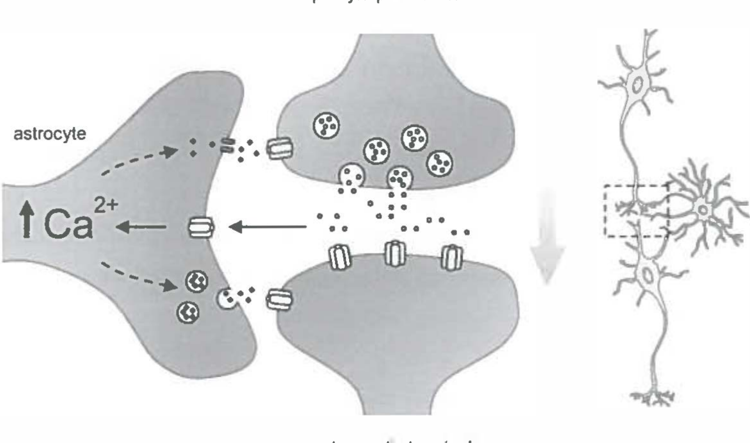
Astrocytes in CNS pathology

The functions of astrocytes in neuropathology may be as versatile as those reported for microglia. Upon neuronal injury, local astrocytes respond rapidly with a series of structural and functional changes (Pekny and Nilsson, 2005; Ridet et al., 1997). This response is accompanied by increased expression of structural proteins such as glial fibrillary acidic protein (GFAP) and results in astrocyte hypertrophy and proliferation, a process termed "reactive astrogliosis". In general, reactive astrogliosis is a beneficial process aimed at the isolation of the lesion site from the rest of the CNS, and simultaneously facilitates the induction of BBB-repair and remodelling of neuronal circuits surrounding the lesion (Pekny and Nilsson, 2005; Ridet et al., 1997). Conversely, reactive astrogliosis may also be a harmful process. In the near vicinity of lesion sites, astrocyte hypertrophy and proliferation may lead to glial scar formation (anisomorphic gliosis), thereby impeding axonal regeneration and neurite outgrowth (Chen and Swanson, 2003; Fawcett and Asher, 1999).

At the same time, reactive astrocytes are a potent source for both anti- and pro-inflammatory factors, which are critically involved in the orchestration of both innate- and adaptive inflammatory responses within the CNS (Dong and Benveniste, 2001; Farina et al., 2007). An important factor in the initial step of local innate immune responses is the release of ATP by reactive astrocytes, which activates and recruits microglia to sites of local injury (Davalos et al., 2005; Nimmerjahn et al., 2005). Moreover, microglial migration has been shown to be dependent on the activation of P2Y12 receptors, while phagocytosis is induced through P2Y6 receptor activation, suggesting an important role for purinergic signaling in the initial response to neuronal injury (Koizumi et al., 2007; Sasaki et al., 2003).

Other mediators expressed by reactive astrocytes include various cytokines, chemokines and neurotrophic factors (Dong and Benveniste, 2001; Farina et al., 2007). Release of neurotrophic factors may promote neuronal survival and repair after injury, while release of cytokines and chemokines may trigger two types of events (Farina et al., 2007): 1) activation of neighbouring cells (astrocytes and microglia), thereby amplifying the initial innate immune response and 2) modification of BBB permeability and recruitment of leukocytes from the periphery, thereby supporting an adaptive immune response. Release of cytokines and chemokines may influence both inflammatory and immunosuppressive processes. A balance between these pathways proves critical for

A neuron-astrocyte (tripartite synapse) communication



B astrocyte-astrocyte communication

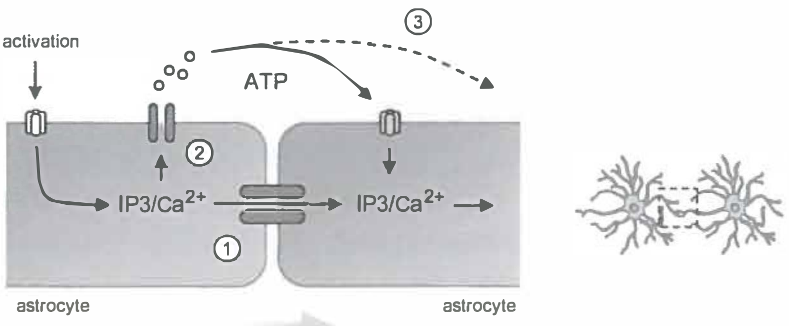


Figure 1.3 Bidirectional communication between neurons and astrocytes (A) and astrocyte-to-astrocyte calcium wave propagation (B). A: astrocytes are closely associated with neuronal synapses (termed "tripartite synapse"). Neurotransmitters released from the presynaptic terminal act on postsynaptic receptors and on astrocytic receptors following "spill-over" from the synaptic cleft. Neurotransmitter-induced calcium transients in astrocytes may trigger the release of gliotransmitters via vesicles and/or via calcium-dependent chloride channels back to the neurons. This may lead to (extrasynaptic) activation of pre- and/or postsynaptic receptors leading to changes in synaptic transmission. **B**: long-range communication through astrocytes may occur through calcium wave propagation. Two mechanisms have been proposed. Calcium waves may be propagated by diffusion of inositol triphosphate (IP3) and calcium (Ca^{2+}) through intercellular gap junctions (1) or through calcium-dependent release of ATP, which subsequently induces intracellular calcium mobilization in neighboring astrocytes through activation of purinergic receptors (2,3). Both mechanisms may occur simultaneously. Adapted from references (Agulhon et al., 2008; Fellin, 2009; Perea and Araque, 2005; Wang and Bordey, 2008).

controlled immune responses in the CNS. Disturbances in this balance may have severe consequences and could lead to the development of chronic neuroinflammatory disease. In contrast to the antigen presenting capacity of microglia, the ability of astrocytes to function as professional APCs in the CNS has been a matter of debate and studies addressing the functional expression of MHC class II and co-stimulatory molecules in astrocytes both *in vivo* as *in vitro* are highly contradictory (discussed in Dong and Benveniste, 2001). However, expression of certain chemokines and inflammatory cytokines by astrocytes may influence T-cell responses within the CNS, suggesting that astrocytes are at least partly involved in the regulation of adaptive immune responses within the CNS.

Taken together, glia cells are intimately involved in CNS homeostasis and participate in various processes associated with CNS development, signal transduction and immune surveillance. Moreover, both astrocytes and microglia have been implicated in the initiation and regulation of neuroinflammatory responses within the CNS (Fig. 1.4). Tight regulation of these immune responses are a prerequisite for neuronal survival under these conditions and therefore requires intimate interactions between neurons, glia cells and (in some cases) peripheral immune cells through cell-cell contact and expression of soluble factors, such as cytokines and chemokines. However, glia reactions in response to CNS injury are not inevitably neurosupportive as excessive or chronic activation of these cells may lead to neurotoxic responses. Identification of the mechanisms underlying supportive and detrimental glia reactions might give important insight in the role of glia cells in acute CNS injury and in the development of chronic CNS diseases.

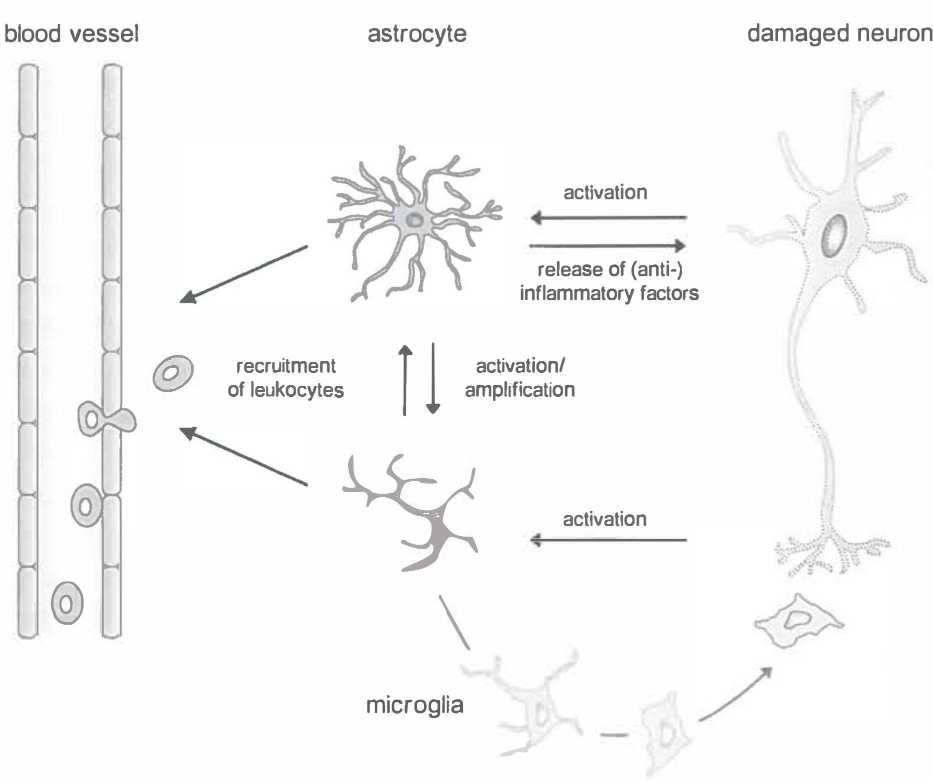


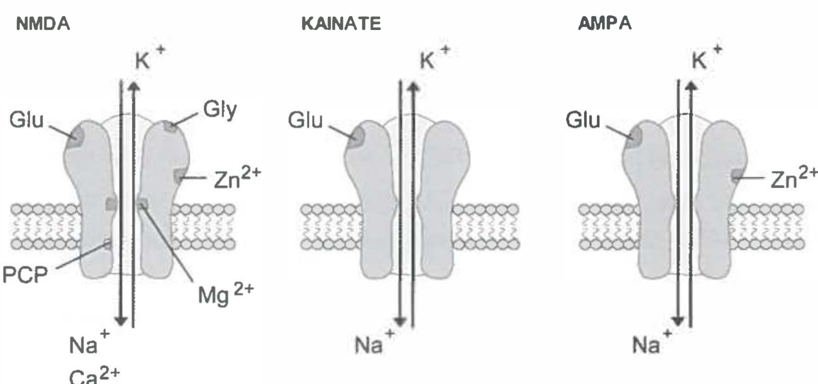
Figure 1.4 Simplified scheme of neuron-glia and glia-glia interactions in response to neuronal damage. Upon neuronal injury, release of neuronal factors and/or disruption of neuron-glia interactions activate both astrocytes and microglia. Microglia respond by retraction of their dendritic processes and migrate to the lesion site, where they remove cellular debris by phagocytosis and release neurotrophic and (anti-) inflammatory factors (cytokines, chemokines, etc.). The net effect of microglia activation can be beneficial or detrimental for neuronal survival, depending on the insult. Activation of local astrocytes may lead to reactive astrogliosis and concomitant expression of neurotrophic and (anti-) inflammatory factors. Expression of glial factors might further activate (cytokines, purines) and/or recruit (chemokines, purines) neighboring glia cells, thereby amplifying the initial innate immune response. Finally, glial- and/or neuron-derived cytokines and chemokines may be presented on the luminal surface of cerebro-vascular endothelial cells in order to activate and recruit leukocytes from the periphery.

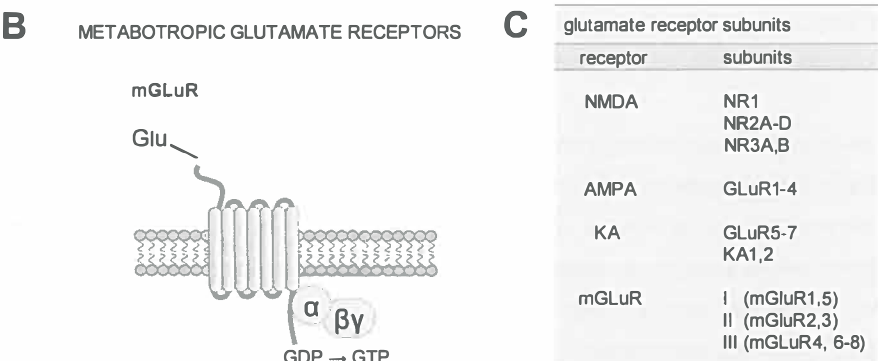
1.2 EXCITOTOXICITY: GLUTAMATE-INDUCED NEURODEGENERATION

Any type of CNS injury inevitably results in the loss of neurons. Although primary and secondary signaling pathways leading to neurodegeneration are difficult to discern, there is strong evidence that excessive glutamate-signaling in the CNS is a major cause for the progressive loss of neurons in many CNS pathologies. Indeed, in the early 1950s it was already recognized that direct exposure to high levels of the neurotransmitter glutamate in the CNS induced seizures in animals (Hayashi, 1952) and resulted in the degeneration of specific brain regions when injected systemically (Lucas and Newhouse, 1957; Olney and Sharpe, 1969). In the following years, these studies were extended by showing that excessive extracellular levels of glutamate were directly and specifically toxic to neurons, an effect that could be mimicked by application of glutamate receptor agonists or counteracted by concomitant application of specific glutamate receptor antagonists, which led to the concept of "*excitotoxicity*", or neurotransmitter-induced neurotoxicity (Choi, 1988; Olney, 1994). Nowadays, it is generally appreciated that excitotoxicity is the primary mechanism leading to neuronal cell death in many acute CNS pathologies including traumatic injury, ischemia and epilepsy (Arundine and Tymianski, 2004; Choi and Rothman, 1990; Vincent and Mulle, 2009). There is also mounting evidence that the process of excitotoxicity-induced neurodegeneration plays a role in more chronic neurological disorders such as Huntington's disease (Fan and Raymond, 2007), MS (Gonsette, 2008), Alzheimer's disease (Walton and Dodd, 2007), Parkinson's disease (Koutsilieri and Riederer, 2007) and amyotrophic lateral sclerosis (ALS) (Van Damme et al., 2005). Although in the last decades research on excitotoxicity has primarily focused on the intracellular pathways leading to acute glutamate-induced neuronal cell death, there is now strong evidence that the activation of glia cells may be a critical factor in the propagation and amplification of this neurodegenerative process.

Glutamate and its receptors in the CNS

Glutamate is the primary excitatory neurotransmitter for neurons in the CNS. Upon release, glutamate induces excitatory responses in postsynaptic neurons via pharmacologically and functionally distinct members of the ionotropic- and metabotropic glutamate receptor families (Fig. 1.5). Ionotropic receptors are ligand-gated ion channels and subsequent activation results in the opening of their channel pores leading to permeability for sodium (Na^+), potassium (K^+) or Ca^{2+}-ions. Three types of ionotropic





glutamate receptor subunits

receptor	subunits
NMDA	NR1 NR2A-D NR3A,B
AMPA	GLuR1-4
KA	GLuR5-7 KA1,2
mGLuR	I (mGluR1,5) II (mGluR2,3) III (mGLuR4, 6-8)

Figure 1.5 Ionotropic- and metabotropic glutamate receptors. Members of the ionotropic glutamate receptor class (**A**) are divided into the NMDA, kainate (KA) and AMPA receptors, which are ligand-gated ion channels. AMPA/KA-receptors have fast kinetics and are highly permeable to extracellular Na^{+}. Activation of AMPA receptors can be potentiated by Zn^{2+}. NMDA receptors are slow gating channels that are highly permeable to extracellular Ca^{2+} and to a lesser extent Na^{+}. NMDA receptors are unique in that they require binding of both glutamate (Glu) and glycine (Gly) for efficient channel opening as well as membrane depolarization. Membrane depolarization (facilitated by Na^{+} influx through AMPA/KA receptors) releases the Mg^{2+} block of the NMDA receptor, allowing glutamate to bind. NMDA receptor activation can be blocked by Zn^{2+} or by drugs that bind to the phencyclidine (PCP) site within the open channel. Metabotropic glutamate receptors (**B**) are seven trans-membrane spanning receptors that couple to heterotrimeric GTP-binding proteins (G-proteins) to induce their action. Activation of the G-protein results in GDP/GTP-mediated dissociation of the complex into an α- and a βγ-subunit, each initiating distinct signaling pathways within the cell. Both ionotropic- and metabotropic receptors can be subdivided into distinct subtypes based on their subunit composition (**C**, table). Adapted from references (Aarts and Tymianski, 2004; Choi, 1988).

receptors have been described, named after their respective affinity for specific agonists: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and kainate (KA) receptors (Fig 1.5A). Each of these ionotropic receptor types can be further divided into differently composed tetramers of glutamate receptor channel subunits which form together the channel-pore (Fig. 1.5C). AMPA and KA receptors have fast kinetics and are permeable for Na^+ and K^+, but are normally impermeable to Ca^{2+} (Hollmann et al., 1991). However, Ca^{2+}-permeable AMPA receptors that specifically lack the GLUR2 subunit have also been described and their expression has been associated with certain CNS disorders (Liu and Zukin, 2007). In contrast, NMDA receptors are slow gating and opening of the channel pore relies on a voltage-dependent removal of magnesium (Mg^{2+}) (Fig 1.5A). Upon activation, NMDA receptors are highly permeable to Ca^{2+} and K^+, and to a lesser extent Na^+ (Hardingham and Bading, 2003). Metabotropic glutamate receptors consist of eight distinct subtypes (mGLUR1-8) and mediate their actions through G-protein dependent signaling leading to Ca^{2+} mobilization from intracellular stores and activation of other downstream signaling pathways (Fig. 1.5B).

Under physiological conditions, glutamate-induced activation of postsynaptic glutamate receptors is tightly regulated both spatially and temporally. Upon release in the synaptic cleft, extracellular glutamate is rapidly sequestered through plasma membrane transporters on the presynaptic terminal and stored within intracellular compartments. In addition, astrocytes actively take up extracellular glutamate via the membrane-associated glutamate transporters GLAST and GLT1 (EAAT1 and EAAT2 in humans, respectively). Once taken up, glutamate is converted into glutamine and is shuttled back to presynaptic terminals where it is used again for glutamate synthesis. In this way, astrocytes are responsible for over 80% of the glutamate uptake in the brain (Yi and Hazell, 2006).

Excessive accumulation of extracellular glutamate causes neurotoxicity

As mentioned before, glutamate has potent neurotoxic activity when extracellular glutamate levels exceed the physiological threshold in the CNS. Excessive accumulation of extracellular glutamate can have several causes, but is most prominent in case of acute CNS injury, in particular traumatic injury and ischemia (Arundine and Tymianski, 2004). Here, sudden deprivation of local energy supplies (oxygen and glucose) causes a rapid loss of intracellular ATP and subsequent depolarization of neurons, resulting in

reduced glutamate re-uptake and excessive synaptic release of glutamate (Nicholls and Attwell, 1990). Moreover, the sudden loss of energy supplies may lead to depolarization of local astrocytes in the centre of the lesion resulting in "glutamate transporter reversal", which is characterized by functional impairment of ATP-dependent astrocytic glutamate transporters, ultimately leading to reduced glutamate uptake and concomitant increase in glutamate release (Yi and Hazell, 2006).

Signaling pathways involved in excitotoxicity

The neurotoxic effect of chronically elevated glutamate levels arises from the strong activation of (in particular) postsynaptic ionotropic glutamate receptors (AMPA-, KA- and NMDA-R), leading to a sudden increase in influx of Na^+ and Ca^{2+} ions (Aarts and Tymianski, 2004; Choi, 1987). This drastic increase of intracellular Na^+ and Ca^{2+} initiates two distinct processes, which differ in time- and receptor-dependency. The first component is an acute swelling of neuronal cell bodies that depends on the persistent activation of AMPA/KA receptors, and to a lesser extent NMDA-receptors. Prolonged opening of these ion channels leads to continuous Na^+ influx and consequent depolarization of the neuronal membrane, leading to passive influx of chloride (Cl^-) and water (Choi, 1987; Olney et al., 1986; Rothman, 1985). This neuronal swelling occurs within minutes after glutamate exposure and can be blocked *in vitro* by removal of extracellular Na^+ and Cl^- (Olney et al., 1986; Rothman, 1985).

The second component is a delayed neurodegeneration, predominantly triggered by a drastic increase in intracellular Ca^{2+} (Fig. 1.6). This excessive increase in cytosolic free Ca^{2+} is predominantly caused by direct influx of Ca^{2+} through activation of NMDA receptors (after removal of their Mg^{2+} block as a result of membrane depolarization), but also through secondary opening of voltage-gated Ca^{2+} channels and reverse operation of Na^+/Ca^{2+} exchangers (NCX) on the membrane (Arundine and Tymianski, 2003; Choi, 1994). It is now believed that this excessive influx of Ca^{2+}, together with any Ca^{2+} release triggered from intracellular stores, exceeds the capacity of Ca^{2+}-sequestering mechanisms, leading to over-activation of ATP-ases and potentially harmful proteins that either directly damage the cell or induce formation of free oxygen radicals that induce neuronal apoptosis (Fig. 1.6) (Arundine and Tymianski, 2003; Choi, 1994). Proteins activated downstream of Ca^{2+} that are potentially harmful for the cell when over-activated include endonucleases (DNA degradation), phospolipases (breakdown of the cell membrane and subsequent formation of arachidonic acid), calmodulin (free radical

formation), proteases such as calpain (cytoskeletal breakdown), and protein kinases (alterations in membrane associated Ca^{2+} channels leading to enhanced Ca^{2+} influx) (Arundine and Tymianski, 2003; Choi, 1994). Finally, cell lysis may result in release of intracellular stored glutamate thus creating a vicious circle.

Several lines of evidence suggest that the persistent activation of NMDA receptors and subsequent intracellular Ca^{2+} overload is the crucial step in glutamate-induced neuronal cell death. In line with this, it has been demonstrated *in vitro* that neuronal cell death induced by excessive glutamate stimulation or oxygen/glucose deprivation is effectively prevented by application of selective NMDA receptor inhibitors, while selective inhibition of AMPA/KA receptors has only minor effects (Choi et al., 1988; Koh and Choi, 1991). Moreover, treatment with selective NMDA receptor antagonists has proven beneficial for neuronal survival in animal models for ischemia and hypoglycemia (Simon et al., 1984; Wieloch, 1985), emphasizing the role of NMDA receptors in excitotoxic-related pathologies. In spite of the beneficial effects of NMDA receptor antagonists in cellular and animal models, these drugs are unfortunately of limited clinical use for several reasons. One reason is the very limited time frame for therapeutic intervention as activation of NMDA receptors may occur to early for effective treatment. Secondly, human clinical trials with various antagonists that have been performed so far were unsuccessful due to significant side effects of the treatments, including psychosis, memory impairment, respiratory problems and even neuronal cell death (Lee et al., 1999; Waxman and Lynch, 2005).
Although the initial process of excitotoxicity-induced neuronal damage at the primary lesion site might prove to be difficult if not impossible to counteract, there is a reasonable time window for potential therapeutic intervention in the first twenty four hours after the primary insult for the following reason. In many acute CNS injuries, and in particular in traumatic brain injury and ischemia, primary neuronal degeneration at the lesion site is often followed by an anterograde/retrograde degeneration of neurons in secondary regions (Horn and Schlote, 1992; Kirino and Sano, 1984). The fact that this type of secondary neuronal damage is relatively resistant to delayed treatment with (non)-selective NMDA antagonists, as demonstrated in an animal model for traumatic brain injury (Bernert and Turski, 1996), suggests that the amplification or propagation of secondary neuronal damage depends on secondary signaling mechanisms other than NMDA.

It is therefore of interest to identify these secondary signaling systems in order to develop therapeutic agents with no or tolerable side effects that have protective potential when administered several hours after the primary insult.

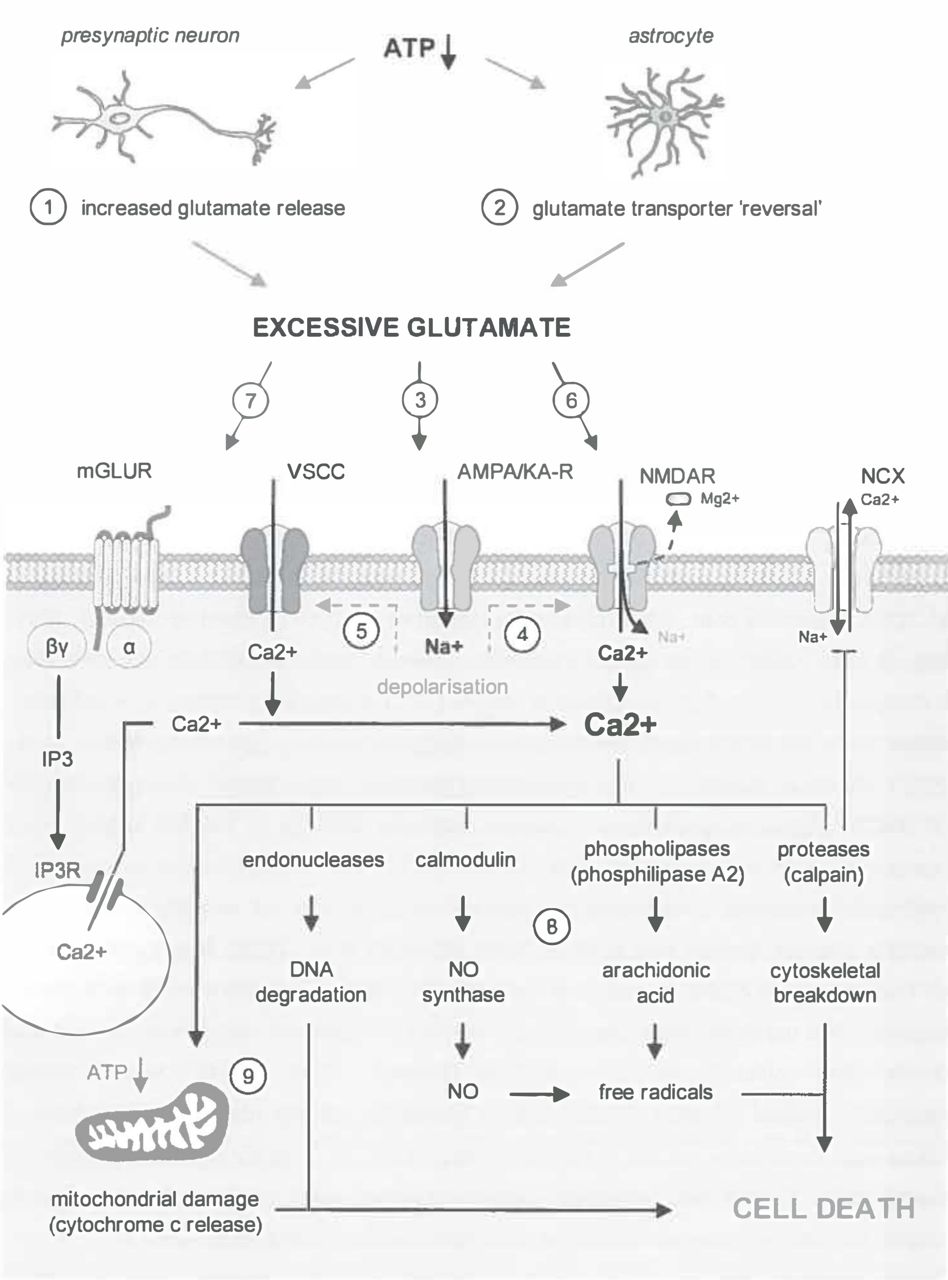
Selective neuronal vulnerability in excitotoxicity: involvement of glia cells?

As mentioned above, an additional feature of excitotoxic-related injuries in the CNS, such as ischemia and hypoglycemia, involves the temporal profile of delayed neuronal damage in selective CNS regions such as the hippocampus, neocortex, striatum and thalamus (Horn and Schlote, 1992; Kirino and Sano, 1984; Rosenblum, 1997). Interestingly, similar patterns of neuronal damage are observed *in vivo* after intracerebral injection with glutamate agonists (Acarin et al., 1996; Schauwecker, 2002; Won et al., 1999). Moreover, within these brain regions a selective order of vulnerability between distinct neuronal subpopulations has been observed, in particular in the neocortex and hippocampus. In the hippocampus, pyramidal neurons of the cornu ammonis (CA) CA1 region are most vulnerable to an ischemic insult followed by neurons from the CA3 region, whereas neurons in the dentate gyrus (DG) region are largely resistant (Schmidt-Kastner and Freund, 1991). In the neocortex, prolonged ischemia results in the spread of neuronal loss specifically in the cortical layers III, V and VI (Pulsinelli et al., 1982). These profiles of selective neuronal vulnerability can be reproduced *in vitro* in organotypic hippocampal and neocortical slice cultures in response to oxygen/glucose deprivation or glutamate (agonist)-induced excitotoxicity (Bernaudin et al., 1998; Kristensen et al., 2001; Tasker et al., 1992; Vornov et al., 1991). Differences in selective vulnerability may occur depending on the type of glutamate agonist used (Kristensen et al., 2001; Vornov et al., 1991). However, reasons for the selective vulnerability of neurons in these regions are not well understood. In line with this, it has been proposed that heterogeneity in NMDA receptor combination and receptor density may correlate with differences in patterns of excitotoxicity (Coultrap et al., 2005; Lynch and Guttmann, 2002; Martens et al., 1998). As the NMDA receptor density is most abundant in the hippocampal CA1 region, this might explain the relative vulnerability of these neurons towards an excitotoxic insult. However, this suggestion was placed into question as neurons in the DG region, which are largely resistant to excitotoxicity, are also rich in NMDA receptor expression. It is therefore likely that additional signaling mechanisms contribute to the selective death of specific neuronal subpopulations. Although differences in endogenous neuronal properties are not excluded, recent evidence suggests that non-neuronal cells,

in particular glia cells, are critically involved in the process of secondary neuronal degeneration.

Like any other type of CNS injury, excitotoxicity-induced neurodegeneration coincides with local reactive astrogliosis and rapid activation and recruitment of microglia, as demonstrated *in vivo* (Acarin et al., 1996; Jorgensen et al., 1993; Marty et al., 1991) and *in vitro* in organotypic slice cultures (Bernaudin et al., 1998). As discussed before, activation of microglia significantly contributes to the development and progress of CNS injury. Microglia activation can be either beneficial or detrimental for neuronal survival, depending on the type of signals and injury involved (Hanisch and Kettenmann, 2007; van Rossum and Hanisch, 2004). Previous research concerning the role of microglia in excitotoxicity-related pathologies has suggested that microglia activation in this type of injury might be detrimental for neuronal survival (Dehghani et al., 2003; Dehghani et al., 2004; Hailer et al., 2005; Tikka et al., 2001; Tikka and Koistinaho, 2001; Yrjanheikki et al., 1998). Upon excitotoxic injury, activated microglia release pro-inflammatory factors such as TNFα, IL-1, NO, inducible nitric oxide synthase (iNOS) and cycloxygenase-2 (COX2) that are all damaging to neurons when expressed at large quantities (Aloisi, 2001; Barone and Feuerstein, 1999; Campuzano et al., 2008).

Figure 1.6 (right) Signaling pathways leading to neuronal cell death in excitotoxicity: involvement of calcium (Ca^{2+}). Increased energy metabolism (ATP ↓) as a result of loss of oxygen supply or loss of ion-homeostasis may lead to dysregulation of the presynaptic vesicle release machinery resulting in excessive release (and reduced uptake) of glutamate from presynaptic terminals (*1*). This build-up of toxic extracellular glutamate levels may be enhanced by neighbouring astrocytes as ATP-deprivation leads to impaired function/downregulation of membrane-associated glutamate transporters GLAST and GLT-1 (*2*), resulting in a decrease in glutamate uptake and an increase in glutamate release (glutamate transporter 'reversal'). High levels of extracellular glutamate in the synaptic cleft persistently activate AMPA- and KA receptors (*3*) located at the postsynaps, leading to membrane depolarization through strong influx of Na^+ (*4*). AMPA/KA receptor-mediated membrane depolarization opens voltage-sensitive calcium channels (VSCC, *5*) and removes the Mg^{2+} block from NMDA-receptors (*6*), allowing glutamate-induced activation of the NMDA-receptor resulting in excessive influx of calcium. Additionally, calcium release from intracellular stores through activation of metabotropic glutamate receptors (mGLURs) might contribute to the excessive increase in intracellular calcium (*7*). Excessive increases of intracellular calcium may over-activate potentially harmful proteins such as endonucleases (DNA degradation), proteases (cytoskeletal/NCX breakdown), phospholipases and calmodulin (free radical formation), protein kinases and others (*8*). Additionally, excessive calcium leads to mitochondrial calcium overload (*9*), resulting in impaired ATP production and release of cytochrome *c*, which is involved in the activation of caspase-mediated pathways leading to programmed cell death (apoptosis). NCX: Na^+/Ca^{2+} exchanger. Adapted from references (Anderson and Swanson, 2000; Arundine and Tymianski, 2003; Baskys and Blaabjerg, 2005; Choi, 1988; Choi, 1994; Estrada Sanchez et al., 2008; Sheldon and Robinson, 2007; Yi and Hazell, 2006).



postsynaptic neuron

In line with these findings, it was demonstrated that treatment with an anti-inflammatory compound (minocycline) reduced infarct size following focal ischemia, a process which coincided with reduced proliferation and production of inflammatory mediators in microglia (Yrjanheikki et al., 1998; Yrjanheikki et al., 1999). Moreover, treatment with minocycline inhibited activation and proliferation of microglia *in vitro* and protected mixed (neurons + glia) spinal cord cultures against NMDA-induced neurotoxicity (Tikka et al., 2001; Tikka and Koistinaho, 2001). However, treatment with minocycline has also been shown to directly protect primary neuronal cultures from glutamate-toxicity, suggesting that the therapeutic range of this drug exceeds that of microglia inhibition alone (Yrjanheikki et al., 1999). Potential neurotoxic effects of microglia activation have also been described in organotypic hippocampal slice cultures exposed to NMDA, while treatment with various drugs that (in)directly inhibit microglia function attenuated neuronal cell death in this paradigm (Dehghani et al., 2003; Dehghani et al., 2004; Hailer et al., 2005; Hailer, 2008).

Conversely, there are several lines of evidence indicating that microglia activation correlates with the reduction of neuronal damage under excitotoxic conditions (Imai et al., 2007; Kitamura et al., 2004; Lalancette-Hebert et al., 2007; Simard and Rivest, 2007; Xapelli et al., 2008). In an animal model for ischemia, selective ablation of proliferating microglia using a transgenic approach resulted in a significant increase in infarct size, which coincided with a significant increase in apoptotic neurons (Lalancette-Hebert et al., 2007). Similarly, depletion of microglia from organotypic hippocampal slice cultures prior to NMDA exposure resulted in increased neuronal damage in the DG region when compared to control conditions (Kohl et al., 2003). The neuroprotective properties of microglia in ischemia *in vivo* have also been demonstrated in rats and Mongolian gerbils using a different approach (Imai et al., 2007; Kitamura et al., 2004). Ventricular injection of exogenous microglia in rats and subsequent migration of these cells to ischemic lesions in the cerebral cortex and striatum resulted in significant neuroprotection in these areas when compared to control animals (Kitamura et al., 2004). Using a similar approach, arterial injection of microglia in Mongolian gerbils resulted in migration of these cells to ischemic lesions in the hippocampus and an increase in local expression of neurotrophic factors was observed, which coincided with an increase in surviving neurons in this region when compared to control conditions (Imai et al., 2007).

Taken together, the role of microglia in excitotoxic-related injuries remains elusive. However, results obtained so far suggest that microglia activation per se is beneficial in

excitotoxicity as full ablation of these cells results in increased neuronal loss. Conversely, the fact that immunosuppressive agents reduce excitotoxicity-induced neurodegeneration by (in)direct inhibition of microglia function suggests that microglia acquire an inflammatory profile that is detrimental for neuronal survival under these conditions. Identification of the signals leading to this apparent inflammatory microglia profile under excitotoxic conditions might prove beneficial for the development of therapeutic strategies aimed to suppress or redirect microglia function in order to reduce neuronal loss and promote neuronal regeneration.

1.3 CHEMOKINES: VERSATILE MESSENGERS IN THE CNS

Chemokines are small chemotactic cytokines of approximately 8-12 kDa and are predominantly known for their ability to facilitate peripheral immune cell trafficking (Baggiolini, 1998; Moser and Loetscher, 2001; Rot and von Andrian, 2004). Numerous studies have demonstrated that chemokines and their receptors are also expressed within the CNS, where they play a crucial role in a variety of functions both under physiological and pathological conditions. Not only are chemokines involved in CNS development and homeostasis, neuronal transmission and nociception, but they also seem to function as key regulators in the communication between neurons and glia cells in response to CNS injury and disease-associated neuroinflammation (Bertollini et al., 2006; Charo and Ransohoff, 2006; Limatola et al., 2000; Ma et al., 1998; Oh et al., 2001; Rostene et al., 2007; Ubogu et al., 2006). Understanding the mechanism of chemokine regulation in the injured CNS may provide essential insight in the initiation and/or development of several neuropathologies.

Chemokine nomenclature and classification

The first molecule with chemotactic properties (IL-8 or CXCL8) was identified in 1987 (Yoshimura et al., 1987) and since then the chemokine family has expanded rapidly (Rossi and Zlotnik, 2000). The family now consists of nearly 50 chemokines (Laing and Secombes, 2004) and almost 20 chemokine receptors (Murphy, 2002), but more are likely to be discovered (Fig. 1.7).

The family of chemokines has been classified into four subfamilies (CC, CXC, CX_3C and C), based on the number and spacing of their highly conserved cysteine residues in the amino (NH_2)-terminus. The two major chemokine subfamilies are the CXC- (or α) and

CC- (or β) chemokines, where the two cysteines are either separated by one amino acid or are adjacent to each other, respectively (Fernandez and Lolis, 2002; Rossi and Zlotnik, 2000). The CXC-chemokines are further divided into two subclasses based on a distinct Glu-Leu-Arg (ELR)-motif that is located between the N-terminus and the first cysteine (Rollins, 1997). The two smaller chemokine subfamilies are the C- (or γ) chemokines, which contain only one cysteine in the N-terminal region, and the CX_3C- (or δ) subfamily, which has only one member, namely CX_3CL1, in which the first two cysteines are separated by three other amino acid residues (CX_3C or δ chemokines) (Rossi and Zlotnik, 2000).

Chemokine receptors and chemokine promiscuity

Chemokines exert their effects through surface receptors that belong to the superfamily of seven-transmembrane spanning G-protein coupled receptors (GPCRs). The nomenclature of the chemokine receptors follows that of the chemokine subfamily they preferentially bind to, namely CCR, CXCR, XCR and CX_3CR (Murphy, 2002). Accordingly, CC-chemokines bind to CC-receptors, etcetera. However, certain promiscuity exists between chemokines and their receptors as multiple receptors can recognize more than one ligand and, vice versa, various ligands can bind to more than one receptor (Bajetto et al., 2002). A few specific chemokine-chemokine receptor pairs like CCR6-CCL20, CCR9-CCL25, CXCR4-CXCL12, CXCR5-CXCL13, CXCR6-CXCL16 and CX_3CR1-CX_3CL1 appear to be monogamous (Bajetto et al., 2002). Although most chemokines recognize more than one receptor it seems to be a general rule that CC-receptors only bind CC-chemokines, whereas CXC-chemokines are recognized by CXC-receptors. Accordingly, there is no cross reactivity between members of the four chemokine subclasses (Murphy et al., 2000). One exception to this rule is chemokine CCL21, which not only binds CCR7, but also has been shown to bind a member of the CXCR-family, namely CXCR3 (see below) (Biber et al., 2001; Dijkstra et al., 2004; Rappert et al., 2002; Soto et al., 1998).

Chemokine receptor signaling

GPCRs mediate their action through activation of scaffolding proteins (e.g. β-arrestin 1 and 2) and through activation of various membrane-associated heterotrimeric G-proteins ($G_{i/0}$, G_s, G_q and others), which consist of a $G_α$- and $G_{βγ}$-subunit complex (Defea, 2008;

CC family (β)

Receptor	Agonist
CCR1	CCL3
	CCL5
	CCL7
	CCL9
	CCL14
	CCL15
	CCL16
	CCL23
CCR2	CCL2
	CCL7
	CCL12-13
	CCL16
CCR3	CCL5
	CCL7-8
	CCL11
	CCL13
	CCL15
	CCL24
	CCL26
	CCL28
CCR4	CCL17
	CCL22
CCR5	CCL3-5
	CCL8
	CCL14
CCR6	CCL20
CCR7	CCL19
	CCL21
CCR8	CCL1
	CCL4
	CCL17
CCR9	CCL25
CCR10	CCL27
	CCL28

CXC family (α)

Receptor	Agonist
CXCR1	CXCL1
	CXCL6
	CXCL8
CXCR2	CXCL1-3
	CXCL5-8
CXCR3	CXCL9-11
	CCL21
CXCR4	CXCL12
CXCR5	CXCL13
CXCR6	CXCL16

C family (δ)

Receptor	Agonist
XCR1	XCL1
	XCL2

CX3C family (γ)

Receptor	Agonist
CX3CR1	CX3CL1

Figure 1.7 Classification of chemokine receptors and their agonists, based on the structural characteristics of their relative chemokine ligands (images). C: cysteine, X: an amino acid other than cysteine. Current list is adapted from the NC-IUPHAR (International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification), www.iuphar-db.org/index.jsp, updated 2008-12-03. Images were adapted from (Bajetto et al., 2002; Rostene et al., 2007).

Neves et al., 2002). Activation of G-proteins results in GDP/GTP-mediated dissociation of the heterotrimeric complex into the $G_{\beta\gamma}$- and G_{α}-subunit, each initiating distinct signaling cascades within the cell (Mellado et al., 2001). In general, chemokine receptors are associated with G_i-proteins as the majority of chemokine-induced responses are inhibited by *pertussis toxin* (PTX), which prevents the dissociation of these heterotrimeric protein complexes (Murphy, 1996). Intracellular signaling pathways induced through activation of G_i-proteins involve $G_{\alpha i}$-mediated downregulation of cyclic adenosine monophosphate (cAMP) and $G_{\beta\gamma}$-mediated mobilization of Ca^{2+} from intracellular stores through activation of phospholipase C (PLC) (Thelen, 2001). Ca^{2+} is vital for the cell as it functions as a second messenger in a plethora of signaling cascades. Moreover, induction of intracellular Ca^{2+} transients is one of the most characteristic effects in response to chemokine stimulation and has been widely used to test chemokine receptor functionality in response to various ligands (Bajetto et al., 1999; Gillard et al., 2002; Thelen, 2001). Other main signaling pathways induced by chemokine receptor activation (Fig 1.8) include the mitogen-activated protein kinase (MAPK)-cascade and activation of phosphatidylinositol-3-kinases (PI3Ks) (Balkwill, 1998; Bondeva et al., 1998; Mellado et al., 2001; Murdoch and Finn, 2000; Thelen, 2001). Chemokine-induced activation of PI3Ks leads to accumulation of phosphatidyl (3,4,5)-triphosphate (PIP3), which in its turn activates protein kinase B (Akt/PKB) and small GTPases such as Rac, Rho and Cdc42, which are required for cell polarization and cytoskeletal re-organization necessary for cellular migration (Fig 1.8) (Bondeva et al., 1998; Hu et al., 2008; Muessel et al., 2008; Thelen, 2001). Furthermore, chemokine stimulation may lead to cell proliferation and differentiation through activation of MAPKs (Fig. 1.8), specifically through activation of extracellular signal- regulated kinase 1/2 (ERK1/2) and protein tyrosine kinase 2 (PY2K) (Bajetto et al., 2001a; Del et al., 2001; Tilton et al., 2000). Taken together, these findings show that chemokine receptor activation results in the induction of various (parallel) signaling pathways and, depending on the ligand, may result in the induction of various processes, including cell proliferation, differentiation, directed migration and/or cell adhesion (Fig 1.8).

Moreover, the effective outcome of chemokine receptor signaling might be influenced by secondary signaling events. For example, it has been shown that certain chemokine receptors may form homo- or heterodimers, which has been described for the chemokine receptors CCR2, CCR5, CXCR2 and CXCR4 (Babcock et al., 2003; Rodriguez-Frade et al., 1999; Rodriguez-Frade et al., 2001; Trettel et al., 2003; Vila-Coro et al., 1999).

Interestingly, heterodimer formation of CCR2-CCR5 may lead to changes in signaling properties. Whereas ligand binding to either of the receptors alone would result in directed migration mediated by $G_{\alpha i}$-proteins, heterodimerization introduces a PTX-insensitive rise in intracellular Ca^{2+} concentration and instead of inducing directed migration, it induces cell adhesion (Mellado et al., 2001).

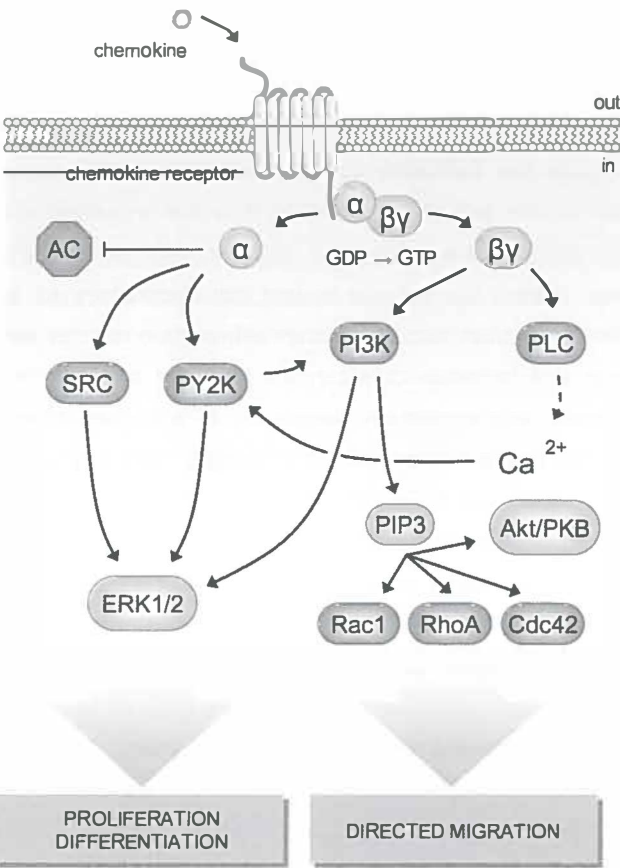


Figure 1.8. Simplified scheme of main signaling pathways and downstream effects as a result of chemokine receptor activation. AC, adenylyl cyclase; Akt/PKB, protein kinase B; α/βγ, heterotrimeric G-protein complex; Ca2+, calcium; Cdc42, cell division cycle 41; ERK1/2, extracellular signal-regulated kinase; GDP, guanine diphosphate; GTP, guanine triphosphate; PI3K, phosphoinositide 3-kinase; PLC, phospholipase C; PY2K, protein tyrosine kinase 2; Rac1, Ras-related C3 botulinum toxin substrate 1; RhoA, Ras-homolog gene family member A; SRC, sarcoma tyrosine kinase. Adapted from Bajetto et al.,2002 and Cartier et al., 2005.

Furthermore, it has been described that homo- and heterodimerization may augment the sensitivity of target cells as it was shown that cells expressing CCR5 and CCR2 display a lower threshold for chemokine-induced Ca^{2+} signaling (Mellado et al., 2001). In conclusion, homo- and heterodimerization between chemokine receptors may further increase the complexity of chemokine signaling as this leads to changes in downstream effects.

Chemokines in the periphery

In the last two decades, research has focused primarily on the role of chemokines in peripheral immune surveillance as these molecules have proven to be crucial in the facilitation of immune cell trafficking and the recirculation of leukocyte populations between lymphoid organs and target tissues both under physiological as pathological conditions (Cyster, 2005; Kunkel and Butcher, 2002; von Andrian and Mempel, 2003).
However, in recent years it has become evident that chemokines are more than mere facilitators of cellular migration. Nowadays, chemokines have not only been implicated in early development and formation of secondary lymphoid organs, but also in wound healing, angiogenesis and angiostasis, regulation of adhesion molecule expression, development of T cell helper 1 and 2 (Th1/Th2) profiles, tumour growth and metastasis (Benelli et al., 2006; Charo and Ransohoff, 2006; Kunkel, 1999; Mackay, 2001; Murray et al., 2006; Pease and Williams, 2006; Rittner and Brack, 2006; Rossi and Zlotnik, 2000; Zlotnik, 2006). Thus, from being molecules thought to solely orchestrate migration of immune cells, chemokines are now considered as versatile messengers with the ability to control the interaction between a wide diversity of cell types.

Expression of chemokines and their receptors in the CNS

Expression of chemokines in CNS-resident cells was described for the first time in the early 1990s (Aloisi et al., 1992; Ransohoff et al., 1993). In the same period, chemokine expression in the CNS was found to be enhanced in an animal model for MS, showing for the first time a disease-associated increase in chemokine expression by distinct subsets of CNS-residing cells (Hulkower et al., 1993; Ransohoff et al., 1993). Since then, many *in vitro* and *in vivo* studies on functional expression of chemokines and their receptors in the CNS have been published and its findings have been reviewed extensively (Ambrosini and Aloisi, 2004; Bajetto et al., 2001b; Bajetto et al., 2002; Biber et al., 2006; Cartier et al., 2005).

Taken these findings together, it is now appreciated that all CNS-residing cell types (including microglia, astrocytes, oligodendrocytes, neurons and endothelial cells) functionally express chemokine receptors, making them potential targets for the action of chemokines (Ambrosini and Aloisi, 2004; Bajetto et al., 2002; Biber et al., 2006; Cartier et al., 2005).

In the healthy human CNS, constitutive expression of chemokine receptors CCR2, CCR5, CXCR2, CXCR3, CXCR4 and CX_3CR1 has been observed, indicating a function for these receptors in normal CNS homeostasis (Biber et al., 2006; Cartier et al., 2005; Rostene et al., 2007; Tran and Miller, 2003). Moreover, expression of these receptors is enhanced under various pathological conditions in the human CNS, including stroke (Trebst et al., 2003a) and neurodegenerative diseases like HIV-associated dementia (Lavi et al., 1997; McManus et al., 2000; Petito et al., 2001; Sanders et al., 1998; Tong et al., 2000; Vallat et al., 1998; van der Meer et al., 2000), Alzheimer's disease (Xia et al., 1997; Xia et al., 1998), and multiple sclerosis (MS) (Balashov et al., 1999; Hulshof et al., 2003; Simpson et al., 2000a; Simpson et al., 2000b; Sorensen et al., 1999; Trebst et al., 2001). These findings are corroborated by *in vitro* studies, which show that chemokine receptor expression in human CNS cell types can be enhanced by stimulation with inflammatory mediators IFNγ, IL-1β and TNFα (Aloisi et al., 1992; Flynn et al., 2003; Guillemin et al., 2003).

Similar to chemokine receptors, expression of chemokines in the CNS can be roughly divided into two categories: 1) "constitutive" chemokines which are either developmentally regulated or constitutively expressed in the healthy CNS and 2) "inducible" chemokines which are only expressed in case of CNS injury or in response to inflammatory stimuli. Overlap may exist as some constitutive chemokines are shown to be expressed at higher levels in response to pathological conditions. Constitutive chemokines, although few in number, have been implicated in CNS development and homeostasis (Rostene et al., 2007; Tran and Miller, 2003), while inflammatory chemokines seem to play a critical role in the communication between neurons, glia cells and infiltrating leukocytes under various pathological conditions (see below) (Ambrosini and Aloisi, 2004; Biber et al., 2006; De Haas et al., 2007).

Astrocytes and microglia are the main chemokine-producing cells under pathological conditions (Ambrosini and Aloisi, 2004). Depending on the stimulus or pathological conditions, both types of glia cells have been demonstrated to express chemokines CCL2, CCL3, CCL4, CCL5, CXCL8 and CXCL10, while *in vivo* expression of CXCL9,

$CXCL12$ and CX_3CL1 has been observed in astrocytes only (for review see (Biber et al., 2006)). However, expression of "inducible" chemokines is not restricted to glia cells as neurons have also been reported to express chemokines under certain neurodegenerative conditions in the human CNS, such as CXCL10, CXCL12 and CX_3CL1 in HAD/HIV-encephalitis (Rostasy et al., 2003; Sui et al., 2004; Tong et al., 2000), CCL2 in amyotrophic lateral sclerosis (ALS) (Baron et al., 2005) and CX_3CL1 in MS (Hulshof et al., 2003).

Chemokines in CNS development and homeostasis

Although constitutive expression of chemokine receptors can be found throughout the human CNS, there is little evidence for constitutive expression of chemokines in the healthy brain. So far, only a few chemokines have been reported in the healthy human CNS such as CCL2 (Meng et al., 1999), CCL4 (Sanders et al., 1998; Xia et al., 1998), CXCL12 (Rostasy et al., 2003) and CX_3CL1 (Hulshof et al., 2003). *In vivo* experiments with rodents and *in vitro* experiments with rodent- and human primary cells suggest a role for these chemokines in CNS development. Especially CXCL12 and its receptor CXCR4 seem to be important regulators of CNS development as they are expressed at high levels at the early stages of the developing CNS (McGrath et al., 1999; Rostasy et al., 2003; Tham et al., 2001). Indeed, it has been shown that CXCL12/CXCR4 signaling facilitates axonal guidance and migration of neuronal progenitor cells during hippocampal and cerebellar neurogenesis (Arakawa et al., 2003; Bagri et al., 2002; Belmadani et al., 2005; Chalasani et al., 2003b; Pujol et al., 2005). Moreover, it has been shown that mice lacking expression of CXCL12 or CXCR4 (CXCL12- and CXCR4-deficient mice) die prematurely and display abnormal development of specific brain regions, in particular the cerebellum and the hippocampus, emphasizing the importance of CXCL12-CXCR4 signaling in CNS development (Lu et al., 2002; Ma et al., 1998; Zhu et al., 2002).

Other suggested roles for chemokines in the developing CNS are the facilitation of astrocyte proliferation (CXCL12, CCL2, CCL3 and CCL5) (Bajetto et al., 2001a; Bakhiet et al., 2001; Rezaie et al., 2002), migration of microglia precursor cells to the CNS (CCL2 and CCL5) (Rezaie and Male, 1999), regulation of oligodendrocyte maturation and myelination of the spinal cord (CXCL1) (Robinson et al., 1998; Robinson and Franic, 2001; Tsai et al., 2002; Wu et al., 2000), and neuronal survival (CXCL12) (Chalasani et

al., 2003a; Vlahakis et al., 2002). Whether these proposed functions for chemokines also play a role in the development of the human CNS remains to be established.

A recently described feature of chemokine function in the adult CNS might be the modulation of neuronal signaling (Rostene et al., 2007). Several chemokines (including CXCL1, CXCL8, CXCL10, CXCL12, CX_3CL1 and several CC-chemokines) have been shown to either modulate synaptic activity, neurotransmitter release, neuronal excitability or induction of Ca^{2+} transients in various primary neuronal cultures (Gillard et al., 2002; Giovannelli et al., 1998; Gosselin et al., 2005; Limatola et al., 2000; Liu et al., 2003; Meucci et al., 1998; Nelson and Gruol, 2004; Oh et al., 2002; Puma et al., 2001; White et al., 2005). Depending on the chemokine and neuronal cell type, the nature of these modulatory effects can be either excitatory or inhibitory. Similar neuromodulatory effects of chemokines (including CXCL10, CXCL12 and CX_3CL1) have also been described in brain slices (Bertollini et al., 2006; Dong and Xiong, 2006; Guyon et al., 2005; Guyon et al., 2006; Limatola et al., 2000; Limatola et al., 2005; Liu et al., 2003; Ragozzino et al., 2002; Vlkolinsky et al., 2004). However, the presence of glia cells in these slices makes it difficult to determine whether the neuromodulatory effects of chemokines are induced through chemokine receptors on neurons and/or on glia cells, since electrophysiological changes in neurons might also be due to chemokine-induced transmitter release from glia cells (Limatola et al., 2000; Ragozzino et al., 1998; Bertollini et al., 2006). In line with this, it has been shown that CXCL12 indirectly regulates neuronal excitability of cerebellar Purkinje cells by enhancing glutamate release from astrocytes through a TNFα-dependent mechanism (Bezzi et al., 2001; Limatola et al., 2000). Although these findings underline the importance of chemokine-signaling in the developing- and adult CNS under physiological conditions, there is now ample evidence that chemokines might be of equal importance in the development of several CNS-related pathologies.

Chemokines in CNS pathology

The expression of "inducible" chemokines within the CNS is primarily associated with neuropathological conditions. Indeed, upregulation of chemokine expression by CNS-residing cells (including neurons, astrocytes, microglia and endothelial cells) has been described in various pathologies and/or inflammatory processes associated with human CNS disease (see also table 1.1) (Ambrosini and Aloisi, 2004; Biber et al., 2006; Cartier et al., 2005; De Haas et al., 2007; Gerard and Rollins, 2001). Interestingly, cellular expression of these chemokines was predominantly observed within or in close vicinity of

primary CNS lesions. As specific chemokines have shown to trigger glia cell activation and/or migration of leukocytes *in vitro*, it has been hypothesized that chemokines in the injured CNS may facilitate local immune responses through activation of local glia cells and by recruitment of blood leukocytes to local sites of CNS injury.

Chemokines in CNS pathology: leukocyte attraction and extravasation

Under physiological conditions, the BBB acts as a structural barrier between the blood stream and the CNS parenchyma limiting the entry of leukocytes and immune-related molecules into the CNS (Bechmann et al., 2007; Galea et al., 2007). From a classical point-of-view, the isolating capacity of the BBB diminishes under pathological conditions such as MS, ischemia and infection, thereby allowing infiltration of activated immune cells into the CNS. The recruitment and infiltration of leukocytes from the blood into the CNS tissue requires a multi-step process, involving tethering, rolling and adhesion to the cerebrovascular endothelium, followed by activation and transmigration of the leukocytes into the CNS parenchyma. Various steps in this process are facilitated by chemokines (Baggiolini, 1998; Engelhardt, 2008; Johnston and Butcher, 2002; Moser and Loetscher, 2001).

The initial process of leukocyte extravasation is facilitated by interaction with various types of adhesion molecules expressed on the cerebrovascular endothelium. Tethering and rolling of leukocytes along the endothelium is mediated through interaction with selectins and their corresponding carbohydrate receptors expressed at the endothelial surface. At the same time, chemokines produced by the inflamed neuronal tissue are transported intracellularly to the luminal site of the endothelium, where they are immobilized through glycosaminoglycans (GAGs) and presented to the "rolling" leukocytes. Upon recognition, the leukocytes undergo a rapid activation step that upregulates integrin affinity and avidity, resulting in arrest and firm adhesion to the endothelium through interaction with endothelial adhesion receptors. Once adhered, chemokine gradients guide the leukocytes across the endothelium towards the site of inflammation. Subsequent infiltration of eosinophils and at a later time-point macrophages and lymphocytes into the damaged/inflammatory area may lead to perivascular inflammatory reactions, in which the BBB plays an active role by production of inflammatory mediators and the presentation of chemotactic substances.

Taken together, chemokine expression by CNS-residing cells in response to pathological

Table 1.1 *in vivo* expression of chemokines in neurons and glia cells associated with human CNS disease. Adapted from (Biber et al., 2006; De Haas et al., 2007).

CNS pathology	chemokine	cell type(s)
Aicardi-Goutières	CXCL10	astrocytes
Alzheimer's disease	CCL2	microglia
	CCL3	microglia, neurons
	CCL4	astrocytes
	CXCL10	astrocytes
Amyotrophic Lateral Sclerosis	CCL2	neurons
HIV-associated dementia	CXCL10	astrocytes
	CXCL12	astrocytes, neurons
	CX3CL1	astrocytes
HIV-encephalopathy	CCL2	astrocytes, microglia
	CCL3	astrocytes, microglia
	CCL4	astrocytes, microglia
	CCL5	microglia
	CXCL8	astrocytes
	CXCL10	astrocytes, neurons
	CX3CL1	neurons
Multiple sclerosis	CCL2	astrocytes
	CCL3	microglia
	CCL4	microglia
	CCL5	astrocytes
	CCL7	astrocytes
	CCL8	astrocytes
	CXCL9	astrocytes
	CXCL10	astrocytes, microglia
	CX3CL	neurons

conditions and the subsequent presentation of these chemokines at the luminal side of the BBB cerebrovascular endothelium provides a regulatory mechanism for the recruitment of specific leukocyte subtypes to the site of injury. This feature may be important in the development of autoimmune diseases in the CNS such as MS, which is characterized by the destruction of myelin sheaths by infiltrating myelin specific auto-reactive T cells and macrophages leading to axonal damage and loss of neurons.

Indeed, studies in an animal model for MS, known as experimental autoimmune encephalitis (EAE), have indicated an important role for chemokine-receptor couples such as CCL2-CCR2 and CXCL10-CXCR3 in the development of MS-like pathologies. Mice lacking expression of CCL2 ($CCL2^{-/-}$) or CCR2 ($CCR2^{-/-}$) develop strongly reduced EAE pathology when compared to wild type littermates, which coincides with a strong

reduction in infiltrating macrophages in the CNS (Babcock et al., 2003; Fife et al., 2000; Huang et al., 2001; Izikson et al., 2000). Conversely, CXCL10 expression in EAE and MS has been associated with the attraction of CXCR3-positive Th1 cells into the CNS. However, studies using CXCL10-neutralizing antibodies and studies in CXCL10- and CXCR3-knockout mice do not give a uniform answer regarding the role of this chemokine-receptor couple in the development of EAE pathology. Administration of CXCL10-neutrilizing antibodies has shown to decrease the infiltration of CD4+ T cells as well as the clinical and histological disease incidence in EAE (Fife et al., 2001). In contrast, induction of EAE in CXCL10- or CXCR3-knockout mice provoked more severe clinical and histological symptoms and earlier onset compared to healthy controls (Klein, 2004; Liu et al., 2006; Muller et al., 2007). Moreover, the number of infiltrating T cells in these knockout studies was similar to that observed wild type conditions, suggesting a compensatory mechanism (Klein, 2004; Liu et al., 2006; Muller et al., 2007). Taken together, targeting CXCL10 as a therapeutic strategy in MS should be addressed with caution (Liu et al., 2005).

Endogenous role for CXCL10-CXCR3 signaling in CNS injury

Expression of CXCL10 in the CNS has been associated with many neuropathologies, including ischemia (Wang et al., 1998), Alzheimer's disease (Galimberti et al., 2006; Xia et al., 2000), MS (Balashov et al., 1999; Simpson et al., 2000b; Sorensen et al., 2002), and HIVE (Cinque et al., 2005; Sanders et al., 1998). Depending on the pathology, CXCL10 was predominantly found in astrocytes surrounding primary lesions (Balashov et al., 1999; Omari et al., 2005; Simpson et al., 2000b; Tanuma et al., 2006; Wang et al., 1998; Xia et al., 2000), but several reports have also demonstrated CXCL10 expression in neurons (Rappert et al., 2004; Wang et al., 1998). As mentioned previously, initial research was focused on the role of CNS-expressed CXCL10 in the recruitment of leukocytes from the periphery under inflammatory conditions, as infiltrating T cells were found to express its corresponding receptor CXCR3 (Boztug et al., 2002; Liu et al., 2000; Simpson et al., 2000b; Sorensen et al., 1999; Sorensen et al., 2002; Trebst et al., 2003b). However, knockout studies in CXCL10$^{-/-}$ and CXCR3$^{-/-}$ mice could not univocally prove this concept (Klein, 2004; Liu et al., 2006; Muller et al., 2007). In addition to T cells, expression of CXCR3 has also been reported in CNS-residing cells, in particular microglia (Biber et al., 2001; Biber et al., 2002; De Haas et al., 2008; Dijkstra et al., 2004; Li et al., 2006; Rappert et al., 2004; Tanuma et al., 2006) and reactive astrocytes

(Goldberg et al., 2001; Omari et al., 2005; Simpson et al., 2000b; Tanuma et al., 2006), suggesting that CXCL10 may activate and/or recruit local glia cells to the site of injury. This suggestion is corroborated by *in vitro* findings showing that CXCL10 induces CXCR3-mediated migration of primary microglia (Biber et al., 2001; Biber et al., 2002; Rappert et al., 2002) and proliferation of primary astrocyte cultures (Flynn et al., 2003). Nonetheless, the proposed role and consequences of CXCL10/CXCR3-signaling between neurons and/or glia cells under pathological conditions are far from understood. The importance of CXCR3-signaling in the activation of microglia *in vivo* has been demonstrated recently in the entorhinal cortex lesion (ECL) model (Rappert et al., 2004). In this model, *in vivo* axotomy of the neuronal fibers connecting the entorhinal cortex and the hippocampus leads to axonal degeneration and subsequent loss of neuronal dendrites in the middle and outer layer of the DG, a process which coincides with local activation of astrocytes and recruitment of microglia to the site of axonal damage. In CXCR3$^{-/-}$ mice, microglia activation and subsequent recruitment to sites of axonal degeneration was impaired after ECL when compared to wild type conditions, while aberrations in astrocyte activation were not observed (Rappert et al., 2004). Moreover, it was demonstrated that, in contrast to wild type conditions, dendrites of denervated DG interneurons in CXCR3$^{-/-}$ mice were maintained. These findings not only show that CXCR3 is important in the recruitment of microglia to sites of neuronal injury, but also suggest that this recruitment may be important for neuronal reorganization after injury (Rappert et al., 2004). However, a direct link between microglia recruitment and neuronal survival in this paradigm, as well as which CXCR3 ligands are involved here, remains to be established.

CCL21-CXCR3, an alternative communication pathway between endangered neurons and glia cells?

Previously, we have shown in a middle cerebral artery occlusion (MCAO) mouse model for CNS ischemia that cortical neurons in the penumbra zone surrounding the ischemic core rapidly express the chemokine CCL21 (Biber et al., 2001). Since expression of CCL21 was not detected in healthy brain tissue, it was assumed that CCL21 is specifically expressed by endangered neurons (Biber et al., 2001). This finding was rather surprising as CCL21 is well known for its constitutive expression in the periphery, where it controls the homing of CCR7-positive mature dendritic cells and naïve T- and B cells to secondary lymphoid organs (Dieu et al., 1998; Forster et al., 2008; Lira, 2005;

Sallusto et al., 1998; Stein et al., 2000; Yanagihara et al., 1998). Thus, CCL21 expression in the periphery was generally considered as a homeostatic feature. Recently, another group has demonstrated CCL21 expression in dorsal horn neurons after spinal cord injury, supporting a role for CCL21 in neuronal pathology (Zhao et al., 2007). Moreover, CCL21 expression was demonstrated *in vitro* in cortical neuronal cultures and in neurons in hippocampal slice cultures when exposed to glutamate-induced excitotoxicity (Biber et al., 2001; De Jong et al., 2005). Taken together, it is suggested that injury-induced expression of CCL21 in endangered neurons reflects a specific role for this chemokine in CNS pathology. This assumption is corroborated by findings in transgenic mice in which CCL21 was expressed ectopically in various tissues (Chen et al., 2002b; Chen et al., 2002a). Transgenic expression of CCL21 in the CNS of intact mice induced severe neuropathology and premature death (Chen et al., 2002a), whereas CCL21 expression in the skin was not accompanied by any alterations (Chen et al., 2002b).

Several lines of evidence suggest that rapid induction of CCL21 in endangered neurons might provide a stress signal that recruits surrounding glia cells (Biber et al., 2001; De Jong et al., 2005; Dijkstra et al., 2004; Rappert et al., 2002; Zhao et al., 2007). This assumption is based on *in vitro* findings, showing activation and migration of both human and mouse microglia in response to recombinant CCL21 (Biber et al., 2001; Dijkstra et al., 2004; Rappert et al., 2002). Moreover, it has been demonstrated very recently that injection of CCL21 into the thalamus of intact mice results in transient activation of local microglia (Zhao et al., 2007). An interesting feature here is that the activation of microglia in response to CCL21 *in vitro* has been shown not to be dependent on its native receptor CCR7, but on the presence of CXCR3 (Biber et al., 2001; Dijkstra et al., 2004; Rappert et al., 2002). Cross-desensitization experiments with CXCR3-ligand CXCL10 prevented CCL21-induced activation of both human and mouse microglia, and microglia derived from CXCR3$^{-/-}$ mice did not migrate in response to CCL21, while microglia derived from CCR7$^{-/-}$ mice did migrate in response to this chemokine (Biber et al., 2001; Dijkstra et al., 2004; Rappert et al., 2002). Taken together, these results suggest that neuron-derived CCL21 might provide a signaling system between endangered neurons and CXCR3-expressing glia cells that is associated with a neuroimmune response. However, whether CCL21-induced activation of glia cells triggers a neuroprotective or a neurotoxic response in these cells remains to be elucidated.

1.4 THESIS OUTLINE

Summary

Any type of neuronal injury in the CNS inevitably results in the activation of local glia cells, in particular microglia and astrocytes. Activation of these cells is generally considered a beneficial process, aimed at the protection and restoration of normal CNS homeostasis. However, chronic or excessive glia activation might lead to uncontrolled responses with detrimental consequences for neuronal survival under pathological conditions.

Neuronal excitotoxicity is a process associated with many CNS pathologies and is characterized by progressive loss of neurons as a result of excessive glutamate release. This process of glutamate-induced neurodegeneration coincides with reactive astrogliosis and local accumulation of highly activated microglia. Studies addressing the role of microglia in excitotoxicity are controversial as inhibition of microglia function in this paradigm proved to be either beneficial or detrimental for neuronal survival. The aim of the first part of this thesis was to get more insight in the role of microglia under excitotoxic conditions.

In **Chapter 2** we took a closer look at the neuroprotective/neurotoxic properties of microglia in the process of excitotoxicity-induced neurodegeneration. For this purpose, we set up an *ex vivo* model for excitotoxicity using mouse organotypic hippocampal slice cultures. Subsequently, we selectively removed the microglia population from these slice cultures by treatment with liposome-encapsulated clodronate and determined its effect on excitotoxicity-induced neurodegeneration by means of immunohistochemistry and confocal imaging.

The second part of this thesis was focused on the role of specific chemokines as potential messengers between injured neurons and local glia cells under excitotoxic conditions. A prominent candidate is the chemokine CXCL10, which mediates its actions through its receptor CXCR3. Expression of CXCL10 and CXCR3 in both glia and neuronal cells has been associated with the clinical signs of several excitotoxicity-related CNS disorders. Interestingly, interference with CXCL10/CXCR3 signaling in various CNS disease models has been shown to alter the progression and severity of disease symptoms. However, the mechanism and cell-types involved in CXCL10/CXCR3 signaling under pathological conditions are far from understood. In **Chapter 3** the role of

CXCL10 and its receptor CXCR3 in excitotoxicity-induced neurodegeneration has been examined using mouse hippocampal slice cultures as a model. The expression and localization of CXCL10 and CXCR3 were determined by RNA- and protein analysis of whole slice culture lysates, flow cytometry and *in situ* hybridisation in combination with immunohistochemistry. To address the role of CXCL10/CXCR3-signaling in hippocampus excitotoxicity, we determined the degree of NMDA-induced neuronal cell death and glia activation in wild type hippocampal slice cultures and compared these findings with those acquired in slice cultures derived from CXCL10$^{-/-}$ and CXCR3$^{-/-}$ mice.

Another chemokine that might play a role in CNS-excitotoxicity is CCL21. CCL21 expression *in vivo* has been observed in ischemic cortical neurons and in dorsal horn neurons after spinal cord injury. Neuronal expression of CCL21 *in vitro* has been observed in hippocampal slice cultures and in primary neuronal cultures when exposed to toxic levels of glutamate. Moreover, CCL21 was demonstrated to induce activation of microglia both *in vivo* and *in vitro*, suggesting a role for this chemokine in the communication between injured neurons and glia cells. However, the consequence of CCL21-signaling in the injured CNS remains to be elucidated. In **Chapter 4** we have addressed this question by comparing excitotoxicity-induced neuronal cell death in hippocampal slice culture preparations derived from wild type- and CCL21/CCL19 (*plt*)-deficient mice. In addition, the involvement of its receptors CCR7 and CXCR3 are addressed.

Previously, it has been demonstrated that CCL21 induces activation and migration of both human and mouse microglia *in vitro*, a process dependent on the presence of CXCR3. It has been well established that astrocytes, like microglia, functionally express CXCR3. However, effects of CCL21-signaling on astrocytes have not been investigated yet. Therefore, we have examined the effect of CCL21 on intracellular calcium mobilization and proliferation in primary mouse astrocytes and compared these findings with the effects observed in response to CXCR3-ligand CXCL10. The results are presented in **Chapter 5**.

Chapter 6 provides a summary of all the results presented in this thesis and their interpretations are discussed.

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CHAPTER 2

Neuroprotective activity of ramified microglia in hippocampal excitotoxicity

Hilmar R.J. van Weering *(1)*; Hendrikus W.G.M. Boddeke *(1)*; Nico van Rooijen *(2)*; Knut P.H. Biber *(1,3)*

(1) Department of Neuroscience, Section Medical Physiology, University Medical Center Groningen (UMCG), Rijksuniversiteit Groningen (RUG), Groningen, The Netherlands. *(2)* Department of Molecular Cell biology, Free University Medical Center (VUMC), Amsterdam, The Netherlands. *(3)* Molecular Psychiatry, Psychiatry and Psychotherapy, University of Freiburg, Freiburg, Germany.

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ABSTRACT

Upon any type of central nervous system (CNS) injury microglia become rapidly activated, a process which has long been thought to be detrimental for neuronal survival. However, recent findings suggest that microglia display neuroprotective properties in various CNS pathologies. In the present study we determined the effect of microglia depletion on excitotoxicity-induced neurodegeneration using mouse organotypic hippocampal slice cultures. Treatment of slice cultures with 10-50 µM *N*-methyl-D-aspartic acid (NMDA) induced a region-specific increase in neuronal cell death with CA1 neurons being most vulnerable to NMDA-exposure, followed by CA3 and DG neurons, respectively (CA1>CA3>DG). This selective neuronal vulnerability strongly correlated with activation of local microglia. At 10 µM NMDA, microglia activation was restricted to the CA1 region, while in the CA3 region changes in microglia morphology were only evident at 15 µM NMDA or higher. As DG neurons were most resistant to NMDA-excitotoxicity, microglia activation in this region was only observed at NMDA-concentrations of 25 µM or higher. Interestingly, ablation of microglia by treatment of slice cultures with liposome-encapsulated clodronate, and subsequent exposure to NMDA, resulted in severely enhanced neuronal cell death in the CA3 and DG region. Particularly, the resistance of DG and CA3 neurons to NMDA-induced toxicity in the presence of ramified microglia, and the severely increased vulnerability of these neurons in the absence of microglia, suggests that ramified microglia contribute to the protection of neurons under pathological conditions.

INTRODUCTION

Brain tissue is highly vulnerable to injury because of its restricted regenerative capacity. From the outside, the brain is protected by the skull and the blood-brain barrier (Bechmann et al., 2007). Within the central nervous system (CNS), microglia are the first line of defense and are activated upon any type of brain injury (Kreutzberg, 1996; Rossum and Hanisch, 2004; Hanisch and Kettenmann, 2007; Streit, 2002). The original definition of microglia activation as a stereotypic and graded process (Kreutzberg, 1996; Streit, 2002) has been challenged in the last couple of years (Hanisch and Kettenmann, 2007). The current view is that in response to activating stimuli, microglia rapidly change their morphology. They first direct their dendritic processes towards the injury in order to shield it before they retract their processes and become motile cells that migrate to the site of injury. Microglia activation can also be accompanied by proliferation (Hailer et al., 1999; O'Donnell et al., 2002). It is now clear that microglia respond with a variety of different reactions by integrating multifarious inputs (Hanisch, 2002; Hanisch and Kettenmann, 2007; Schwartz et al., 2006). In line with this, microglia responses are not inevitably neurotoxic as long been thought. Various neuroprotective effects of activated microglia have been demonstrated recently *in vivo*. Microglia were found beneficial in a model of nitric oxide-dependent excitotoxicity (Turrin and Rivest, 2006) and in stroke (Lalancette-Hebert et al., 2007). Moreover, protective microglia activity was described in mouse models of amyotrophic lateral sclerosis (Boillee et al., 2006) and Alzheimer's disease (El Khoury et al., 2007). However, microglial neurotoxicity can occur in case of overshooting and uncontrolled stimulation of microglia (Cardona et al., 2006; Rossum and Hanisch, 2004) or when microglia function is impaired (Boillee et al., 2006; Neumann and Takahashi, 2007; Streit, 2006). Proper facilitation of microglia activation is therefore of crucial importance for the survival of neurons under pathological conditions.

It is important to note that the studies mentioned above all focus on the functions of morphologically activated (non-ramified) microglia. Although it is clear today that ramified microglia can not be considered anymore as "resting", as it has been shown *in vivo* that these cells actively screen their microenvironment with highly motile dendritic processes (Davalos et al., 2005; Haynes et al., 2006; Nimmerjahn et al., 2005), very little is known about possible other functions of ramified microglia in the CNS.

In order to study the role of ramified microglia we made use of a mouse organotypic hippocampal slice culture (OHSC) model, in which microglia maintain their ramified

morphology comparable to the *in vivo* situation. Since microglia can be specifically eliminated from these slice cultures by treatment with the biphosphonate clodronate (Kohl et al., 2003) without affecting other cell-types (Marin-Teva et al., 2004; Markovic et al., 2005), this model provides an ideal system to analyze the function of microglia in their ramified state. Here, we provide evidence that the presence of ramified microglia is essential for the survival of dentate gyrus (DG) and CA3 neurons in *N*-methyl-D-aspartic acid (NMDA)-induced excitotoxicity, strongly indicating that ramified microglia, next to their monitoring function, display neuroprotective properties.

EXPERIMENTAL PROCEDURES

Animals

All experiments have been approved by the Dutch animal experimental committee. The C57BL/6J mice (Harlan) were housed and handled in accordance with the guidelines of the central animal laboratory (CDL) facility of Groningen.

Chemicals

Culture media and supplements were all obtained from GIBCO® (Invitrogen Corporation; Breda, The Netherlands), unless mentioned otherwise. Multi-lamellar CL_2MDP (clodronate)-liposomes (Lip-CL) were obtained from the Department of Molecular Cell Biology of the Free University of Amsterdam, The Netherlands (for an extensive preparation protocol see (Van Rooijen and Sanders, 1994). Clodronate was a gift of Roche Diagnostics (Mannheim, Germany), phosphatidylcholine (Lipoid EPC) was obtained from Lipoid (Ludwigshafen, Germany) and cholesterol was purchased from Sigma (USA).

Preparation of organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures (OHSCs) were prepared as described (Stoppini et al., 1991) with minor modifications. In brief, OHSCs were prepared from 2-3 day old C57BL/6J mouse pups under sterile conditions. After decapitation, the brains were removed and the hippocampi from both hemispheres were acutely isolated in ice cold serum-free Hank's Balanced Salt Solution (HBSS), supplemented with 0.5% glucose (Sigma) and 15 mM HEPES. Isolated hippocampi were cut into 375 µM thick slices using a tissue chopper (McIlwain) and were transferred to 0.4 µM culture plate inserts

(Millipore, PICM03050). These culture plate inserts, containing four to six slices, were placed in six-well plates containing 1.2 ml of culture medium per well. Culture medium (pH 7.2) consisted of 0.5x minimum essential medium containing 25% heat-inactivated horse serum, 25% BME basal medium without L-glutamine, 2 mM glutamax and 0.65% glucose (Sigma). The slice cultures were kept at 35°C in a humidified atmosphere (5% CO_2) and the culture medium was refreshed the first day after preparation and every consecutive two days.

Induction of excitotoxicity in OHSCs

After six days in culture, OHSCs were placed in culture medium containing various concentrations of the glutamate receptor-agonist *N*-methyl-D-aspartic acid (NMDA; Sigma) for 4 hours to induce excitotoxicity and subsequently the medium was replaced with standard culture medium. NMDA-treated OHSCs were kept in culture for 24 hours after the NMDA challenge. Vehicle-treated OHSCs and OHSCs pre-treated with NMDA-antagonist MK-801 (30 µM) served as controls.

Immunohistochemistry

For immunohistochemical analysis, control and NMDA-challenged OHSCs were shortly rinsed in phosphate-buffered saline (PBS, 35°C) and fixated with 4% paraformaldehyde overnight at 4°C. After fixation, the slice cultures were rinsed in PBS and pre-incubated with 5% normal goat serum (NGS; Vector) in PBS containing 0.3% Triton X-100 (PBS^+) for at least one hour. Subsequently, the slice cultures were incubated with the appropriate primary antibodies overnight in 1% NGS/PBS^+ at 4°C. The following primary antibodies were used: rabbit-anti-Iba1 (1:1000; Wako 019-19741) for detection of microglia, mouse-anti-GFAP (1:600; Chemicon MAB3402) for detection of astrocytes and mouse-anti-NeuN (1:1000; Chemicon MAB377) for detection of neuronal nuclei. Secondary antibodies used were donkey-anti-mouse-Alexa488 (Molecular Probes) for NeuN, donkey-anti-rabbit-Alexa633 (Molecular Probes) for Iba1 and goat-anti-mouse-Cy3 (Jackson IR Laboratories) for GFAP. Analysis of the slice cultures was done by confocal imaging using a Leica SP2 AOBS system (Leica Microsystems, Heidelberg, Germany).

Quantification of neuronal cell death

To quantify neuronal cell death in response to NMDA-induced excitotoxicity OHSCs were incubated with 5 µg/ml propidium iodide (PI; Sigma) during and after the NMDA-challenge (Pozzo Miller et al., 1994; Vornov et al., 1991). PI-positivity was restricted to the neuronal layers CA1, CA3 and DG and co-localized with the neuronal nuclear marker NeuN (see also figure 1). Confocal images of the neuronal layers were taken mid-section at 40x magnification and double positive cells (NeuN/PI) were quantified manually using ImageJ software. The percentage of neuronal cell death was determined by the number of double positive cells (NeuN/PI) divided by the total number of NeuN-positive cells per neuronal layer.

Depletion of microglia from OHSCs using clodronate-liposomes

To deplete microglia from slice cultures, freshly prepared OHSCs were placed on culture plate inserts and incubated with approximately 0.5 mg/ml Lip-CL solution (1:10 liposome dilution in standard slice culture medium) for 24 hours at 35°C. Subsequently, the slice cultures were carefully rinsed in PBS (35°C) to wash away residual liposomes and placed on fresh culture medium. After depletion the medium was refreshed every two days.

Statistical Analysis

Data are represented as the mean ± standard error of the mean (SEM). Statistical comparison between groups was performed using one-way analysis of variance (ANOVA) with Gabriel's posthoc test and *p*-values smaller than 0.05 were considered significant. All statistical tests were performed in SPSS version 14.0.2 (SPSS Inc., Chicago).

RESULTS

NMDA-induced excitotoxicity in mouse organotypic hippocampal slice cultures

To examine the effects of NMDA-induced excitotoxicity on neuronal degeneration and microglial activation, we established a mouse organotypic hippocampal slice culture (OHSC) model. After six days in culture, the neuronal layers CA1, CA3 and DG were well-preserved under standard culture conditions and neuronal cell death, as determined by PI-uptake, was minimal (<1%; Fig. 2.1A/B control). Treatment with 10, 15, 25 and 50

μM NMDA induced a concentration-dependent, region-specific increase in neuronal cell death in the slice cultures, as determined by NeuN/PI positivity (Fig. 2.1A/C). Highest vulnerability towards NMDA was observed in the CA1 region with 67.1 ± 16.3% cell death at 10 μM NMDA. At concentrations of 15-50 μM NMDA neuronal cell death in the CA1 reached maximum levels (97.1 ± 1.1–100%). In contrast, CA3 neurons were less susceptible to NMDA as almost no cell death was detected at 10 μM NMDA (1.9 ± 0.8%) and pronounced cell death was only detected at concentrations of 15 μM NMDA (41.7 ± 11.3%), 25 μM NMDA (79.9 ± 8.6%) and 50 μM NMDA (96.4 ± 1.5%).

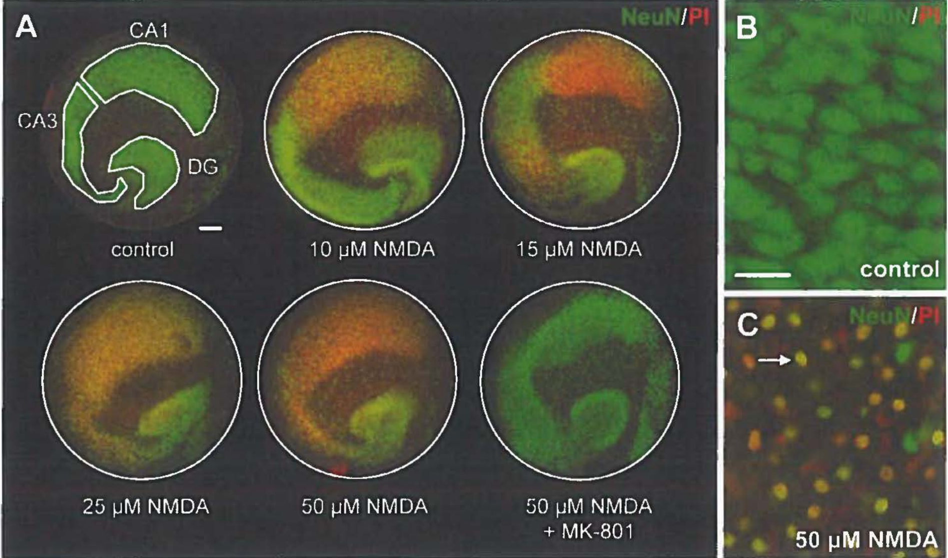


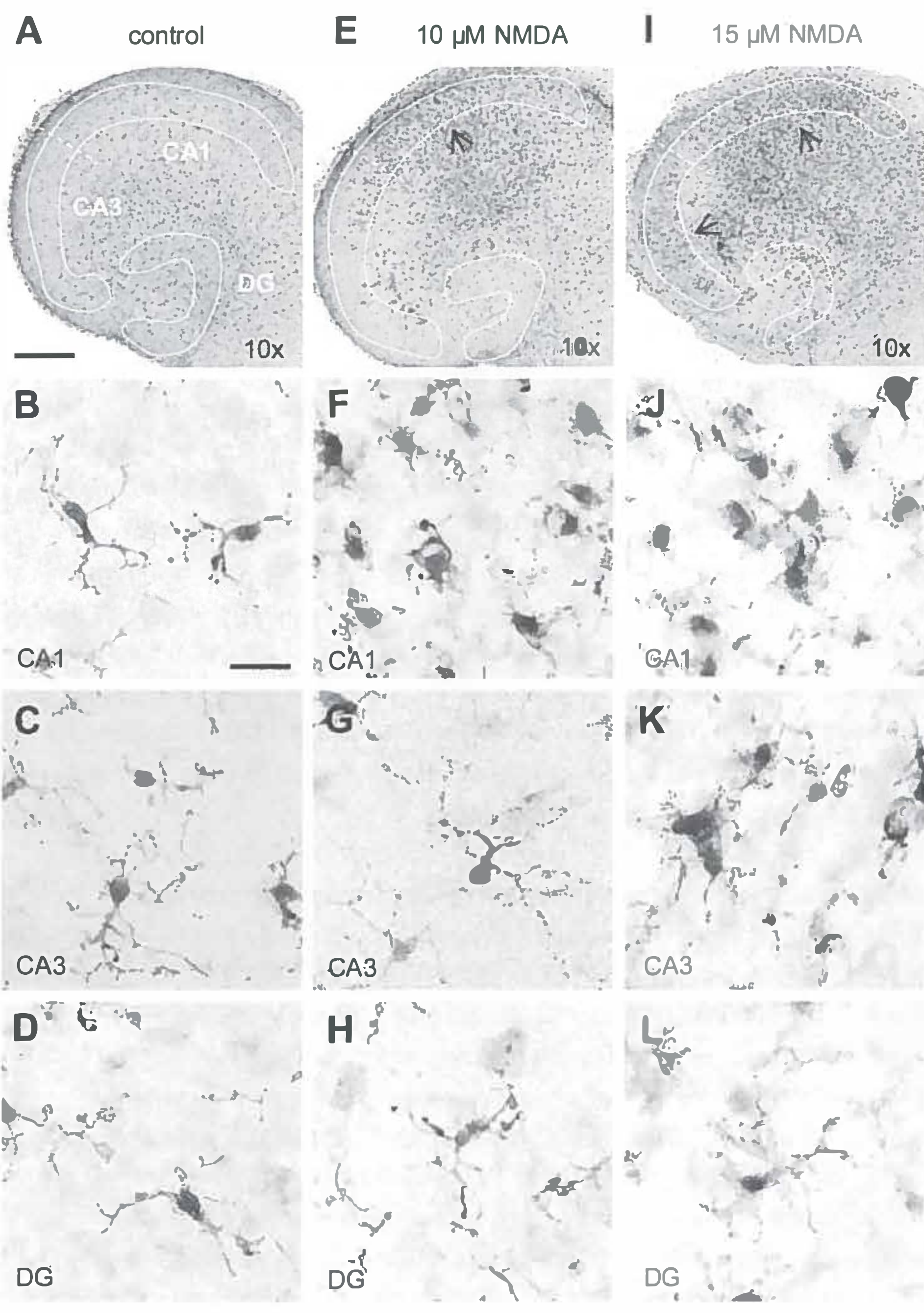
Figure 2.1. NMDA-induced neurodegeneration in mouse hippocampal slice cultures. After six days in culture, hippocampal slice cultures were treated with increasing concentrations of 0 (control), 10, 15, 25 and 50 μM NMDA. Treatment with NMDA clearly induced cell death in the slice cultures as determined by propidium iodide uptake (PI; red), which co-localized with the neuronal nuclear marker NeuN (**C**, white arrow), indicating that NMDA specifically induced neuronal cell death. A concentration-dependent vulnerability towards NMDA was observed as neurons of the CA1 region were most sensitive to the NMDA-treatment (with 67.1% cell death at 10 μM NMDA), followed by the CA3 (41.7% at 15 μM NMDA) and finally the DG, which showed relatively low levels of cell death even at 50 μM NMDA (30.7%). Control slice cultures showed hardly any cell death (<1%, **B**). Treatment of slice cultures with the NMDA-antagonist MK-801 (30 μM) for one hour prior to NMDA-treatment completely blocked NMDA-induced neuronal cell death. The percentages of neuronal cell death per neuronal cell layer (DG/CA3/CA1) were quantified and are represented in figure 4. Scale bars indicate 100 μm (**A**) and 25 μm (**B,C**).

The DG region was least sensitive to NMDA with 6.5 ± 2% cell death at 10 μM NMDA, 12.7 ± 1% at 15 μM NMDA, 23.5 ± 2.1% at 25 μM NMDA and 30.7 ± 4.1% at 50 μM NMDA. NMDA-concentrations lower than 10 μM did not induce significant cell death in any of the neuronal regions (data not shown). Pre-treatment for one hour with the NMDA-antagonist MK-801 prior to NMDA-treatment completely inhibited NMDA-induced neuronal cell death (Fig. 2.1A) in all regions and percentages of neuronal cell death were equal to control conditions (DG; 2.3 ± 1%, CA3; 4.0 ± 0.5, CA1; 1.4 ± 0.6%).

Microglial activation in response to excitotoxicity coincides with selective neuronal vulnerability towards NMDA

Next, we determined the effects of NMDA-induced excitotoxicity on microglial activation. After six days in culture, microglia were evenly distributed throughout the slice culture (Fig. 2.2A) and showed typical ramified morphology with small somata and long processes with secondary and tertiary branches (Fig. 2.2B, CA1; C, CA3; D, DG). Treatment of slice cultures with toxic concentrations of NMDA caused pronounced microglia activation (Fig. 2.2E-L). Treatment with 10 μM NMDA induced accumulation and activation of microglia in the CA1 region (Fig. 2.2E, arrow) as evidenced by rounded morphology and retraction of dendrites (Fig. 2.2F). In contrast, in the CA3 region (Fig. 2.2G) and the DG region (Fig. 2.2H) microglial accumulation was not detected and microglia morphology predominantly resembled the ramified phenotype as seen in control slice cultures. Treatment with 15 μM NMDA (Fig. 2.2I) induced microglia activation and accumulation in both the CA1 region (Fig. 2.2J) and the CA3 region (Fig. 2.2K). Microglial accumulation in the DG region however was minimal in response to 15 μM NMDA, and microglia displayed a typical "hypertrophic" phenotype with thickened and shortened dendrites (Fig. 2.2L).

Figure 2.2 (right). Microglial activation coincides with selective neuronal vulnerability towards excitotoxicity. Confocal images of microglia in control (**A-D**), 10 μM (**E-H**) and 15 μM (**I-L**) NMDA-treated slice cultures, as determined by Iba1-immunohistochemistry. After six days in culture (**A**), microglia were evenly distributed throughout the slice cultures and displayed a typical ramified morphology (**B**:CA1, **C**:CA3, **D**:DG). At 10 μM NMDA (**E**), changes in the CA1 region were clearly visible as numerous microglia accumulated at the site of injury (**E**, arrow). Morphologically, these microglia displayed an "activated" phenotype (**F**, CA1) with enlarged somata and loss of secondary and tertiary branching. In contrast, accumulation of microglia did not occur in the CA3 (**G**) and DG (**H**) and these cells retained their ramified phenotype. At 15 μM NMDA, pronounced accumulation of morphologically activated microglia (**I**, arrows) was observed in both CA1 (**J**) and CA3 (**K**). In contrast, microglia in the DG (**L**) showed only mild activation and accumulation of microglia was minimal in this region. Scale bars indicate 300 μM (overviews) and 25 μM (magnifications). Confocal images were gray-scaled and inversed.


A
control
E
10 μM NMDA
I
15 μM NMDA
CA1
CA3
DG
10x
10x
10x
B
CA1
F
CA1
J
CA1
C
CA3
G
CA3
K
CA3
D
DG
H
DG
L
DG

Treatment with 25 µM NMDA and 50 µM NMDA resulted in pronounced accumulation of morphologically activated microglia in all three neuronal regions (data not shown). As expected, the morphological activation and recruitment of microglia in the three hippocampal layers coincided with the selective vulnerability of neurons towards NMDA-induced excitotoxicity.

Depletion of microglia from OHSCs using liposome-encapsulated clodronate

Next, we developed a protocol to specifically deplete microglia from mouse OHSCs using liposome-encapsulated clodronate (Lip-CL) to study the role of these cells in NMDA-induced excitotoxicity. Lip-CL has been shown to successfully deplete microglia from mouse organotypic slice cultures, without affecting other cell-types (Marin-Teva et al., 2004; Markovic et al., 2005). Overnight treatment with 0.5 mg/ml Lip-CL directly after slice culture preparation and subsequent culturing in standard culture medium for six days reduced the microglia population to less than 5% compared to its original quantity (Fig. 2.3A/D). In line with previous findings, both astrocytes (Fig. 2.3E) and neurons (Fig. 2.3F) were not affected by the Lip-CL treatment and the number and morphology of these cells did not differ from those in untreated controls (Fig. 2.3B/C). Treatment with liposomes without clodronate (Lip-PBS) did not result in microglia depletion (data not shown). Although treatment with Lip-PBS resulted in mild activation of microglia, no neuronal cell death was observed and the total number of microglia did not change compared to untreated control conditions.

Ablation of microglia results in severe neuronal loss in response to excitotoxicity

To determine the role of microglia in excitotoxicity, control and microglia-depleted slice cultures were treated with 0, 10, 15, 25 and 50 µM NMDA (Fig. 2.4). Already at a low concentration of NMDA (10 µM) a severe increase in neuronal cell death was observed in microglia-depleted slice cultures, when compared to control conditions (Fig. 2.4A-C). Especially in the DG (Fig. 2.4A) neuronal cell death was severely enhanced in the absence of microglia with 30.9 ± 5.1% cell death at 10 µM NMDA (compared to 6.5 ± 2% in controls), 66.0 ± 4.8% at 15 µM NMDA (compared to 12.7 ± 1% in controls), 74.7 ± 2.5% at 25 µM NMDA (compared to 23.5 ± 2.1% in controls) and 89.4 ± 4.5% at 50 µM NMDA (compared to 30.7 ± 4.1% in controls). Neuronal cell death in the CA3 region (Fig. 2.4B) was also significantly enhanced in microglia-depleted slice cultures in response to 10 µM NMDA (33.0 ± 8.8% compared to 1.9 ± 0.8% in controls) and 15 µM NMDA (94.0

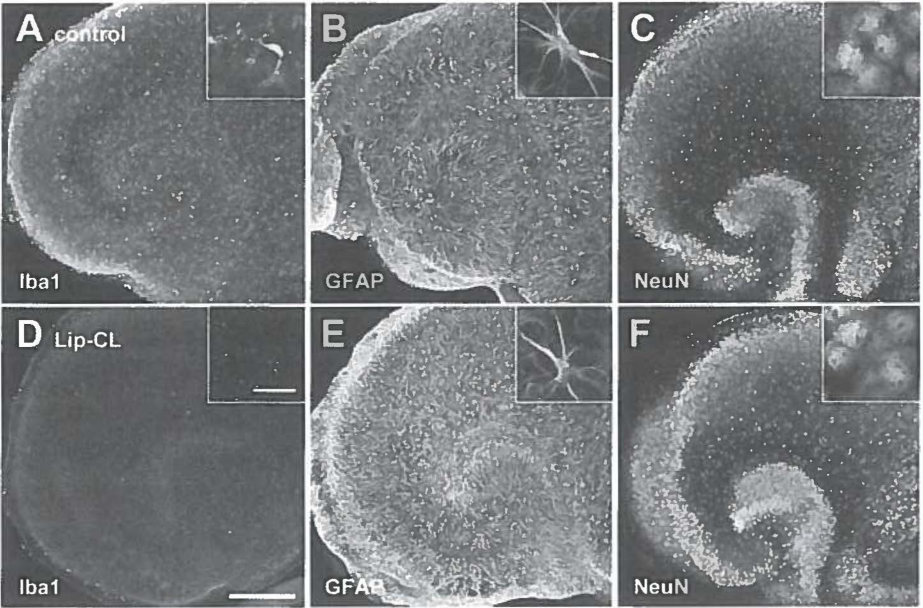


Figure 2.3. Liposomic clodronate specifically depletes microglia from hippocampal slice cultures. Untreated mouse hippocampal slice cultures showed preserved organotypic structure with ramified microglia (**A**, Iba1), astrocytes (**B**, GFAP) and neuronal layers CA1/CA3/DG (**C**, Neun). Overnight treatment with liposome-encapsulated clodronate (Lip-CL) directly after slice culture preparation resulted in specific depletion of microglia (**D**), while astrocytes (**E**) and neurons (**F**) remained unaffected. After six days in culture, the microglia population in Lip-CL treated slice cultures was reduced to less then 5% (**D**). Scale bars indicate 300 μM (overviews) and 25 μM (inserts). Confocal images were grey-scaled.

± 2.6% compared to 41.7 ± 11.3% in controls). At concentrations of 25 μM and 50 μM NMDA differences in neuronal cell death were minimal and reached maximum levels under both conditions (99.6 ± 0.3% and 100% compared to 79.9 ± 8.6% and 96.4 ± 1.5% respectively in controls). In the CA1 region, no significant differences in neuronal cell death were observed between control and microglia-depleted slice cultures as treatment with 10 μM NMDA already resulted in severe neuronal cell death in both conditions (Fig. 2.4C, 95.2 ± 1.4% compared to 67.1 ± 16.3% in controls). Treatment of microglia-free slice cultures with NMDA-concentrations lower than 10 μM did not induce neuronal cell death in any of the three hippocampal regions (data not shown). Taken together, these results show a clear neuroprotective property for microglia in NMDA-induced excitotoxicity as depletion of resident microglia results in severely enhanced neuronal vulnerability towards NMDA-treatment.

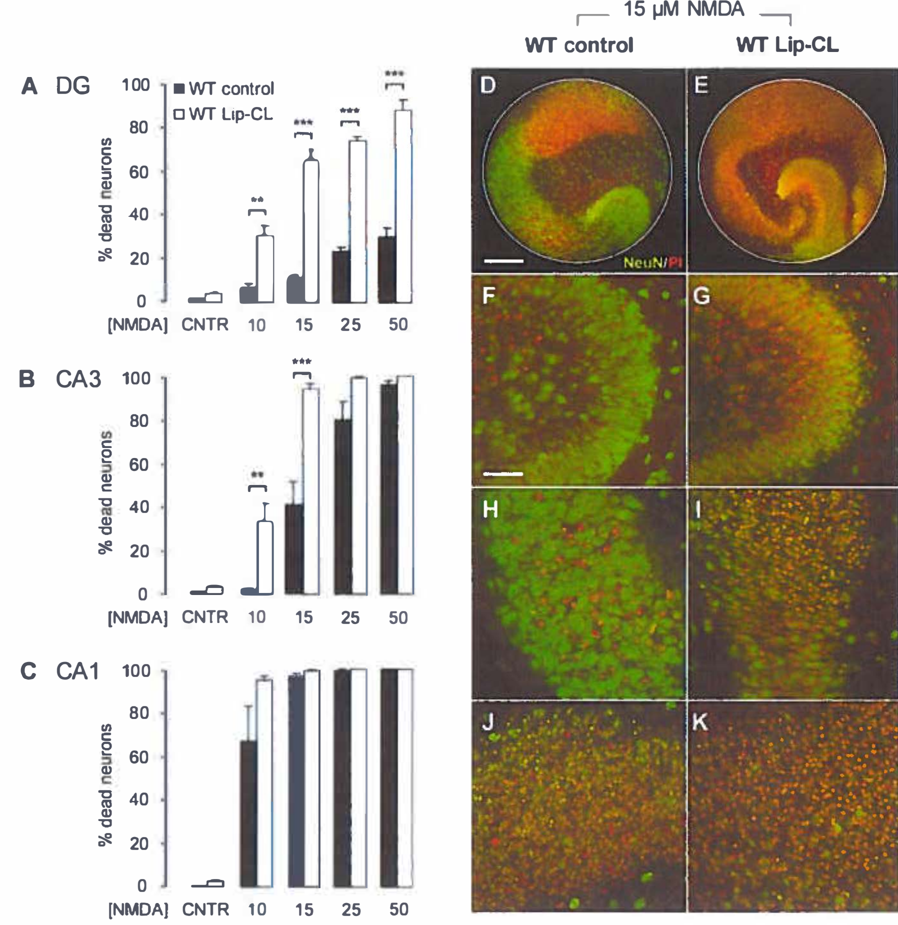


Figure 2.4. Depletion of microglia leads to severely enhanced loss of neurons in response to excitotoxicity. Graphs represent the percentages of neuronal cell death per hippocampal sub-region (**A** to **C**) in response to 0, 10, 15, 25 and 50 µM NMDA in untreated (WT control) and microglia-depleted slice cultures (WT Lip-CL). Microglial depletion alone did not lead to a significant increase in neuronal cell death (**A**-**C**; CNTR). However, in the absence of microglia, neuronal cell death in response to NMDA-induced excitotoxicity was severely enhanced in the DG (**A**) and CA3 (**B**). Confocal images clearly show the effect of microglial depletion on neuronal degeneration in response to 15 µM NMDA (**D-K**). Here, in the absence of microglia, neuronal cell death was significantly enhanced in the DG from 12.7% to 66.0% (**F**,**G**) and in the CA3 from 41.7% to 94.0% (**H**, **I**). In the CA1 (**J**,**K**) no significant effect in response to 15 µM NMDA was observed between control and Lip-CL treated slice cultures (97.1% versus 99.8%, respectively). Data are a summary of three individual experiments with at least 6 slice cultures per condition. Bars indicate mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, ANOVA. Scale bars indicate 300 µM (**D**,**E**) and 75 µM (**F**-**K**).

Interestingly, the selective neuronal vulnerability to NMDA as observed in control slice cultures was partly abolished in microglia-depleted slice cultures. Both CA3 and CA1 neurons were equally affected upon treatment with 15-50 µM NMDA and cell death in the DG was severely enhanced in response to these concentrations, resulting in an attenuation of the selective neuronal vulnerability between the various hippocampal regions. At 50 µM NMDA, this selectivity was even completely abolished as all three hippocampal regions were equally affected in terms of neuronal cell death. Clearly, the region-dependent hippocampal vulnerability as observed in this model is at least partly dependent on the presence of microglia.

DISCUSSION

We have investigated the effect of microglia-depletion on the survival of neurons during NMDA-induced excitotoxicity in a mouse hippocampal slice culture model. Our results show that morphological activation of microglia coincides with local neuronal cell death in the hippocampal regions CA1, CA3 and DG. However, in the absence of microglia, neuronal vulnerability in response to NMDA-excitotoxicity is severely enhanced, suggesting that microglia are neuroprotective under these conditions.

Selective hippocampal neuronal vulnerability in excitotoxicity: involvement of microglia
In our system neurons of the hippocampal regions CA1, CA3 and DG showed selective vulnerability towards NMDA-excitotoxicity with CA1 neurons being most susceptible to NMDA followed by CA3 and DG neurons, respectively. Similar patterns of selective neuronal vulnerability towards excitotoxicity or (hypoxic-) ischemic insults in the hippocampus have been observed previously both *in vivo* (Kirino and Sano, 1984; Horn and Schlote, 1992; Acarin et al., 1996; Schauwecker, 2002; Won et al., 1999) and *in vitro* in organotypic slice cultures (Gee et al., 2006; Boscia et al., 2006; Cronberg et al., 2005; Keynes et al., 2004; Kristensen et al., 2001; Strasser and Fischer, 1995; Vornov et al., 1991), corroborating our findings. Reasons for this selective neuronal vulnerability in the hippocampus are not well understood. Variability in glutamate receptor (-subtype) expression (Coultrap et al., 2005; Martens et al., 1998) and/or endogenous properties of the distinct neuronal populations in the CA1, CA3 and DG regions could (in part) explain their selective vulnerability towards excitotoxicity (Chen et al., 1999; Cronberg et al., 2005; Grishin et al., 2004; Sakaguchi et al., 1997). In line with this, it has been shown

that CA1 neurons express relatively high levels of AMPA- and NMDA-receptor (-subtypes), while neurons in the CA3 region express relatively high levels of kainate-receptors (Martens et al., 1998; Coultrap et al., 2005). Accordingly, it has been demonstrated that CA1 neurons are most vulnerable to glutamate- and NMDA-induced insults, whereas CA3 (and DG) neurons are most sensitive to the excitotoxin kainic acid (Kristensen et al., 2001; Schauwecker, 2002; Vornov et al., 1991). Moreover, selectivity towards NMDA has been shown to be independent of an intact hippocampal neuronal circuitry (Ikegaya and Matsuki, 2002) as isolated CA3, CA1 and DG slice cultures still respond with a selective vulnerability towards NMDA, with the CA1 and CA3 regions being more susceptible to NMDA than the DG region.
Here, we provide evidence that selective vulnerability is not solely based on endogenous neuronal properties. In our slice culture model, the absence of microglia did not simply sensitize neurons for NMDA-toxicity since concentrations below 10 μM NMDA did not induce neuronal cell death, irrespective of the presence of microglia in the slice cultures. Moreover, the differences in neuronal sensitivity to NMDA between the three hippocampal regions were attenuated in the absence of microglia. Without microglia, neurons from both the CA3 and CA1 region were equally affected upon treatment with 15-25 μM NMDA. Treatment of microglia-free slice cultures with 50 μM NMDA even fully abrogated selective vulnerability as all three hippocampal regions (CA1, CA3 and DG) were equally affected in terms of neuronal cell death. These results suggest that microglia are at least partly involved in the selective vulnerability of hippocampal neurons towards NMDA. How the impact of microglia on the region-specific vulnerability of neurons is achieved is not yet clear. Whether neuron-microglia signaling in the hippocampus shows region-specific properties (Biber et al., 2007) or whether different subpopulations of microglia can be found in the various hippocampal layers remains to be established.

Evidence for neuroprotective properties of ramified microglia upon excitotoxicity

In our slice culture model morphological activation of microglia coincided with the selective neuronal vulnerability to NMDA and was restricted to sites of neuronal cell death. This is a well-known feature of microglia and has been reported *in vivo* (Acarin et al., 1996) and *in vitro* in OHSCs (Bernardino et al., 2005). We show that ablation of microglia severely increased the vulnerability of CA3 and DG neurons upon NMDA-induced excitotoxicity, suggesting that microglia in these neuronal regions have profound

neuroprotective properties. These results are in line with other reports describing neuroprotective properties for microglia upon excitotoxic or ischemic injury (Imai et al., 2007; Kitamura et al., 2004; Lalancette-Hebert et al., 2007; Turrin and Rivest, 2006). Interestingly, our results suggest that a morphological activation of microglia is not required for their neuroprotective function. At 10 µM NMDA we did not observe neuronal loss in the CA3 and DG region and therefore no morphological microglia activation in these regions. From the classical point of view one could assume that microglia are not active here. However, neuronal loss was profound in these regions in the absence of microglia, clearly indicating that also ramified (morphologically non-activated) microglia have the capacity to support neurons in excitotoxicity. In line with this observation, recent reports have shown that ramified microglia express neurotrophic factors (Elkabes et al., 1996; Xapelli et al., 2008; Shein et al., 2008), supporting our hypothesis. Our findings, together with the observations that ramified microglia *in vivo* continuously scan their environment for homeostatic irregularities (Davalos et al., 2005; Haynes et al., 2006; Nimmerjahn et al., 2005) and express neurotrophic factors (Elkabes et al., 1996; Shein et al., 2008; Xapelli et al., 2008), clearly shows that ramified microglia contribute to the protection of neurons both under healthy and pathological conditions. In conclusion, one should not regard ramified microglia as solely inactive “resting” cells, but as active contributors in the protection of CNS neurons.

Acknowledgements

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CHAPTER 3

CXCL10/CXCR3 signaling in glia cells differentially affects NMDA-induced cell death in CA and DG neurons of the mouse hippocampus

Hilmar R.J. van Weering *(1)*; Hendrikus W.G.M. Boddeke *(1)*; Jonathan Vinet *(1)*; Nieske Brouwer *(1)* Alexander H. de Haas *(1)*; Nico van Rooijen *(2)*; Allan R. Thomsen *(3)*; Knut P.H. Biber *(1,4)*

(1) Department of Neuroscience, Section Medical Physiology, University Medical Center Groningen (UMCG), Rijksuniversiteit Groningen (RUG), Groningen, The Netherlands. *(2)* Department of Molecular Cell biology, Free University Medical Center (VUMC), Amsterdam, The Netherlands. *(3)* Institute of International Health, Immunology and Microbiology, University of Copenhagen, Copenhagen. *(4)* Molecular Psychiatry, Psychiatry and Psychotherapy, University of Freiburg, Freiburg, Germany.

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ABSTRACT

The chemokine CXCL10 and its receptor CXCR3 have been implicated in various CNS pathologies and their expression has been demonstrated in both neurons and glia cells. Interference with CXCL10/CXCR3 signaling alters the onset and progression of pathological signs in various CNS disease models. However, the mechanism and cell-types involved in CXCL10/CXCR3 signaling under pathological conditions are far from understood. Here, we investigated the potential role for CXCL10/CXCR3 signaling in neuronal cell death and glia activation in response to *N*-methyl-D-aspartic acid (NMDA)-induced excitotoxicity in mouse organotypic hippocampal slice cultures (OHSCs). Our findings demonstrate that astrocytes rapidly express CXCL10 in response to NMDA-induced excitotoxicity. To determine the effect of CXCL10 on NMDA-induced neuronal cell death we prepared OHSCs derived from CXCL10-deficient ($CXCL10^{-/-}$) and CXCR3-deficient ($CXCR3^{-/-}$) mice and subsequently exposed these slice cultures to 0 (control), 10, 15, 25 or 50 µM NMDA. In the absence of CXCL10 or CXCR3, neuronal cell death in the hippocampal regions CA1 and CA3 was attenuated in response to NMDA-treatment when compared to wild type conditions. In contrast, neuronal cell death in the DG region was enhanced in both $CXCL10^{-/-}$ and $CXCR3^{-/-}$ OHSCs in response to a high (50 µM) NMDA-concentration. Furthermore, we show that in the absence of microglia these differential changes in neuronal vulnerability between $CXCR3^{-/-}$ and wild type OHSCs are fully abrogated, suggesting a prominent role for microglia in this process. Taken together, our results identify a region-specific role for CXCL10/CXCR3 signaling in neuron-glia and glia-glia interactions under pathological conditions.

INTRODUCTION

Excitotoxicity is a pathological process in the central nervous system (CNS) by which persistent activation of glutamate receptors by high extracellular glutamate levels leads to the progressive loss of neurons (Arundine and Tymianski, 2003; Choi and Rothman, 1990). Excitotoxicity has been associated with several neurological disorders such as ischemia (Arundine and Tymianski, 2004), multiple sclerosis (MS) (Gonsette, 2008), Parkinson's disease (Henchcliffe and Beal, 2008), Alzheimer's disease (Walton and Dodd, 2007), Huntington's disease (Fan and Raymond, 2007), amyotrophic lateral sclerosis (Van Damme et al., 2005) and epilepsy (Vincent and Mulle, 2009). Persistent activation of neuronal glutamate receptors provokes a lethal increase in intracellular calcium levels which results in activation of numerous enzymes that damage neuronal structure and induces formation of free radicals, ultimately leading to cell death (Arundine and Tymianski, 2003; Choi, 1988).

For a long time, the concept of excitotoxicity-induced neuronal cell death has been viewed as a result of solely endogenous processes but recent data suggest a major role for glial cells in excitotoxic insults. Microglia, the immunocompetent cells of the CNS, have been shown to rapidly respond to excitotoxicity by proliferation and morphological changes enabling them to migrate towards sites of neuronal injury to exert their function (Acarin et al., 1996; Heppner et al., 1998). Interestingly, inhibition of microglia activity has been shown to considerably affect the extent of excitotoxicity-induced neurodegeneration (Kohl et al., 2003; Lalancette-Hebert et al., 2007; Dehghani et al., 2003; Tikka and Koistinaho, 2001), suggesting a prominent role for these cells in excitotoxicity. However, the source and type of signals for excitotoxicity-induced microglial activation are largely unknown.

In the last years, chemokines have emerged as potential modulators of glia activity under pathological conditions (Cartier et al., 2005; De Haas et al., 2007). Chemokines are small cytokines (8-12 kDa) that are predominantly known for their ability to mediate directed migration of various cell-types. Of interest here is the chemokine CXCL10, which mediates its actions through chemokine receptor CXCR3 (Loetscher et al., 1998). CXCL10 is a well known chemo-attractant for CXCR3-positive immune cells in the periphery including specific subsets of circulating NK cells and macrophages, dendritic cells and activated $CD4^{+}$ T-cells and has therefore been implicated in the modulation of both innate- and adaptive immune responses under pathological conditions (Neville et

al., 1997; Liu et al., 2005). In the CNS, enhanced expression of CXCL10 has been demonstrated in various pathologies such as ischemia (Wang et al., 1998), Alzheimer's disease (Xia et al., 2000; Galimberti et al., 2006), MS (Balashov et al., 1999; Simpson et al., 2000; Sorensen et al., 1999), and human immunodeficiency virus (HIV)-encephalitis (Sanders et al., 1998; Cinque et al., 2005). Depending on the type of insult, CXCL10 has been predominantly found expressed in neurons (Rappert et al., 2004; Wang et al., 1998) and in astrocytes surrounding primary lesions (Balashov et al., 1999; Omari et al., 2005; Simpson et al., 2000; Tanuma et al., 2006; Wang et al., 1998; Xia et al., 2000), while CXCR3 expression has been demonstrated in microglia (Biber et al., 2001; Biber et al., 2002; De Haas et al., 2008; Dijkstra et al., 2004; Li et al., 2006; Rappert et al., 2004; Tanuma et al., 2006) and in reactive astrocytes (Goldberg et al., 2001; Omari et al., 2005; Simpson et al., 2000; Tanuma et al., 2006). Moreover, CXCL10 has been shown to induce activation and migration of CXCR3-positive microglia *in vitro* (Biber et al., 2002; Rappert et al., 2002) and proliferation in primary astrocyte cultures (Flynn et al., 2003). These findings suggest that CXCL10 might provide a signal in the CNS that is involved in the activation and/or recruitment of local glia cells under pathological conditions.

Interestingly, interfering with CXCL10/CXCR3 signaling in various CNS disease models has been shown to alter the progression and severity of disease symptoms (reviewed in Liu et al., 2005). Blocking of CXCL10 with specific antibodies reduced neuronal apoptosis in spinal cord injury (Glaser et al., 2006), suggesting a pro-inflammatory role for CXCL10. In line with these findings, it has been shown that administration of anti-CXCL10 decreased the clinical and histological disease incidence in a model for MS, known as experimental autoimmune encephalomyelitis (EAE) (Fife et al., 2001). In contrast, induction of EAE in knockout animals for CXCL10 or CXCR3 provoked more severe clinical and histological symptoms and earlier onset compared to wild type controls (Klein et al., 2004; Liu et al., 2006; Muller et al., 2007). Apart from variations in experimental design, these conflicting observations suggest that CXCL10 and its receptor CXCR3 have multiple functions in the development of CNS pathologies.

In summary, expression of CXCL10 and CXCR3 in both glia and neuronal cells has been associated with the clinical signs of several excitotoxicity-related CNS disorders. Furthermore, *in vitro* findings suggest a role for this chemokine-receptor couple in the communication between damaged neurons and glia cells. However, the proposed role for CXCL10 in neuron-glia and glia-glia signaling under pathological conditions is far from understood. Therefore, we have studied the implication of CXCL10/CXCR3

signaling on neuronal cell death and glia activation in an *ex vivo* model for excitotoxicity, known as *N*-methyl-D-aspartic acid (NMDA)-induced excitotoxicity, in mouse hippocampal slice cultures.

EXPERIMENTAL PROCEDURES

Animals

All experiments have been approved by the Dutch animal experimental committee. The C57BL/6J (Harlan) and CXCR3-deficient (CXCR3$^{-/-}$) mice were housed and handled in accordance with the guidelines of the central animal laboratory (CDL) facility of Groningen. The CXCR3$^{-/-}$ mice were kindly provided by B. Lu, MD, and Prof. C. Gerard, MD/PhD (Children's Hospital, Harvard Medical School, Boston (MA). The CXCL10-deficient (CXCL10$^{-/-}$) mice were kindly provided by Prof. A.R. Thomsen, MD/PhD (Experimental Virology, Department of International Health, Immunology & Microbiology, University of Copenhagen, Copenhagen, Denmark).

Chemicals

Culture media and supplements were all obtained from GIBCO® (Invitrogen Corporation; Breda, The Netherlands), unless mentioned otherwise. Recombinant murine CXCL10 was obtained from PeproTech® (London, United Kingdom).

Preparation of organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures (OHSCs) were prepared as described with minor modifications (Stoppini et al., 1991). In brief, OHSCs were prepared from 2-3 day old C57BL/6J, CXCL10$^{-/-}$ and CXCR3$^{-/-}$ mouse pups under sterile conditions. After decapitation, the brains were rapidly removed and the hippocampi from both hemispheres were acutely isolated in ice cold serum-free Hank's Balanced Salt Solution (HBSS), supplemented with 0.5% glucose (Sigma) and 15 mM HEPES. Isolated hippocampi were cut into 350-375 µM thick slices using a tissue chopper (McIlwain) and were transferred to 0.4 µM culture plate inserts (Millipore, PICM03050). These culture plate inserts, containing four to six slices, were placed in six-well plates containing 1.2 ml of culture medium per well. Culture medium (pH 7.2) consisted of 0.5x minimum essential medium containing 25% heat-inactivated horse serum, 25% BME basal medium without L-glutamine, 2 mM glutamax and 0.65% glucose (Sigma). The slice

cultures were kept at 35°C in a humidified atmosphere (5% CO_2) and the culture medium was refreshed the first day after preparation and every consecutive two days.

Depletion of microglia from OHSCs using clodronate-liposomes

Multi-lamellar CL_2MDP (clodronate)-liposomes (Lip-CL) were prepared as described elsewhere (Van Rooijen and Sanders, 1994). Clodronate was a gift of Roche Diagnostics (Mannheim, Germany), phosphatidylcholine (Lipoid EPC) was obtained from Lipoid (Ludwigshafen, Germany) and cholesterol was purchased from Sigma (USA). Depletion of microglia was achieved by incubation of freshly prepared OHSCs with approximately 0.5 mg/ml Lip-CL solution (1:10 liposome dilution in standard slice culture medium) for 24 hours at 35°C. Subsequently, the slice cultures were carefully rinsed in PBS (35°C) to wash away residual liposomes and placed on fresh culture medium. After depletion the medium was refreshed every two days. Liposomes containing PBS (Lip-PBS) and PBS served as negative controls.

Induction of excitotoxicity in OHSCs

After 6 days in culture, OHSCs were placed in culture medium containing 0, 10, 15, 25 or 50 µM *N*-methyl-D-aspartic acid (NMDA; Sigma) for 4 hours to induce excitotoxicity and subsequently the medium was replaced with standard culture medium. NMDA-treated OHSCs were kept in culture for maximally 48 hours after the NMDA challenge, depending on the experiment.

Immunohistochemistry

For immunohistochemical analysis, control and NMDA-challenged OHSCs were shortly rinsed in phosphate-buffered saline (PBS, 35°C) and fixated with 4% paraformaldehyde overnight at 4 °C. After fixation, the slice cultures were rinsed in PBS and pre-incubated with 5% normal goat serum (NGS; Vector) in PBS containing 0.3% Triton X-100 (PBS^+) for at least one hour. Subsequently, the slice cultures were incubated with the appropriate primary antibodies overnight in 1% NGS/PBS^+ at 4 °C. The following primary antibodies were used: rabbit-anti-Iba1 (1:1000; Wako 019-19741) for detection of microglia, mouse-anti-GFAP (1:600; Chemicon MAB3402) for detection of astrocytes and mouse-anti-NeuN (1:1000; Chemicon MAB377) for detection of neuronal nuclei. Secondary antibodies used were donkey-anti-mouse-Alexa488 (Molecular Probes) for NeuN, donkey-anti-rabbit-Alexa633 (Molecular Probes) for Iba1 and goat-anti-mouse-

Cy3 (Jackson IR Laboratories) for GFAP. Analysis of the slice cultures was done by confocal imaging using a Leica SP2 AOBS system (Leica Microsystems).

Quantification of neuronal cell death

To determine neuronal cell death in response to NMDA-induced excitotoxicity, OHSCs were incubated with 5 µg/ml propidium iodide (PI) during and up to 24 hours after the NMDA-challenge (Pozzo Miller et al., 1994; Vornov et al., 1991). Confocal images of the neuronal layers were taken mid-section at 40x magnification and both $NeuN^+$ and $NeuN^+/PI^+$ cells were quantified manually using ImageJ software. The percentage of neuronal cell death was determined by the number of $NeuN^+/PI^+$ cells divided by the total number of $NeuN^+$ cells per neuronal layer.

Quantitative polymerase chain reaction (QPCR)

For RNA analysis, OHSCs (6 slice cultures per condition) were lysed in guanidinium isothiocyanate/ monopthiolglycerol buffer (both from Sigma) and total RNA was extracted and precipitated with a one-chloroform-phenol step (Chomczynski and Sacchi, 1987). The quality and quantity of the total RNA were determined with the Agilent 2100 bioanalyzer and the NanoDrop® ND-1000. RNA integrity number (RIN) values were between 9.9 and 8.8 and the 260/280 nm ratios around 1.9. 1 µg of total RNA was transcribed into cDNA as described previously (Biber et al., 1997). Amplification reactions were performed in a total volume of 10 µl containing 0.8 µl cDNA solution, 5.0 µl TaqMan® universal PCR master mix, 0.5 µl TaqMan® primer-probe (Applied biosystems: β-actin, Mm00607939_s1 and CXCL10, Mm00445235_m1) and 3.7 µl pure water (Sigma). Reactions were run in triplets on the ABI Prism® 7900 HT real time PCR instrument. After 15 minutes at 95°C, the cDNA samples were run for 40 cycles, consisting of 15 seconds at 95°C for denaturation and 1 min at 60°C for annealing and elongation. Threshold cycles were determined manually by setting thresholds for fluorescence intensity. Relative gene expression levels were analysed by the 2-ΔΔCT method, using β-actin as a reference gene (Livak and Schmittgen, 2001). The amplification efficiency was verified by linear regression with the fluorescence per cycle number (Ramakers et al., 2003).

In situ hybridisation (ISH) followed by immunohistochemistry

The CXCL10 PCR product (147 bp) was cloned into a dual PCRII vector (Invitrogen) and linearized. CXCL10 sense- and antisense probes were synthesised by run-off transcription with SP6 or T7 RNA polymerase and digoxigenin (DIG)-conjugated UTP according to manufaturers protocol (Roche).

Control and NMDA-challenged OHSCs were shortly rinsed in phosphate-buffered saline (PBS, 35°C) and fixated with 4% paraformaldehyde (PFA) in 0.1 borate buffer (pH 9.5) overnight at 4°C. After rinsing in ice-cold KPBS, fixated OHSCs were dehydrated with 20% sucrose in KPBS for 10 hours. Then, the OHSCs were carefully removed from the culture plate inserts and cut in 10 µM sections using a Leica CM3050 S cryostat (Leica Microsystems). Subsequently, the sections were mounted on APES (Sigma)-coated glass slides, air-dried and post-fixated with 4% PFA for 30 minutes at 37°C. After post-fixation, the sections were rinsed in KPBS and digested with 0.5 µg/ml proteinase K (Sigma) for 30 minutes at 37°C. Then, the sections were rinsed in 2x SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0), dehydrated in an ethanol serie and air-dried. Subsequently, the sections were hybridized overnight at 60°C in 1x Denhardt's solution, containing 10 mM Tris (pH 8.0), 0.3 M NaCl, 50% formamide, 1 mM EDTA, 0.05% tRNA, 10% dextran sulfate and 0.6 ng/µl of the DIG-coupled RNA probe. After hybridization, the sections were treated with 10 µg/ml RNase A (Sigma) at 37°C for 30 minutes and washed in 0.1x SSC at 60°C. For immunodetection of the DIG-labelled RNA-RNA complexes, the slides were pre-incubated with 0.1 M Tris/0.15 M NaCl (buffer 1; pH 7.5) containing 2% blocking reagent (Roche) and subsequently labelled with anti-DIG-alkaline phosphatase (AP; Roche, 1:500) for 2 hours at room temperature (RT) in buffer 1, containing 1% blocking reagent. After labelling, the slides were thoroughly rinsed and equilibrated in an alkaline buffer solution (0.1M Tris, 0.1 M NaCl, 0.05 M $MgCl_2.6H_2O$; pH 9.5). The AP-conjugates were visualized by adding 0.34 mg/ml nitroblue tetrazoleum and 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phospatase (Roche) in alkaline buffer solution, supplemented with 0.24 mg/ml levamisole (Sigma). Finally, the slides were mounted with glass coverslips using glycerol jelly and brightfield images were taken with an Olympus BX50 system, equipped with an Olympus DP70 camera.

For CXCL10 ISH combined with immunohistochemistry, the cover slips were removed by gentle shaking in H_2O and incubated overnight with primary antibodies against microglia (Iba1, Wako), astrocytes (GFAP, Chemicon) or neurons (NeuN, Chemicon) in PBS/1%NGS at 4°C. After short rinsing steps in PBS, the sections were incubated with

the appropriate biotinylated secondary antibodies (1:400, Vector) for one hour at RT. Antibody-antigen reactions were detected using the biotin-streptavidin method (Vectastain® ABC-kit, Vector Labs) and the complex was visualized with diaminobenzidin (DAB)/H_2O_2.

Western blot analysis

After a short rinse in PBS (35°C), both control and NMDA-challenged OHSCs (12 slice cultures per condition) were lysed at set time-points in 200 µl ice-cold RIPA buffer (PBS, containing 0.1% Nonidet P40, 0.5% sodium deoxylate, 0.1% SDS and proteinase inhibitors (Complete Mini; Roche)). Subsequently, one volume of 2x sample buffer (175mM Tris-HCl (pH 6.8) with 4% SDS, 4M urea, 4.5% glycerol, 0.05M DTT and 0.01% bromophenol) was added and the samples were heated for 5 minutes at 95°C. 30 µg of total protein was loaded on a 10% SDS polyacrylamide gel and transferred to a Hybond-ECL nitrocellulose membrane (Amersham Biosciences). Next, the membranes were blocked with Odyssey® Blocking Buffer (OBB) in PBS (1:1) for one hour at RT and subsequently incubated with rabbit-anti-mouse CXCL10 (1:1000, Peprotech) and mouse-anti-ß-actin (1:8000, Abcam) in 1:1 OBB/PBS, containing 0.1% Tween-20 (overnight/4°C).

After four rinsing steps with PBS/0.1% Tween-20 (PBS-T), the membranes were incubated with donkey-anti-rabbit IRDey™ 800CW and donkey-anti-mouse IRDey™ 680 (both 1:8000; LI-COR®) in PBS-T for one hour at RT. Labeled proteins were visualized by paired exposure at 700 nm and 800 nm using the Odyssey® Infrared Imaging System (LI-COR® Biosciences GmbH).

Rapid isolation of microglia and flow cytometry

To isolate microglia from OHSCs, the slice cultures were removed from the culture membrane using a Pasteur pipette and directly triturated in HBSS, containing 0.1% glucose and 15 mM HEPES. Single-cell suspensions were filtered using a 70 µm cell strainer (BD Biosciences) and rinsed in PBS. Then, the cells were pelleted at 1000 x g for 10 min at 4ºC. These cell pellets were resuspended in 3.3 ml ice-cold 75% Percoll (GE Healthcare 17-0891) in PBS and transferred into 12 ml polystyrene tubes. Then, the cell suspension was gently over-layered with 5 ml ice-cold 25% Percoll and subsequently with 3 ml PBS. The density gradient was centrifuged at 800 x g (with minimal acceleration and brake) for 25 min at 4ºC. After centrifugation, the myelin-containing

layer at the 0-25% interface was carefully removed with a pipette and the cells at the 25-75% interface were collected with a fresh pipette. To pellet these cells, the collected suspension was diluted threefold with PBS and centrifuged at 1000 x g for 10 min at 4ºC. The cell pellet was resuspended in PBS, containing 10% normal rat serum (Invitrogen, 10710C) and anti-CD16/32 (eBioscience 14-0161, 1:100) for flow cytometric analysis. Next, the microglia were stained for phycoerythyrin-coupled monoclonal anti-mouse CXCR3 (R&D, FAB1685P) or concentration-matched isotype IgG2A control (eBioscience, 12-4321) for 20 minutes at 4ºC in PBS containing 1% FCS. Cell-size, granularity and fluorescence intensity were analyzed by flow cytometry using a 488-nm laser FACSCalibur (Becton Dickinson), supported by CellQuest software (Becton Dickinson) and the acquired data was analyzed using WinMDI version 2.9 (Joseph Trotter).

Statistical Analysis

Data are represented as the mean ± standard error of the mean (SEM). Statistical comparison between groups was performed using one-way analysis of variance (ANOVA) with Gabriel's posthoc test and *p*-values smaller than 0.05 were considered significant. All statistical tests were performed in SPSS version 14.0.2 (SPSS Inc., Chicago).

RESULTS

NMDA-induced excitotoxicity in mouse hippocampal slice cultures

In order to determine the role of CXCR3 and its ligand CXCL10 in excitotoxicity we devised a model for NMDA-induced excitotoxicity using mouse organotypic hippocampal slice cultures (OHSCs). After six days in culture, slice cultures showed preserved hippocampal structure with intact neuronal layers CA1, CA3 and DG (Fig. 3.1A). While control slice cultures showed almost no cell death in the three neuronal regions (CA1; 0.7 ± 0.2%, CA3; 0.8 ± 0.3%, DG; 1.6 ± 0.3%, see also Fig. 3.1B), treatment with NMDA for 4 hours and subsequent culturing for 24 hours in standard culture medium clearly induced cell death, as determined by PI-uptake. PI^+-cells co-localized with the neuronal marker NeuN, showing that NMDA specifically induced cell death in neurons (Fig. 3.1C,

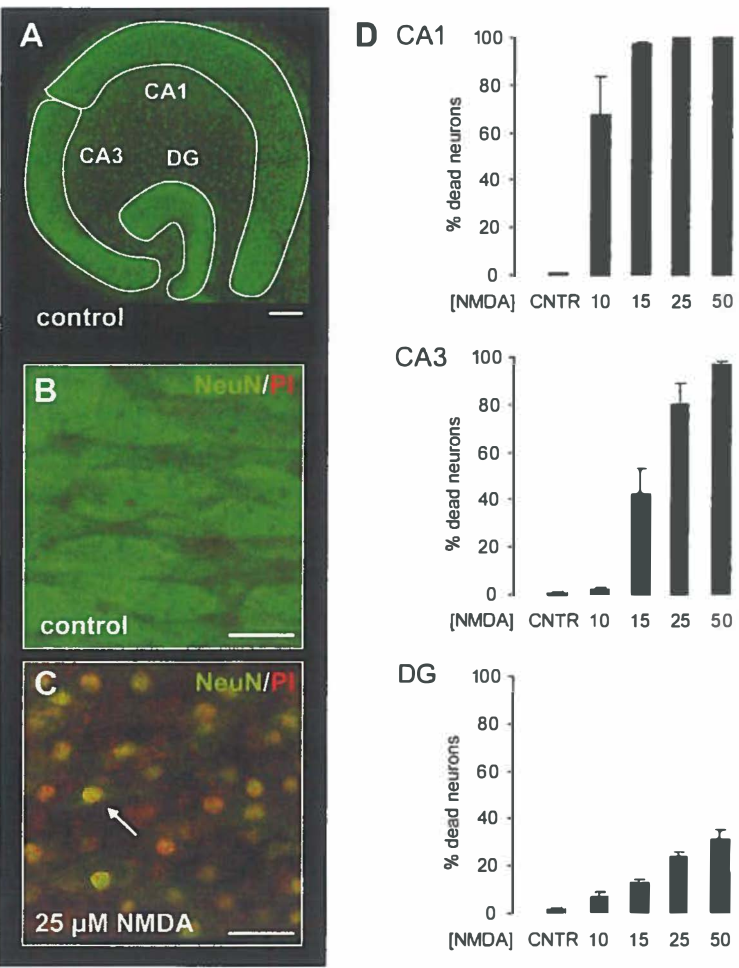


Figure 3.1. NMDA-induced excitotoxicity in mouse hippocampal slice cultures. Hippocampal slice cultures were challenged with increasing concentrations of 0 (control), 10, 15, 25 and 50 μM NMDA to induce excitotoxicity. After six days in culture, control slice cultures showed preserved hippocampal structure with intact neuronal layers CA1, CA3 and DG as determined by neuronal nuclear marker NeuN (**A**, in green). Cell death was determined by propidium iododide (PI, in red) uptake. Whereas almost no cell death was detected in control slice cultures (>1%, **B**), treatment with NMDA for 4 hours and subsequent culturing for 24 hours clearly induced cell death (**C**), which was restricted to the neuronal layers CA1, CA3 and DG and co-localized with NeuN (**C**, white arrow). The percentages of neuronal cell death per subregion (CA1/CA3/DG) were quantified as the number of $NeuN^{+}/PI^{+}$ cells divided by the total number of $NeuN^{+}$ cells with the various NMDA concentrations in μM on the x-axis. Note that individual hippocampal regions showed selective vulnerability towards NMDA-treatment with CA1>CA3>DG, which was dependent on the concentration used. Data are a summary of three separate experiments with at least five slice cultures per condition. **p*<0.05, ***p*<0.01, ****p*<0.001, ANOVA. Bars indicate mean ± SEM. Scale bars indicate 150 μm (**A**) and 25 μm (**B**,**C**).

arrow). The percentage of neuronal cell death in response to 0, 10, 15, 25 and 50 µM NMDA was quantified as the number of $NeuN^+/PI^+$ cells divided by the total number of $NeuN^+$ cells per region (Fig. 3.1D). Note that neurons from the different regions showed selective vulnerability towards NMDA-induced excitotoxicity (CA1>CA3>DG).

NMDA-excitotoxicity induces CXCL10 expression in reactive astrocytes

Next, we determined the expression of CXCL10 in NMDA-exposed slice cultures. Real-time PCR (*rt*-PCR) analysis of whole slice culture total RNA lysates showed clear upregulation of CXCL10 mRNA in response to NMDA-treatment (Fig. 3.2A) with a significant twelve-fold increase 8 hours after induction (p=0.002). CXCL10 mRNA levels were back at control levels 24 hours after the NMDA-treatment (p=1.000). Western Blot analysis of whole slice culture protein lysates (Fig. 3.2B) showed clear upregulation of CXCL10 protein as soon as 4 hours up to 24 hours after excitotoxicity-induction with a clear peak at 10 hours. CXCL10 protein was almost non-detectable 48 hours after the NMDA-treatment. To localize the cellular source for CXCL10 we combined non-radioactive *in situ* hybridization (ISH) for CXCL10 mRNA with immunohistochemistry for NeuN (neurons), Iba1 (microglia) and GFAP (astrocytes). As *rt*-PCR analysis revealed a significant increase in CXCL10 mRNA expression 8 hours after excitotoxicity-induction (Fig. 3.2A), we used slice cultures which were fixated at this time-point. ISH for CXCL10 mRNA alone revealed multiple $CXCL10^+$-cells in NMDA-treated slice cultures (Fig. 3.2C, white arrowheads). These $CXCL10^+$-cells were predominantly located in the stratum radiatum, stratum lacunosum-moleculare and the hilar area (Fig. 3.2E, closed arrows), although some $CXCL10^+$-cells were also observed inside the suprapyramidal and granular cell layers. Slice cultures hybridized with a sense probe were devoid of signal (Fig. 3.2D). Interestingly, ISH for CXCL10 mRNA (Fig. 3.2E, closed arrows) in combination with GFAP-immunohistochemistry showed 100% overlap (Fig. 3.2F, open arrows), demonstrating that the $CXCL10^+$-cells are astrocytes. CXCL10-positivity was pre-dominantly found in morphologically "reactive" astrocytes, as determined by morphology and upregulation of GFAP expression (Fig. 3.2F). In contrast, co-localization for CXCL10 mRNA with NeuN- or Iba1 immunohistochemistry was never observed (data not shown).

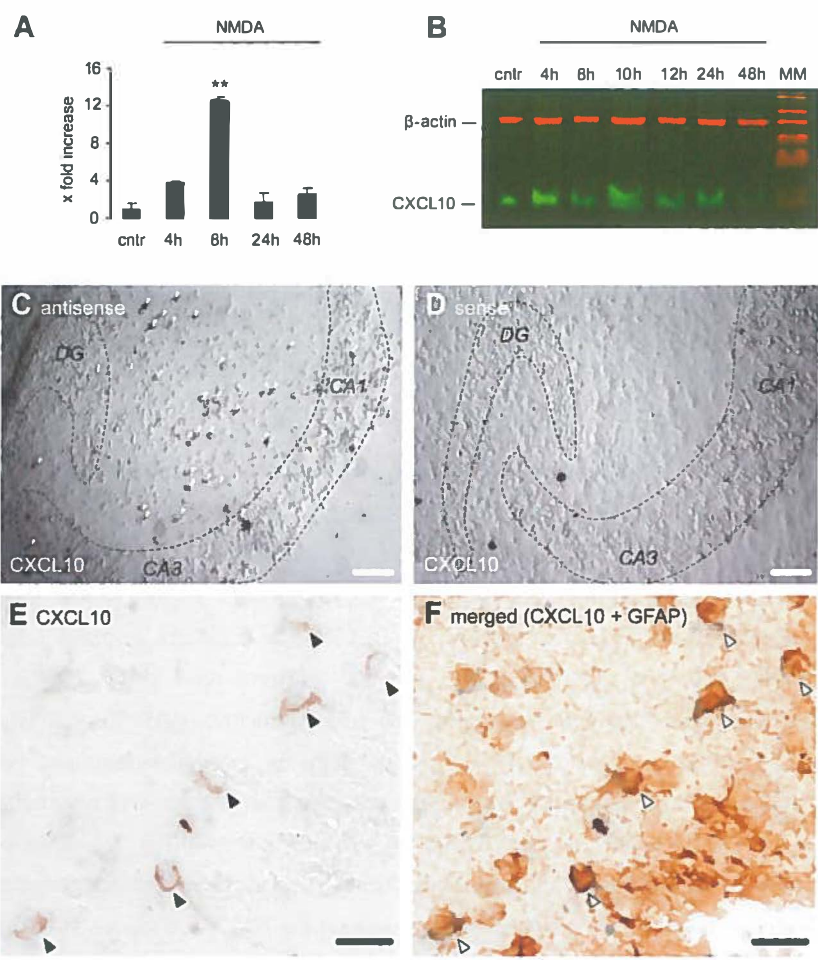


Figure 2. NMDA-excitotoxicity induces CXCL10 expression in astrocytes. (A) Real-time PCR analysis of whole slice culture lysates reveals upregulation of CXCL10 mRNA in response to NMDA-excitotoxicity as soon as 4 hours after induction with a twelve fold increase at 8 hours (p=0.002). 24 hours after the NMDA-challenge CXCL10 mRNA expression was reduced to control levels (p=1.000). Data are a summary of four individual experiments. Bars indicate mean ± SEM, $^{**}p<0.01$, Student's t-test. **(B)** Western blot analysis of whole slice culture lysates shows clear upregulation of CXCL10 protein (in green) from 4 hours up to 24 hours in response to the NMDA-treatment. CXCL10 expression was reduced to almost non-detectable levels at 48 hours after the challenge. Expression of β-actin (in red) was stable in all samples and therefore served as a positive control. Western blot results are a representation of three individual sets of experiments. **(C-F)** ISH experiments revealed multiple CXCL10^{+} cells in NMDA-treated slice cultures **(C**, white arrows) which were scattered throughout the slice culture. Slice cultures hybridized with a sense probe were devoid of signals **(D)**. ISH experiments for CXCL10 **(E**, black arrows) in combination with immunohistochemistry for GFAP **(F)** reveals 100% overlap **(F**, open arrows), showing that these CXCL10^{+} cells are astrocytes. **(C)** and **(D)** are phase contrast images, scale bars indicate 100 µm **(C, D)** and 25 µm **(E, F)**.

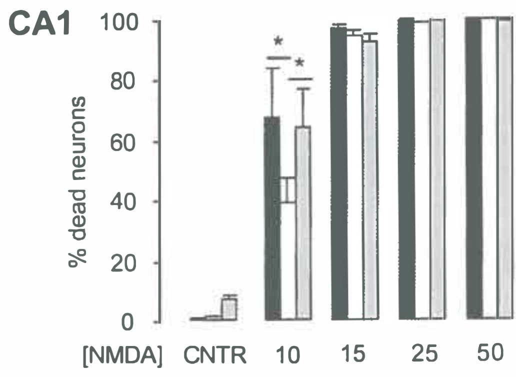
Figure 3.3 (right). Regional differences in cell death in NMDA-treated CXCL10$^{-/-}$ slice cultures compared to wild types. Graphs represent the percentages of neuronal cell death per hippocampal region (CA1, CA3, DG) in response to 0, 10, 15, 25 and 50 µM NMDA. CXCL10$^{-/-}$ OHSCs (open bars) showed significantly lower percentages in cell-death in response to 10 µM NMDA in the CA1 region ($p=0.035$), and in response to 15 µM NMDA ($p=0.001$) and 25 µM NMDA ($p<0.001$) in the CA3 region compared to wild types (closed bars). Treatment with 50 µM NMDA resulted in significantly higher cell death in the DG of CXCL10$^{-/-}$ OHSCs compared to wild types ($p=0.041$). Administration of 10^{-8} M recombinant CXCL10 (grey bars) directly after NMDA-treatment "rescued" the effects seen in CXCL10$^{-/-}$ OHSCs as it resulted in an increase in cell death in the CA1 and CA3, but not in the DG. This CXCL10-mediated increase in cell-death in CXCL10$^{-/-}$ OHSCs equalled wild type conditions. Data are a summary of three separate experiments with at least five slice cultures per condition. Bars indicate mean ± SEM. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, ANOVA.

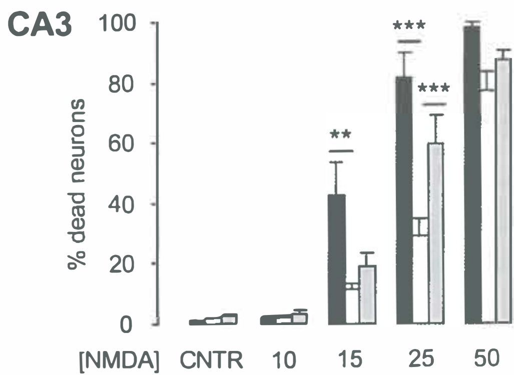
Regional changes in NMDA-induced cell death in CXCL10$^{-/-}$ slice cultures

To determine the role of astrocytic CXCL10 on NMDA-induced neuronal cell death, we prepared hippocampal slice cultures from CXCL10-deficient (CXCL10$^{-/-}$) mice and exposed these slice cultures to 0 (control), 10, 15, 25 and 50 µM NMDA to induce excitotoxicity. After six days in culture, CXCL10$^{-/-}$ slice cultures showed preserved hippocampal structure, and the basal rate of neuronal cell death in the three hippocampal regions was equal to control wild type conditions (CA1; 1.2 ± 0.3%, CA3; 1.6 ± 0.3%, DG; 1.9 ± 0.5%). Interestingly, in response to NMDA-treatment, regional differences in neuronal cell death were observed when compared to wild type conditions (Fig. 3.3). In the CA1 and CA3 region of CXCL10$^{-/-}$ slice cultures (Fig. 3.3, white bars) a significant decrease in neuronal vulnerability to NMDA was observed compared to wild types (Fig. 3.3, black bars), which was dependent on the NMDA-concentration. Neuronal cell death in response to 10 µM NMDA was significantly lower in the CA1 (38.8 ± 8.3%) compared to wild type conditions (67.1 ± 16.3%, $p=0.035$). In the CA3 region a decrease in neuronal cell death was observed in response to 15 µM NMDA (11.2 ± 1.9% versus 41.7 ± 11.3% in wild types, $p=0.001$) and in response to 25 µM NMDA (28.6 ± 5.6% versus 79.9 ± 8.6% in wild types, $p<0.001$).

Surprisingly, the detrimental effect of CXCL10 observed in the CA1 and CA3 regions was not detected in the DG in response 10-25 µM NMDA (Fig. 3.3; DG). In contrast, we observed a significant increase in neuronal cell death in the DG in response to 50 µM NMDA (53.3 ± 7.3% versus 30.7 ± 4.1% in wild types, $p=0.041$), suggesting that the presence of CXCL10 in wild type slice cultures has a protective effect on DG neurons at a high (50 µM) NMDA concentration.

■ wild type □ CXCL10-/- □ + rec. mCXCL10





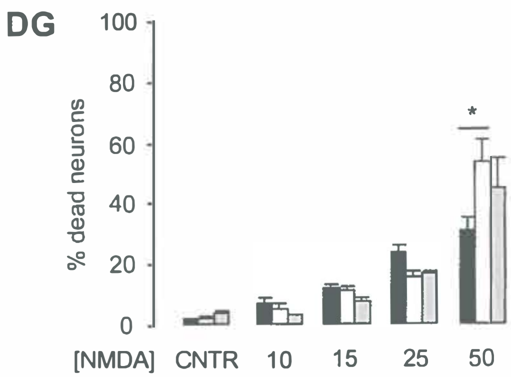


Figure 3.4 (right). Regional differences in cell death in NMDA-treated CXCR3$^{-/-}$ slice cultures compared to wild types. Graphs represent the percentages of neuronal cell death per hippocampal region (CA1, CA3, DG) in response to 0, 10, 15, 25 and 50 μM NMDA. Like in CXCL10$^{-/-}$ slice cultures, CXCR3$^{-/-}$ slice cultures (open bars) show significantly lower percentages in cell death in the CA1 region in response to 10 μM NMDA (*p*<0.001), and in the CA3 region in response to 15 μM NMDA (*p*<0.001) and 25 μM NMDA (*p*=0.038) compared to wild types (closed bars). Also here, a significant increase in neuronal cell death was observed at 50 μM NMDA in the DG region of CXCR3$^{-/-}$ OHSCs (*p*<0.001). Administration of 10^{-8} M recombinant CXCL10 directly after NMDA-treatment had no effect in CXCR3$^{-/-}$ OHSCs at any NMDA-concentration. Data are a summary of three separate experiments with at least five slice cultures per condition. Bars indicate mean ± SEM. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, ANOVA.

Application of CXCL10 to CXCL10$^{-/-}$ slice cultures mimics wild type conditions

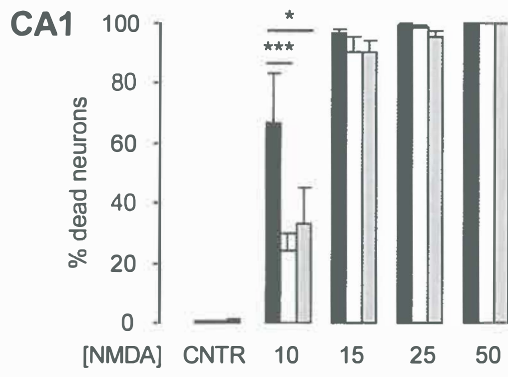
Application of 10^{-8} M recombinant CXCL10 to CXCL10$^{-/-}$ slice cultures directly after the NMDA-challenge (in the aim to "mimic" wild type conditions in response to NMDA) partly "rescued" the differences observed in CXCL10$^{-/-}$ slice cultures compared to wild type conditions (Fig. 3.3, grey bars). In the CA1 region, application of CXCL10 directly after treatment with 10 μM NMDA resulted in a significant increase (*p*=0.015) in neuronal cell death in the CXCL10$^{-/-}$ slice cultures, which was equivalent to wild type conditions (64.2 ± 12.3% versus 67.1 ± 16.3% in wild types, *p*=1.000). In the CA3 region, the effect of CXCL10 application was less pronounced after treatment with 15 μM NMDA in CXCL10$^{-/-}$ slice cultures (18.5 ± 4.5% versus 41.7 ± 11.3% in wild types, *p*=0.132). However, application of CXCL10 after treatment with 25 μM resulted in a strong increase (*p*<0.001) in neuronal cell death in the CA3 region of CXCL10$^{-/-}$ slice cultures, which again was equivalent to wild type conditions (58.2 ± 9.7% versus 79.9 ± 8.6% in wild types, *p*=0.277).

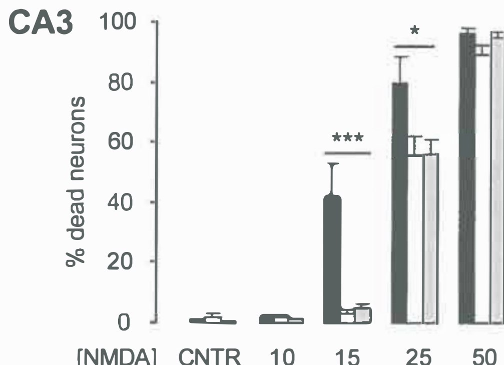
In the DG region, application of CXCL10 to CXCL10$^{-/-}$ slice cultures directly after treatment with 10-25 μM NMDA had no significant effect on neuronal cell death in this region (Fig 3.3). However, neuronal cell death in the DG induced by 50 μM NMDA in CXCL10$^{-/-}$ slice cultures (46.5 ± 5.4%) was slightly decreased by application of CXCL10 (44.7 ± 9.8%) and did not differ significantly anymore from wild type conditions (30.7 ± 4.1%, *p*=0.265).

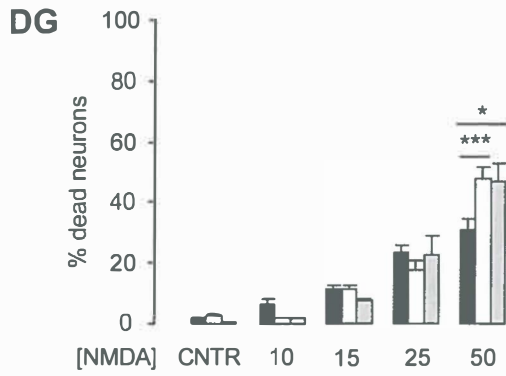
Regional changes in NMDA-induced cell death in CXCR3$^{-/-}$ slice cultures

To verify that the effects of CXCL10 on NMDA-induced excitotoxicity observed in the CXCL10$^{-/-}$ slice cultures are mediated through CXCR3 we performed experiments in

■ wild type □ CXCR3 -/- ▩ + rec. mCXCL10







CXCR3$^{-/-}$ slice cultures (Fig. 3.4). Also here, a decrease in neuronal sensitivity towards NMDA was observed in the CA1 and CA3 region, which was similar compared to the effects seen in the CXCL10$^{-/-}$ slice cultures. In response to 10 µM NMDA, neuronal cell death in the CA1 of CXCR3$^{-/-}$ slice cultures (open bars, 24.5 ± 5.5%) was significantly lower compared to wild types (black bars, 67.2 ± 16.3%, p<0.001). In addition, neuronal cell death in the CA3 was significantly lower upon treatment with 15 µM NMDA (3.3 ± 1.4% versus 41.7 ± 11.3% in wild types, p<0.001) and with 25 µM NMDA (55.7 ± 6.6% versus 79.9 ± 8.6% in wild types, p=0.038). Similar to CXCL10$^{-/-}$ slice cultures, an increase in cell death was observed in the DG of CXCR3$^{-/-}$ slice cultures in response to 50 µM NMDA (Fig. 3.4; 47.9 ± 3.7% versus 30.7 ± 4.1% in wild types, p<0.001).
Application of 10^{-8} M recombinant CXCL10 directly after the NMDA-insult (grey bars) for 24 hours had no effect on the amount of neuronal cell death in CXCR3$^{-/-}$ slice cultures at any condition, suggesting that the effects observed in CXCL10$^{-/-}$ slice cultures in response to CXCL10 application are mediated through CXCR3.

Activation of microglia coincides with regional differences in cell death in CXCR3$^{-/-}$ slice cultures

Acutely isolated microglia from wild type slice cultures (6 DIV) clearly expressed CXCR3 surface protein as determined by flow cytometry (Fig. 3.5A). Previous research has shown that microglia *in vitro* respond to CXCL10 with intracellular calcium transients and migration, processes both mediated through CXCR3 (Biber et al., 2001; Biber et al., 2002; Dijkstra et al., 2004; Rappert et al., 2004). Therefore, we hypothesized that abrogation of CXCR3-signaling could result in impaired microglia activation in CXCR3$^{-/-}$ slice cultures, partly explaining the differences in neuronal cell death observed in CXCR3$^{-/-}$ and wild type slice cultures. Treatment with 10 µM NMDA resulted in prominent cell death and local accumulation of morphologically activated microglia in the CA1 region of wild type slice cultures (Fig. 3.5B). As discussed above, neurons in the CA1 region of CXCR3$^{-/-}$ slice cultures were less affected by treatment with 10 µM NMDA when compared to wild type conditions. The lower level of neuronal cell death in CXCR3$^{-/-}$ slice cultures coincided with lower numbers of activated microglia in this region (Fig. 3.5C). At concentrations of 15-50 µM NMDA no distinction could be made in the number and activation of microglia in the CA1 region between wild type and CXCR3$^{-/-}$ slice cultures (data not shown). Similar effects were observed in the CA3 region in response to 15 µM NMDA, where a decrease in neuronal cell death in CXCR3$^{-/-}$ slice cultures (Fig.

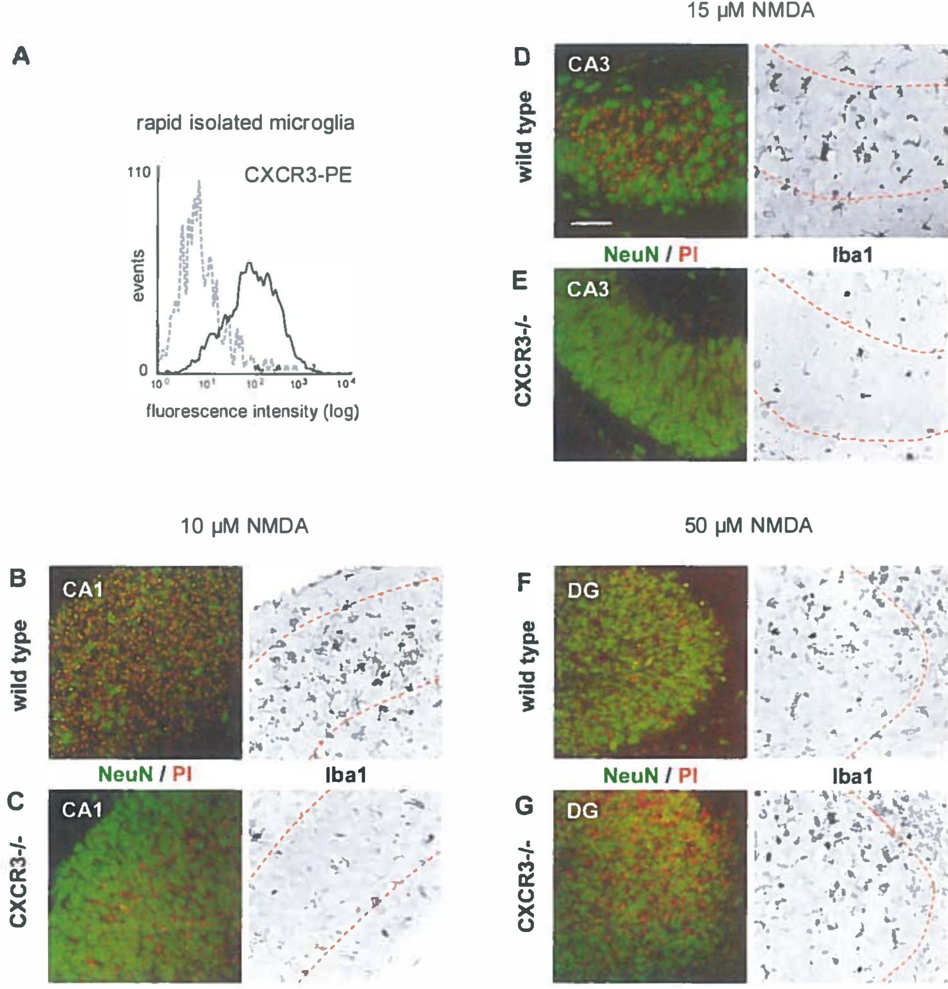


Figure 3.5. Differences in neuronal cell death in CXCR3$^{-/-}$ slice cultures coincide with differences in microglia activation. Rapidly isolated microglia from OHSCs express CXCR3 surface protein as determined by flow cytometry (**A**). Grey dotted line indicates isotype control, black line indicates CXCR3-PE. Confocal images B-G show the correlation between neuronal cell death (NeuN, green/PI, red) and microglia activation (Iba1, black) in wild type (B,D,F) and CXCR3$^{-/-}$ slice cultures (C,E,G) at 10 µM NMDA (CA1), 15 µM NMDA (CA3) and 50 µM NMDA (DG). Note that a decrease in neuronal cell death in CXCR3$^{-/-}$ slice cultures in the CA1 region (**C**) in response to 10 µM NMDA and in the CA3 region (**E**) in response to 15 µM NMDA coincides with a decrease in microglia activation compared to wild type conditions (**B**,**D**). In the DG region, no differences in microglia activation could be detected between wild type (**F**) and CXCR3$^{-/-}$ slice cultures (**G**) in response to 50 µM NMDA, although neuronal cell death was higher in CXCR3$^{-/-}$ slice cultures. Scale bar indicates 75 µm. Confocal images for Iba1 were grey-scaled and inversed.

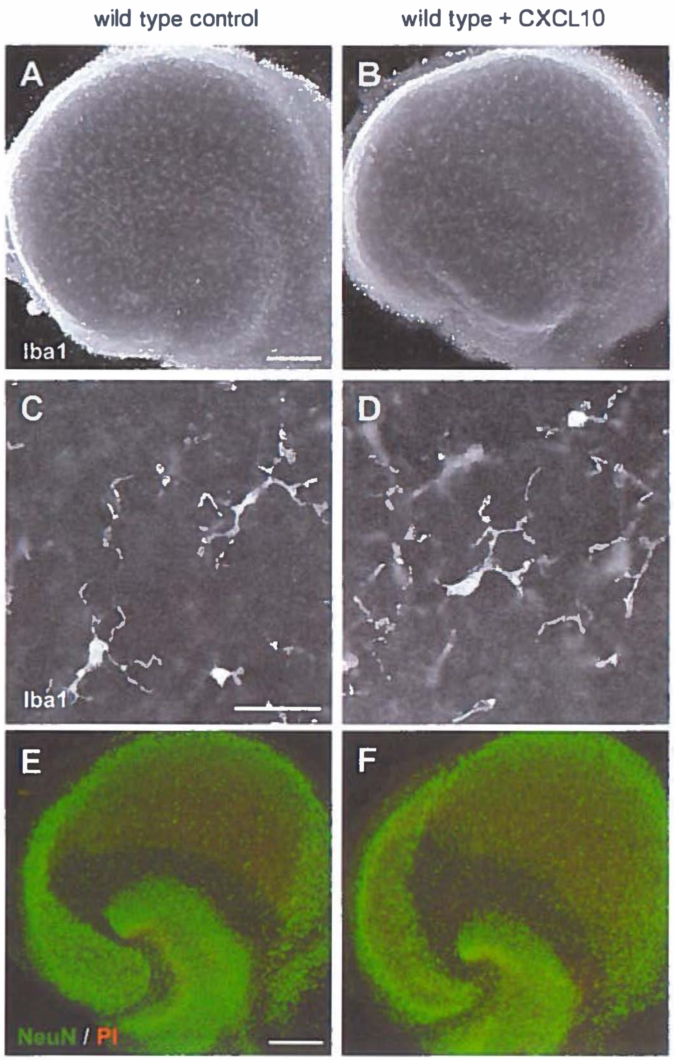


Figure 3.6. Application of CXCL10 alone does not induce microglia activation or neuronal cell death in wild type slice cultures. After 6 DIV, wild type OHSCs were treated with or without 10^{-8} M CXCL10 for 24 hours together with 5 µg/ml PI to asses the effects of CXCL10 on microglia activation and neuronal cell death. In both control (**A**) and CXCL10-treated slice cultures (**B**) no morphological activation of microglia (Iba1) could be detected as all microglia showed typical "ramified" morphology (**C**, **D**). No effect of CXCL10-treatment on neuronal (NeuN) cell death (PI) was observed (**F**) as compared to control conditions (**E**). Scale bars indicate 300 µm (**A**-**B**, **E**-**F**) and 30 µm (**C**, **D**).

3.5E) coincided with a decreased accumulation of microglia compared to wild type conditions (Fig. 3.5D). Also here, no differences in microglia activation were observed in the CA3 region in response to 25 and 50 μM NMDA when compared to wild type conditions (data not shown). In the DG region, no differences in microglia activation were observed between wild type (Fig. 3.5F) and CXCR3$^{-/-}$ (Fig. 3.5G) slice cultures in response to 50 μM NMDA. These results indicate that morphological activation and accumulation of microglia at sites of neuronal cell death were not impaired in CXCR3$^{-/-}$ slice cultures, but rather coincided with the delayed neuronal cell death observed in these slice cultures.

CXCL10 per se does not induce microglia activation or cell death in wild type slice cultures

Next, we determined whether application of CXCL10 alone would induce activation of microglia and/or neuronal cell death in wild type slice cultures. Treatment of wild type slice cultures with 10^{-8} M CXCL10 for 24 hours did not induce morphological activation of microglia (Fig. 3.6B/D) when compared to untreated controls (Fig. 3.6A/C). Microglia in CXCL10-treated slice cultures maintained their ramified morphology with small somata and secondary/tertiary branching, suggesting that CXCL10 per se is not sufficient to induce microglia activation (Fig. 3.6D). In addition, treatment with 10^{-8} M CXCL10 for 24 hours did not induce neuronal cell death in wild type slice cultures (Fig. 3.6F), demonstrating that the presence of CXCL10 *per se*, in the absence of an excitotoxic stimulus, does not induce neurodegeneration in this model.

Differences in neuronal cell death between wild type and CXCR3$^{-/-}$ slice cultures are fully abrogated by ablation of microglia

To further investigate the role of microglia in NMDA-induced excitotoxicity we depleted wild type- and CXCR3$^{-/-}$ slice cultures from their endogenous microglia population by treatment with liposomic clodronate (Lip-CL), without affecting other cell-types (Marin-Teva et al., 2004; Markovic et al., 2005), and subsequently exposed these slice cultures to various concentrations of NMDA (Fig. 3.7). The basal rate of neuronal cell death in microglia-depleted CXCR3$^{-/-}$ slice cultures (CA1; 1.0 ± 0.7%, CA3; 1.2 ± 0.4%; DG; 4.0 ± 2.0%, p=1.000) and wild type slice cultures (CA1; 2.5 ± 0.6%, CA3; 3.1 ± 0.8%; DG; 4.1 ± 1.0%, p=1.000) did not differ from untreated CXCR3$^{-/-}$ and wild type slice conditions,

Figure 3.7 (right). Differences in NMDA-induced cell death between wild type and CXCR3$^{-/-}$ slice cultures are fully abrogated in the absence of microglia. Graphs represent the percentages of neuronal cell death per hippocampal region (CA1, CA3, DG) in response to 0 (CNTR), 10, 15, 25 and 50 µM NMDA in microglia-depleted CXCR3$^{-/-}$ (CXCR3$^{-/-}$ Lip-CL) and wild type (WT Lip-CL) slice cultures. Depletion of the microglia population by treatment with Lip-CL for 24 hours did not induce significant cell death in any of the hippocampal regions and was equal in both conditions (CNTR). In the absence of microglia, the percentage of NMDA-induced neuronal cell death in the CXCR3$^{-/-}$ slice cultures (closed bars) was equivalent to that observed in microglia-depleted wild types slice cultures (open bars) at all concentrations in both CA1, CA3 and DG regions. Data are a summary of three individual experiments with at least six slice cultures per condition. Bars indicate mean ± SEM.

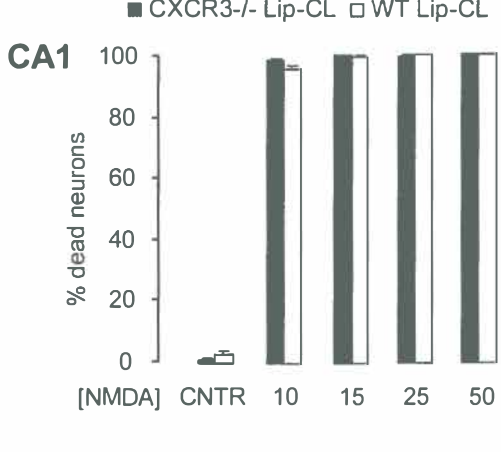
respectively (Fig. 3.7). Note that in response to NMDA-treatment, neuronal cell death was severely enhanced in the absence of microglia in both CXCR3$^{-/-}$ and wild type slice cultures, suggesting a profound neuroprotective role for microglia in excitotoxicity (Fig. 3.7, compare with Fig. 3.4). These results are in accordance with previous findings in our lab and have been discussed elsewhere (Van Weering, manuscript submitted). However, when comparing the percentages of neuronal cell death in microglia-depleted CXCR3$^{-/-}$ slice cultures (Fig. 3.7; black bars) with those in microglia-free wild type slice cultures (Fig. 3.7, white bars) in response to NMDA-treatment, no differences were found at any concentration in any hippocampal region.

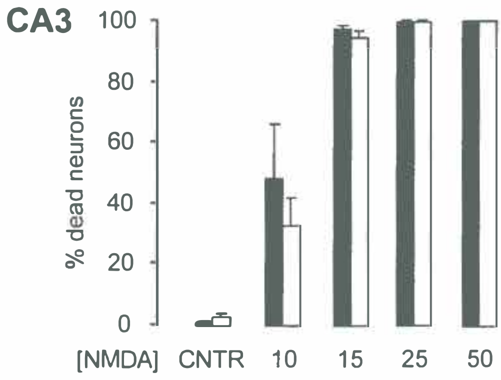
DISCUSSION

Expression of CXCL10 and its receptor CXCR3 in glia cells and/or neurons has been shown to correlate with clinical signs of many if not all CNS pathologies. However, the potential role for CXCL10 in neuron-glia and/or glia-glia signaling under pathological conditions is far from understood. Therefore, we studied the implication of CXCL10/CXCR3 signaling in a model for CNS excitotoxicity in mouse organotypic hippocampal slice cultures.

CXCL10 differently affects CA and DG neurons upon excitotoxicity

Our primary finding here is that CXCL10/CXCR3 signaling differentially affects neuronal survival in the three hippocampal regions upon NMDA-induced excitotoxicity. Accordingly, we demonstrated that neurons in the CA regions displayed a decreased vulnerability to NMDA-treatment in the absence of CXCL10 (CXCL10$^{-/-}$), suggesting that CXCL10 has a negative effect on the survival of these neurons upon excitotoxicity.


CXCR3-/- Lip-CL
WT Lip-CL
CA1
% dead neurons
100
80
60
40
20
0
[NMDA]
CNTR
10
15
25
50


CA3
% dead neurons
100
80
60
40
20
0
[NMDA]
CNTR
10
15
25
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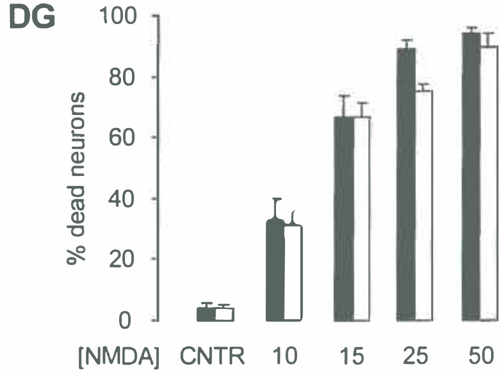
DG
% dead neurons
100
80
60
40
20
0
[NMDA]
CNTR
10
15
25
50

In contrast, abrogation of CXCL10 signaling did not desensitize neurons of the DG region in response to NMDA-induced excitotoxicity, but resulted in an increased vulnerability of these cell to high (50 µM) NMDA-concentrations. Interestingly, this differential neuronal vulnerability to CXCL10 observed in $CXCL10^{-/-}$ slice cultures was similar to that observed in $CXCR3^{-/-}$ slice cultures, suggesting that here CXCL10 mediates its actions through CXCR3. Accordingly, we demonstrated that application of exogenous CXCL10 directly after NMDA-treatment "rescued" the phenotype observed in $CXCL10^{-/-}$ slice cultures, but not in $CXCR3^{-/-}$ slice cultures, supporting these findings.
Our observations that exposure to exogenous CXCL10 only affected neuronal survival in the presence of NMDA and did not lead to neuronal cell death or glia activation in untreated (control) slice cultures suggests that CXCL10 *per se* does not induce neurodegeneration nor does it directly trigger glia activation under healthy conditions. Indeed, it has been shown previously that chronic expression of CXCL10 in the non-challenged CNS *in vivo* did not lead to the development of neuropathological signs (Boztug et al., 2002), corroborating our observations. Since it is not yet known through which cell types CXCL10 affects neuronal survival under excitotoxic conditions (see below) it is difficult at the moment to speculate why CXCL10 does not have any effect in untreated slice cultures. A possible explanation for this finding might be that NMDA treatment renders the slice cultures sensitive for CXCL10. In preliminary experiments we observed an up-regulation of CXCR3 mRNA in response to NMDA treatment (Van Weering et al., unpublished findings), which might corroborate this hypothesis. Alternatively, CXCL10 might only modulate the signaling cascade leading to excitotoxicity-induced neurodegeneration rather than being a direct cause of neuropathological signs in our paradigm. Studies that directly investigate these alternative hypotheses are at the moment ongoing in our laboratory.

Effects of CXCL10 on neurons

Although CXCL10 *per se* did not induce neuronal cell death, it is not unlikely that this chemokine might have direct effects on neurons as several studies have shown CXCR3-expression in these cells (Coughlan et al., 2000; Nelson and Gruol, 2004; Xia et al., 2000). Recently, it has been demonstrated that acute exposure of CXCL10 altered neuronal signaling properties in rat hippocampal neuron-glia cultures (Nelson and Gruol, 2004) and reduced long-term potentiation via CXCR3 in acute adult hippocampal slices (Vlkolinsky et al., 2004). Although in both studies it could not be excluded whether these

CXCL10-mediated neuronal effects were direct or mediated through glia cells, these findings suggest that CXCL10 can have a modulatory effect on neuronal properties in the hippocampus. In line with this, it has been shown very recently that exposure of rat hippocampal neuron-glia cultures to relatively high levels of CXCL10 for nine days resulted in (in)direct downregulation of synaptic proteins GAD65/67, $GABA_BR1$ and $GABA_AR\alpha2$ and upregulation of NMDAR1 and mGluR2/3 in the neuronal population (Cho et al., 2009). Whether such changes may account for the differences we observed in neuronal excitotoxicity between wild type slice cultures and CXCL10-/- and CXCR3-/- slice cultures is not yet clear. However, a CXCL10-induced downregulation of inhibitory synaptic proteins and upregulation of glutamate-subtype receptors in neurons could in part explain the relative insensitivity of CA neurons in $CXCL10^{-/-}$ and $CXCR3^{-/-}$ slice cultures towards NMDA and may also account for the increased vulnerability of these neurons in $CXCL10^{-/-}$ slice cultures when exposed to CXCL10.

CXCL10 is expressed in astrocytes in response to excitotoxicity

We demonstrated that upon NMDA-induced neuronal cell death CXCL10 is expressed in astrocytes, but not in neurons or microglia. Expression of CXCL10 in astrocytes *in vivo* has been observed in various excitotoxicity-related neuropathologies such as in stroke (Wang et al., 1998), encephalitis (Bhowmick et al., 2007), Alzheimer's disease (Xia et al., 2000), MS (Balashov et al., 1999; Omari et al., 2005; Simpson et al., 2000; Tanuma et al., 2006) and in it's experimental animal model EAE (Carter et al., 2007; Ransohoff et al., 1993). Similar to our findings, these studies demonstrated that local upregulation of CXCL10 expression in reactive astrocytes was highly correlated with sites of neuronal injury (Balashov et al., 1999; Carter et al., 2007; Omari et al., 2005; Ransohoff et al., 1993; Simpson et al., 2000; Tanuma et al., 2006; Wang et al., 1998; Xia et al., 2000), suggesting that the process of neurodegeneration itself may provide the signals which trigger CXCL10 expression in these cells. Possible signaling candidates under excitotoxic conditions are (pro-)inflammatory factors such as IFNβ1/2, IFNγ, IL-1α/β and TNFα, which all have been shown to induce CXCL10 expression in astrocytes *in vitro* (Croitoru-Lamoury et al., 2003; Oh et al., 1999; Omari et al., 2005; Salmaggi et al., 2002; Hua and Lee, 2000). However, direct evidence that these factors also stimulate astrocytic CXCL10 expression in response to excitotoxicity *in vivo* remains to be established.

Moreover, astrocytes have also been shown to express CXCR3 both *in vivo* (Omari et al., 2005; Simpson et al., 2000; Tanuma et al., 2006; Goldberg et al., 2001; Van der Meer et al., 2001) as *in vitro* (Biber et al., 2002; Croitoru-Lamoury et al., 2003; Flynn et al., 2003), suggesting that CXCL10 could have an auto-regulatory effect on these cells. However, we did not detect any abnormalities or differences in astrocyte activation in CXCR3$^{-/-}$ and CXCL10$^{-/-}$ slice cultures when compared to wild type conditions (data not shown), indicating that CXCR3 in astrocytes is not required for the activation of these cells under conditions of neuronal excitotoxicity.

Effects of CXCL10 on neuronal survival are most likely mediated through microglia
Microglia are considered to play a prominent role in excitotoxic-related injuries (Kohl et al., 2003; Lalancette-Hebert et al., 2007; Dehghani et al., 2003; Tikka and Koistinaho, 2001). Here, we demonstrated that selective ablation of microglia in hippocampal slice cultures prior to NMDA-treatment resulted in severely enhanced neuronal degeneration, supporting the notion that microglia have a basal neuroprotective role in excitotoxic-related injuries (Imai et al., 2007; Kitamura et al., 2004; Lalancette-Hebert et al., 2007; Turrin and Rivest, 2006; Xapelli et al., 2008). However, there are several lines of evidence that inhibition of microglia function is beneficial after acute CNS injury, supporting a neurotoxic role for microglia in this paradigm (Hailer et al., 2005; Hailer, 2008; Tikka and Koistinaho, 2001; Yrjanheikki et al., 1998). Indeed, it has been demonstrated that upon excitotoxicity activated microglia express pro-inflammatory factors, such as TNFα, IL-1, nitric oxide (NO), inducible nitric oxide synthase (iNOS) and cycloxygenase-2 (COX2), which are all toxic to neurons when expressed in large quantities (Aloisi, 2001; Barone and Feuerstein, 1999; Campuzano et al., 2008). Thus, microglia are not inevitably neuroprotective as certain conditions might trigger a (pro-)inflammatory phenotype in these cells that is detrimental for neuronal survival under excitotoxic conditions, showing the urgent need to identify the factors that control microglia activity.

The data presented here demonstrate that CXCL10/CXCR3 signaling might be one of the factors that affect microglia activity under excitotoxic conditions, leading to region-dependent effects on the survival of CA and DG neurons. Previously, it has been demonstrated that microglia functionally express CXCR3 *in vitro* (Biber et al., 2001; Biber et al., 2002; Dijkstra et al., 2004; Rappert et al., 2002). We confirmed these findings by flow cytometry as acutely isolated microglia from wild type hippocampal slice cultures

expressed CXCR3. The observation that the accumulation of microglia at sites of neuronal cell death was not impaired in CXCR3$^{-/-}$ slice cultures suggests that CXCL10/CXCR3 signaling is not a prerequisite for microglia recruitment in response to excitotoxicity. In line with this evidence, it has been described recently that the migration of microglia towards injured neurons in the hippocampus depends on purinergic signaling, as induced degradation of extracellular ATP/ADP inhibited directed migration of microglia towards sites of neurodegeneration (Kurpius et al., 2007). However, since differences in neuronal cell death observed between wild type and CXCR3$^{-/-}$ slice cultures were fully abrogated in the absence of microglia, it suggests that neuronal survival under excitotoxic conditions is affected through microglial CXCR3, which is in agreement with earlier studies from our group (Rappert et al., 2004). Why CXCL10/CXCR3 signaling is detrimental in the CA regions of the hippocampus and protective in the DG region is not understood. Regional differences in the response of microglia to CXCR3-stimulation might be instrumental here. Alternatively, the differential effects of CXCL10/CXCR3 signaling on DG and CA neurons might depend on regional differences in co-expression of local factors that modulate the pro-inflammatory activity of CXCL10-stimulated microglia. More experiments are necessary to address these concepts.

In summary, our results identify a region-specific role for CXCL10/CXCR3 signaling in neuron-glia and glia-glia interactions under pathological conditions. Since ablation of microglia abrogated the differences in neuronal cell death between wild type and CXCR3-/- slices, an important role for microglial CXCR3 in neuronal excitotoxicity in the hippocampus is suggested.

Acknowledgements

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Chemokine CCL21 negatively affects neuronal survival in a model for hippocampal excitotoxicity

Hilmar R.J. van Weering *(1)*; Hendrikus W.G.M. Boddeke *(1)*; Knut P.H. Biber *(1,2)*

(1) Department of Neuroscience, Section Medical Physiology, University Medical Center Groningen (UMCG), Rijksuniversiteit Groningen (RUG), Groningen, The Netherlands.
(2) Molecular Psychiatry, Psychiatry and Psychotherapy, University of Freiburg, Freiburg, Germany.

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ABSTRACT

Neuronal injury in the CNS inevitably results in the local accumulation of activated microglia, indicating that damaged neurons release signals that activate and/or attract microglia. Recently, it has been demonstrated that neurons rapidly express and release the chemokine CCL21 upon injury. Moreover, it has been demonstrated that CCL21 activates microglia *in vitro,* a process dependent on the presence of chemokine receptor CXCR3. It is therefore suggested that CCL21/CXCR3 might provide a signaling system between neurons and local glia cells that is associated with a neuroimmune response under pathological conditions. However, the effect of CCL21/CXCR3-signaling in the CNS has not been investigated yet. Therefore, we have determined the effect of CCL21-signaling in an *ex vivo* model for *N*-methyl-D-aspartic acid (NMDA)-induced excitotoxicity using mouse organotypic hippocampal slice cultures. Treatment of wild type slice cultures with 10, 15, 25 and 50 μM NMDA for 4 hours and subsequent culturing for 24 hours in standard culture medium resulted in region-selective loss of neurons in the hippocampal regions CA1, CA3 and DG. To determine the role of CCL21 and its receptor CXCR3 in this process, we prepared slice cultures from *plt* (CCL21/CCL19-deficient) and CXCR3-deficient ($CXCR3^{-/-}$) mice and subsequently exposed these slice cultures to NMDA-induced excitotoxicity. Our findings demonstrate that in the absence of CCL21, NMDA-induced neuronal cell death was significantly attenuated in the CA regions when compared to wild type conditions, while concomitant application of 10^{-8} M recombinant CCL21 for 24 hours rescued this phenotype. Similar results were obtained in $CXCR3^{-/-}$ slice cultures, although application of CCL21 had no effect on the rate of NMDA-induced neuronal loss in these slice cultures. Taken together, our data demonstrate that CCL21-signaling in response to excitotoxic injury is associated with a neurotoxic response, a process most likely mediated through CXCR3.

INTRODUCTION

Chemokines and their receptors have been implicated as important mediators in cell-to-cell signaling both in the periphery (Campbell et al., 2003; Rot and von Andrian, 2004) as in the central nervous system (CNS) (Ambrosini and Aloisi, 2004; Cartier et al., 2005; de Haas et al., 2007). Chemokines are small chemotactic cytokines (8-14 kDa) and are classified into four distinct subclasses (CXC, CC, XC and CX3C) based on their conserved cysteine residue structure in the N-terminus (Bacon et al., 2002; Murphy et al., 2000). In general, members of these subclasses mediate their actions through their respective CXC-, CC-, XC- and CX3C receptors. One exception so far is chemokine C-C motif ligand 21 or CCL21 (formerly known as TCA-4, Exodus-2, 6CKine, Secondary Lymphoid-organ Chemokine (SLC)), which not only binds its primary receptor CCR7, but also binds and activates a member of the CXC-chemokine receptor family, namely CXCR3 (Soto et al., 1998).

In the periphery, CCL21 is constitutively expressed in secondary lymphoid organs and controls the homing of mature dendritic cells and of naive T- and B-cells (Dieu et al., 1998; Sallusto et al., 1998; Stein et al., 2000; Yanagihara et al., 1998). Accordingly, it has been demonstrated that mice lacking CCL21 (and CCL19) show aberrations in the recruitment of naive T cells and activated dendritic cells to secondary lymphoid organs (Mori et al., 2001). However, recent findings suggest that CCL21 is also implicated in the development of several CNS pathologies. Elevated levels of CCL21 have been found in the cerebrospinal fluid (CSF) of patients suffering from multiple sclerosis (MS), an auto-immune disease characterized by infiltration of auto-reactive T cells into the CNS (Pashenkov et al., 2003). In line with this, increased expression of CCL21 has been demonstrated in an animal model for MS, known as experimental autoimmune encephalomyelitis (EAE) (Alt et al., 2002; Columba-Cabezas et al., 2003). CCL21 expression has been shown in the endothelium of inflamed blood vessels (Columba-Cabezas et al., 2003) and venules of inflammatory cuffs surrounded by CCR7-positive cell-infiltrates (Alt et al., 2002). As desensitization with CCL21 or blocking of CCL21 with specific antibodies strongly reduced adhesion strengthening of T-cells to inflamed vessels in EAE, it was proposed that CCL21 facilitates the infiltration of T-cells into the CNS under pathological conditions (Alt et al., 2002). However, this suggestion is not without argument as transgenic over-expression of CCL21 in mouse brain was not sufficient to recruit lymphocytes into the CNS (Chen et al., 2002). Although these mice

developed a strong neuropathology characterized by neurobehavioral deficits and reactive gliosis, leukocytic infiltrates into the CNS consisted primarily of neutrophils and eosinophils (Chen et al., 2002).

Recent findings suggest an intrinsic role for CCL21 in the CNS as a communication signal between damaged neurons and surrounding glia cells under neuropathological conditions (Biber et al., 2001; de Jong et al., 2005; Dijkstra et al., 2004; Rappert et al., 2002; Zhao et al., 2007). Generally, any type of neuronal injury induces activation and recruitment of microglia towards the site of injury, suggesting that endangered neurons emit signals that attract/activate local microglia (Biber et al., 2008; de Haas et al., 2007; Hanisch and Kettenmann, 2007). One of these signals might be CCL21 as it is expressed by damaged neurons *in vivo* early after onset of ischemia and in response to spinal cord injury (Biber et al., 2001; Zhao et al., 2007). In addition, neuronal expression of CCL21 has been reported in hippocampal slice cultures and primary neuronal cultures in response to toxic levels of glutamate (de Jong et al., 2005). Interestingly, CCL21 has been shown to induce activation and migration of human and mouse microglia *in vitro* (Biber et al., 2001; Dijkstra et al., 2004; Rappert et al., 2002). These findings are corroborated *in vivo* as injection of recombinant CCL21 into the thalamus of intact mice resulted in transient activation of microglia in this region (Zhao et al., 2007). An interesting feature here is that the activation of microglia in response to CCL21 *in vitro* has been shown not to be dependent on its native receptor CCR7, but on the presence of CXCR3 (Biber et al., 2001; Dijkstra et al., 2004; Rappert et al., 2002).

Taken together, these results suggest that the chemokine CCL21 and its receptor CXCR3 could provide a signaling system between neurons and local glia cells that is associated with a neuroimmune response under pathological conditions. However, the role of CCL21/CXCR3-signaling between neurons and glia cells under neuropathological conditions has not been investigated yet. Therefore, we have investigated the implication of CCL21-signaling in an *ex vivo* excitotoxicity model for neurodegeneration using mouse organotypic hippocampal slice cultures.

EXPERIMENTAL PROCEDURES

Animals

All experiments have been approved by the Dutch animal experimental committee. The C57BL/6J (wild type, Harlan), CXCR3-deficient ($CXCR3^{-/-}$) and CCL21/CCL19-deficient

(*plt*) mice were housed and handled in accordance with the guidelines of the central animal laboratory (CDL) facility of Groningen. The CXCR3$^{-/-}$ mice were kindly provided by B. Lu and C. Gerard (Children's Hospital, Harvard Medical School, Boston, MA).

Chemicals

Culture media and supplements were all obtained from GIBCO® (Invitrogen Corporation; Breda, The Netherlands), unless mentioned otherwise. Recombinant murine CCL21 and CCL19 were obtained from PeproTech® (London, United Kingdom).

Preparation of organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures (OHSCs) were prepared as described with minor modifications (Stoppini et al., 1991). In brief, OHSCs were prepared from 2-3 day old C57BL/6J, *plt* and CXCR3$^{-/-}$ mouse pups under sterile conditions. After decapitation, the brains were rapidly removed and the hippocampi from both hemispheres were acutely isolated in ice cold serum-free Hank's Balanced Salt Solution (HBSS), supplemented with 0.5% glucose (Sigma) and 15 mM HEPES. Isolated hippocampi were cut into 350-375 µm thick slices using a tissue chopper (McIlwain) and were transferred to 0.4 µM culture plate inserts (Millipore, PICM03050). These culture plate inserts, containing four to six slices, were placed in six-well plates containing 1.2 ml of culture medium per well. Culture medium (pH 7.2) consisted of 0.5x minimum essential medium containing 25% heat-inactivated horse serum, 25% BME basal medium without L-glutamine, 2 mM glutamax and 0.65% glucose (Sigma). The slice cultures were kept at 35°C in a humidified atmosphere (5% CO_2) and the culture medium was refreshed the first day after preparation and every consecutive two days.

Induction of excitotoxicity in OHSCs

After 6 days in culture, OHSCs were placed in culture medium containing 0, 10, 15, 25 or 50 µM *N*-methyl-D-aspartic acid (NMDA; Sigma) for 4 hours to induce excitotoxicity and subsequently the medium was replaced with standard culture medium. NMDA-treated OHSCs were kept in culture for 24 hours after the NMDA challenge.

Immunohistochemistry

For immunohistochemical analysis, control and NMDA-challenged OHSCs were shortly rinsed in phosphate-buffered saline (PBS, 35°C) and fixated with 4% paraformaldehyde

overnight at 4°C. After fixation, the slice cultures were rinsed in PBS and pre-incubated with 5% normal goat serum (NGS; Vector) in PBS containing 0.3% Triton X-100 (PBS^{+}) for at least one hour. Subsequently, the slice cultures were incubated with the appropriate primary antibody overnight in 1% NGS/PBS^{+} at 4°C. For detection of neuronal nuclei, mouse-anti-NeuN (1:1000; Chemicon MAB377) was used with donkey-anti-mouse-Alexa488 (Molecular Probes) as a secondary antibody. Analysis of the slice cultures was done by confocal imaging using a Leica SP2 AOBS system (Leica Microsystems).

Quantification of neuronal cell death

To determine neuronal cell death in response to NMDA-induced excitotoxicity, OHSCs were incubated with 5 µg/ml propidium iodide (PI) during and after the NMDA-challenge (Pozzo Miller et al., 1994; Vornov et al., 1991). Confocal images of the neuronal layers were taken mid-section at 40x magnification and both NeuN^{+} and NeuN^{+}/PI^{+} cells were quantified using ImageJ software. The percentage of neuronal cell death was determined by the number of NeuN^{+}/PI^{+} cells divided by the total number of NeuN-positive cells per neuronal layer.

Quantative polymerase chain reaction (QPCR)

For RNA analysis, OHSCs (6 slice cultures per condition) were lysed in guanidinium isothiocyanate/ monopthiolglycerol buffer (both from Sigma) and total RNA was extracted and precipitated with a one-chloroform-phenol step (Chomczynski and Sacchi, 1987). The quality and quantity of the total RNA were determined with the Agilent 2100 bioanalyzer and the NanoDrop® ND-1000. RNA integrity number (RIN) values were between 9.9 and 8.8 and the 260/280 nm ratios around 1.9. 1 µg of total RNA was transcribed into cDNA as described previously (Biber et al., 1997). Amplification reactions were performed in a total volume of 10 µl containing 0.8 µl cDNA solution, 5.0 µl TaqMan® universal PCR master mix, 0.5 µl TaqMan® primer-probe (Applied biosystems: β-actin, Mm006 07939_s1; CXCR3, Mm00438259_m1; CCR7, Mm01301785_m1; CCL21, assay by design: intron-spanning, lot. 439860) and 3.7 µl pure water (Sigma). Reactions were run in triplets on the ABI Prism® 7900 HT real time PCR instrument. After 15 minutes at 95ºC, the cDNA samples were run for 40 cycles, consisting of 15 seconds at 95ºC for denaturation and 1 min at 60ºC for annealing and elongation. Threshold cycles were determined manually by setting thresholds for

fluorescence intensity. Relative gene expression levels were analysed by the 2-ΔΔCT method, using ß-actin as a reference gene (Livak and Schmittgen, 2001). The amplification efficiency was verified by linear regression with the fluorescence per cycle number (Ramakers et al., 2003).

Statistical Analysis

Data are represented as the mean ± standard error of the mean (SEM). In the neuronal cell death experiments statistical comparison between groups was performed using one-way analysis of variance (ANOVA) with Gabriel's posthoc test. In the real-time PCR experiments statistical comparison between groups was performed using the two-tailed Students' *t*-test. In both tests, *p*-values smaller than 0.05 were considered significant. All statistical tests were performed in SPSS version 14.0.2 (SPSS Inc., Chicago).

RESULTS

NMDA-induced neuronal cell death is attenuated in the absence of CCL21/CCL19 (plt)

In order to determine the effects of CCL21 on NMDA-induced neuronal cell death we prepared hippocampal slice cultures from wild type and *plt* (paucity of lymph node T cells) mice, which carry a natural mutation resulting in CCL21/CCL19-expression deficiency (Luther et al., 2000; Nakano and Gunn, 2001; Vassileva et al., 1999), and exposed these slice cultures to 0 (control), 10, 15, 25 and 50 μM NMDA to induce excitotoxicity. After six days in culture, the basal rate of neuronal cell death in (control) *plt* slice cultures (Fig. 4.1; open bars) was equal to control wild type conditions (CA1; 0.7 ± 0.1%, CA3; 1.7 ± 0.4%, DG; 1.4 ± 0.3%, all p=1.000). However, in response to NMDA-treatment clear differences in neuronal cell death were observed between *plt*- and wild type slice cultures in the CA1 (Fig. 4.1A) and CA3 regions (Fig. 4.1B). Neuronal cell death in response to 10 μM NMDA was significantly lower in the CA1 (Fig. 4.1A) of *plt* slice cultures (22.4 ± 6.1%) when compared to wild type conditions (67.1 ± 16.3%, $p<0.001$). Treatment with 15 μM NMDA or higher resulted in maximum levels of neuronal cell death (between 95-100%) in the CA1 region in both *plt* and wild type slice cultures. Similar decreases in neuronal cell death were observed in the CA3 region (Fig. 4.1B) as neuronal cell death was markedly lower in *plt* slice cultures in response to 15 μM NMDA (10.4 ± 3.3% compared to 41.7 ± 11.3% in wild types, $p<0.001$) and in response to 25 μM NMDA (39.0 ± 4.6% compared to 79.9 ± 8.6% in wild types, $p<0.001$).

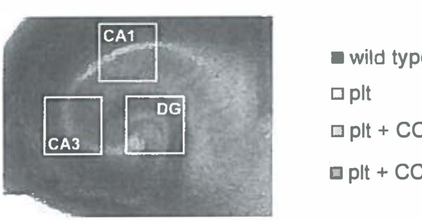
Figure 4.1 (right). CCL21, but not CCL19, negatively affects neuronal survival upon NMDA-induced excitotoxicity. Graphs represent the percentages of neuronal cell death per hippocampal region (**A**; CA1, **B**; CA3, **C**; DG) in response to 0 (CNTR), 10, 15, 25 and 50 µM NMDA in wild type (closed bars) and *plt* slice cultures (open bars) and in *plt* slice cultures treated with 10^{-8} M CCL21 (light-grey bars) or 10^{-8} M CCL19 (dark-grey bars). Top: microscopic image of a hippocampal slice culture showing the localization of the neuronal subregions of interest (CA1, CA3 and DG). In *plt* slice cultures, neuronal cell death induced by NMDA-treatment was significantly lower in the CA1 region (**A**) at 10 µM NMDA (p<0.001) and in the CA3 region (**B**) at 15 µM (p<0.001) and 25 µM NMDA (p<0.001) when compared to wild type conditions. In the DG region (**C**), no differences in neuronal cell death were observed between *plt*- and wild type slice cultures at any NMDA-concentration. Application of 10^{-8} M CCL21 directly after NMDA-treatment for 24 hours partly "rescued" the effects observed in *plt* slice cultures as neuronal cell death in the CA1 region (**A**) at 10 µM (p<0.001) and in the CA3 region (**B**) at 25 µM (p<0.001) was significantly enhanced in the presence of recombinant CCL21. This enhanced NMDA-induced neuronal cell death in the presence of recombinant CCL21 equalled wild type conditions (all p=1.000). Moreover, neuronal cell death in the DG region in the presence of recombinant CCL21 was not only significantly higher in *plt* slice cultures at 25 µM (p<0.001) and at 50 µM NMD (p=0.019), but also higher when compared to wild type conditions (p=0.042 and p<0.001, respectively). Application 10^{-8} M CCL19 directly after NMDA-treatment had no effect on the number of dying neurons in *plt* slice cultures at any NMDA-concentration. Data are a summary of three separate experiments with at least five slice cultures per condition. Bars indicate mean ± SEM. $^{*}p$<0.05, $^{**}p$<0.01, $^{***}p$<0.001, ANOVA.

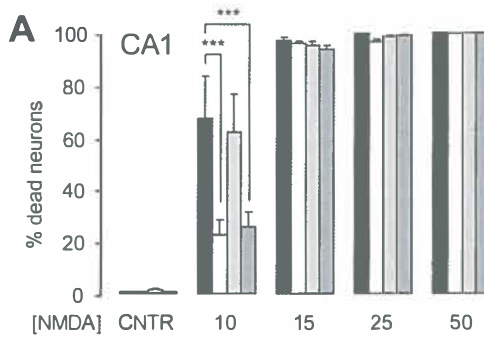
In contrast, in the DG region no significant differences in neuronal cell death were observed between *plt* and wild type slice cultures at any NMDA concentration (Fig. 4.1C).

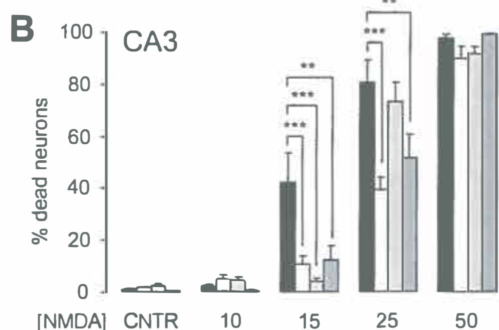
CCL21, but not CCL19, enhances NMDA-induced cell death in plt-derived OHSCs

As a clear decrease in neuronal cell death was observed in the CA regions of NMDA-treated *plt* slice cultures when compared to wild type conditions, we determined whether this effect was due to a lack of CCL21 and/or CCL19. For this purpose we treated *plt*-derived slice cultures with 0 (control), 10, 15, 25 or 50 µM NMDA for 4 hours to induce excitotoxicity and subsequently exposed these slice cultures to either 10^{-8} M recombinant mouse CCL21 or 10^{-8} M CCL19 for 24 hours. After 24 hours the slice cultures were fixated and the percentages of neuronal cell death per hippocampal region were determined.

Application of 10^{-8} M CCL21 alone to *plt*-derived slice cultures did not result in significant changes in the basal rate of neuronal cell death in the three hippocampal regions, when compared to control *plt* conditions (CA1; 1.4 ± 0.6%, CA3; 2.4 ± 0.9%, DG; 0.7 ± 0.2%, all p=1.000). In contrast, application of CCL21 directly after NMDA-treatment clearly enhanced neuronal cell death in these slices cultures.


CA1
DG
CA3
wild type
plt
plt + CCL21
plt + CCL19


A
CA1
% dead neurons
[NMDA]
CNTR
10
15
25
50


B
CA3
% dead neurons
[NMDA]
CNTR
10
15
25
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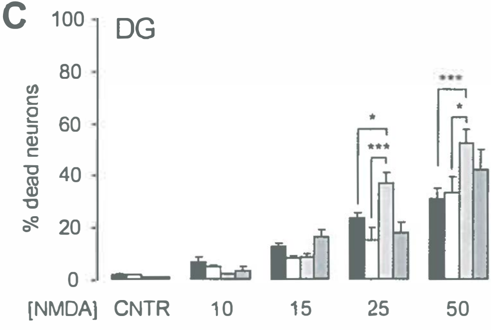

C
DG
% dead neurons
[NMDA]
CNTR
10
15
25
50

In the CA1 region, application of 10^{-8} M CCL21 directly after treatment with 10 µM NMDA (Fig. 4.1A; light-grey bars) resulted in a significant increase in neuronal cell death in *plt*-derived slice cultures (62.0 ± 14.5%, $p<0.001$), which was equivalent to the percentage of cell death observed in wild types (67.1 ± 16.3%, $p=1.000$). In the CA3 region, no significant effect of CCL21 application was observed after treatment with 15 µM NMDA in *plt*-derived slice cultures (4.0 ± 1.0% compared to 10.4 ± 3.3%, $p=0.999$). However, application of CCL21 directly after treatment with 25 µM NMDA resulted in a significant increase in neuronal cell death (72.3 ± 7.5%, $p<0.001$) in the CA3 region when compared to non-treated *plt* slice cultures (39.0 ± 4.5%), which was again equivalent to wild type conditions (79.9 ± 8.6%, $p=1.000$)

Moreover, an effect of CCL21 after NMDA treatment was observed in the DG region (Fig. 4.1C) as application of CCL21 significantly enhanced neuronal cell death after treatment with 25 µM NMDA (36.6 ± 4.3% compared to 14.9 ± 4.8% in *plt* slice cultures, $p<0.001$) and after treatment with 50 µM NMDA (51.9 ± 5.4% compared to 33.1 ± 6.0% in *plt* slice cultures, $p=0.019$). Enhanced neuronal cell death in the DG in the presence of CCL21 in *plt* slice cultures even exceeded percentages of cell death found in wild type slice cultures in response to 25 µM NMDA ($p=0.042$) and 50 µM NMDA ($p<0.001$).

Application of 10^{-8} M CCL19 alone to *plt*-derived slice cultures did not induce neuronal cell death in the various hippocampal regions (CA1; 0.7 ± 0.3%, CA3; 0.3 ± 0.1%, DG; 0.7 ± 0.2%), nor did application of CCL19 influence the percentage of neuronal cell death after treatment with 10-50 µM in these regions (Fig. 4.1; dark-grey bars), indicating that the decreased neuronal cell death observed in *plt*-derived slice cultures is solely due to a lack of CCL21 expression, but not of CCL19.

CXCR3, but not CCR7, is expressed in hippocampal slice cultures

In order to determine which chemokine receptor might be involved here, we analyzed the expression of chemokine receptors CCR7 and CXCR3, which are both known to bind CCL21, in the slice cultures. For this purpose, we analyzed the presence of CXCR3 and CCR7 mRNA by means of real-time PCR in total slice culture RNA lysates of control wild type slice cultures and of NMDA-treated slice cultures at set time-points (0, 4, 8, 24 and 48 hours after induction). CXCR3 mRNA was readily detected in control conditions (Fig. 4.2A) and was present up to 48 hours after the NMDA-treatment. CXCR3 mRNA expression levels did not change significantly at 4 ($p=0.084$), 8 ($p=0.308$), 24 ($p=0.192$)

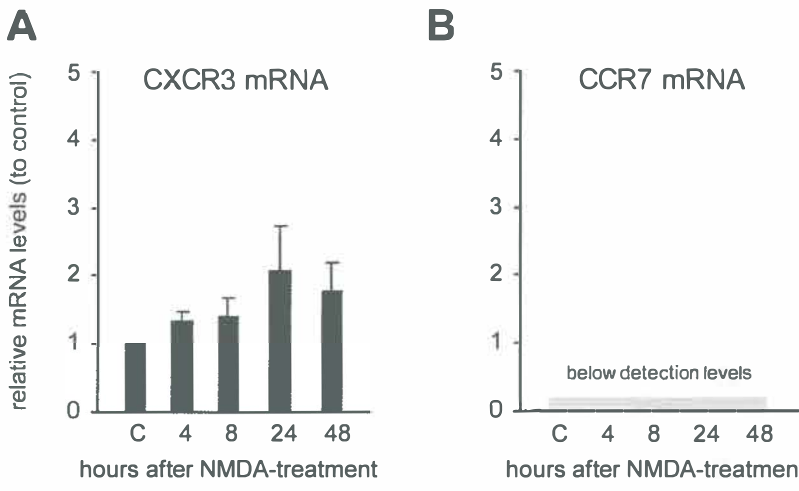


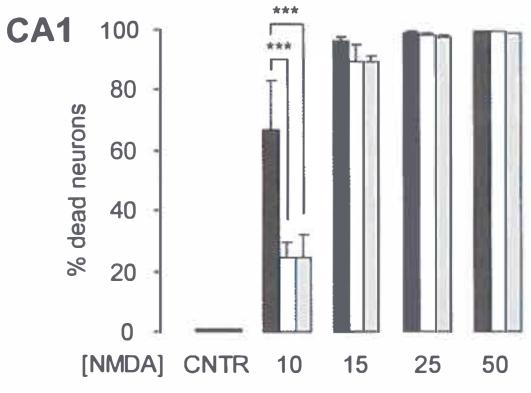
Figure 4.2. Constitutive expression of CXCR3, but not CCR7, in OHSCs. Graphs represent the relative mRNA expression levels of CXCR3 (**A**) and CCR7 (**B**), as determined by QPCR analysis, in whole slice culture lysates of control (c) wild type slice cultures and of slice cultures at 4, 8, 24, and 48 hours after excitotoxicity induction with 50 μM NMDA. Real-time PCR analysis revealed CXCR3 mRNA expression in both control and NMDA-treated slice cultures (**A**). Levels of CXCR3 mRNA did not change significantly at 4 (p=0.084), 8 (p=0.308), 24 (p=0.192) or 48 hours (p=0.172) after excitotoxicity-induction when compared to control levels. In contrast, CCR7 mRNA was below detection levels in control slice cultures (**B**), and could also not be detected in response to NMDA-treatment at any time-point. Relative mRNA expression levels were determined as the expression of CXCR3 or CCR7 normalized to β-actin and related to non-treated control conditions. Data are a summary of four separate experiments. Bars indicate mean ± SEM.

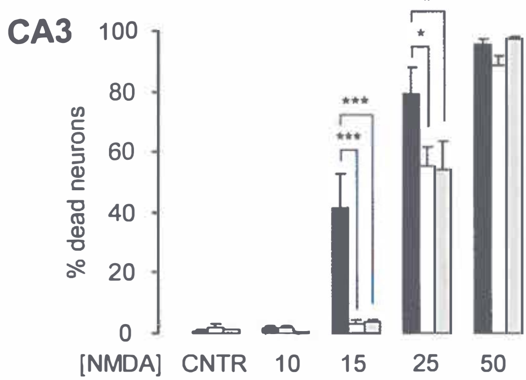
or 48 hours (p=0.172) after the start of the NMDA-treatment. In contrast, CCR7 mRNA could not be detected in control slice cultures (Fig. 4.2B). Neither did we observe CCR7 mRNA in NMDA-treated slice cultures at 4, 8, 24 or 48 hours after induction (Fig. 4.2B), while CCR7 mRNA could be readily detected in thymus tissue, which served as a positive control (data not shown). These results show that CXCR3, but not CCR7, is expressed in our slice culture system.

CCL21-induced effects mediated through CXCR3?

Based on the real-time PCR findings we hypothesized that the observed effects of CCL21 on NMDA-induced neuronal degeneration are mediated through CXCR3 as CCR7 was not detected in the slice cultures. Previously, we have determined the role of

■ wild type □ CXCR3-/- □ CXCR3-/- + CCL21





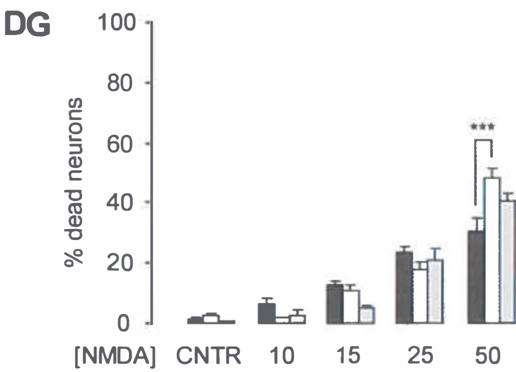


Figure 4.3 (left). Application of CCL21 directly after NMDA-treatment has no effect on excitotoxicity-induced neuronal cell death in CXCR3$^{-/-}$ OHSCs. Graphs represent the percentages of neuronal cell death per hippocampal region (CA1, CA3, DG) in response to 0 (CNTR), 10, 15, 25 and 50 µM NMDA in wild type (closed bars) and CXCR3$^{-/-}$ slice cultures (open bars) and in CXCR3$^{-/-}$ slice cultures treated with 10^{-8} M recombinant CCL21 directly after NMDA-treatment (grey bars). Similar to *plt* slice cultures, neuronal cell death in CXCR3$^{-/-}$ slice cultures was significantly lower in the CA1 region at 10 µM NMDA and in the CA3 region at 15 µM and 25 µM NMDA when compared to wild type conditions. In contrast, neuronal cell death in the DG region was higher in response to 50 µM NMDA in CXCR3$^{-/-}$ slice cultures. Application of CCL21 directly after NMDA-treatment had no significant effect on the percentage of dying neurons observed in CXCR3$^{-/-}$ slice cultures in any hippocampal region. Data are a summary of three separate experiments with at least five slice cultures per condition. Bars indicate mean ± SEM. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, ANOVA.

CXCR3 in NMDA-induced excitotoxicity using slice cultures derived from CXCR3-deficient (CXCR3$^{-/-}$) mice (Van Weering et al., manuscript in press). Interestingly, we observed differences in NMDA-induced neuronal cell death in the absence of CXCR3 in all three hippocampal regions when compared to wild type conditions, which are similar to the differences we observed in the *plt* slice cultures (Fig. 4.3). Like *plt*-derived slice cultures, CXCR3$^{-/-}$ slice cultures showed significantly lower percentages in neuronal cell death in the CA1 region in response to 10 µM NMDA ($p<0.001$), and in the CA3 region in response to 15 µM NMDA ($p<0.001$) and 25 µM NMDA ($p=0.038$) when compared to wild type conditions (Fig. 4.3). Additionally, a significant increase in neuronal cell death was observed at 50 µM NMDA in the DG region of CXCR3$^{-/-}$ slice cultures ($p<0.001$), an effect we did not observe in *plt* slice cultures (see Fig. 4.1C).
Interestingly, in CXCR3$^{-/-}$ slice cultures application of 10^{-8} M CCL21 directly after NMDA-treatment had no effect on neuronal cell death in the CA1 and CA3 regions, nor in the DG region (Fig. 4.3; grey bars), whereas in *plt* slice cultures a clear increase in neuronal cell death was observed in the presence of recombinant CCL21 (see Fig. 4.1).

CCL21 mRNA levels are significantly lower in CXCR3$^{-/-}$ OHSCs

Next, we determined the expression of CCL21 mRNA in both wild type- and CXCR3$^{-/-}$ slice cultures by means of QPCR analysis. CCL21 mRNA was clearly detected in total RNA lysates from untreated (control) wild type slice cultures (Fig. 4.4, closed bars). No changes in CCL21 mRNA levels were observed in response to treatment with 50 µM NMDA at 4 ($p=0.147$), 8 ($p=0.292$), 24 ($p=0.655$) or 48 hours ($p=3.94$) after induction when compared to control conditions. Similar results were observed in CXCR3$^{-/-}$ slice cultures as CCL21 mRNA was detected in untreated control slice cultures (Fig. 4.4, open

bars). Also here, levels of CCL21 mRNA did not change in response to NMDA-treatment at the time-points measured (Fig. 4.4). However, we observed a discrepancy in relative CCL21 mRNA expression levels between CXCR3$^{-/-}$ and wild type slice cultures. Although CCL21 mRNA could be detected in both wild type- and CXCR3$^{-/-}$ slice cultures, the relative expression levels of CCL21 mRNA in CXCR3$^{-/-}$ slice cultures were 50 times lower than observed in wild type slice cultures (Fig. 4.4). Also after NMDA-treatment, levels of CCL21 mRNA remained 50-100 times lower in CXCR3$^{-/-}$ slice cultures than in wild types. These unexpected findings suggest that the expression of CCL21 in these slice cultures is partly dependent on the presence of CXCR3.

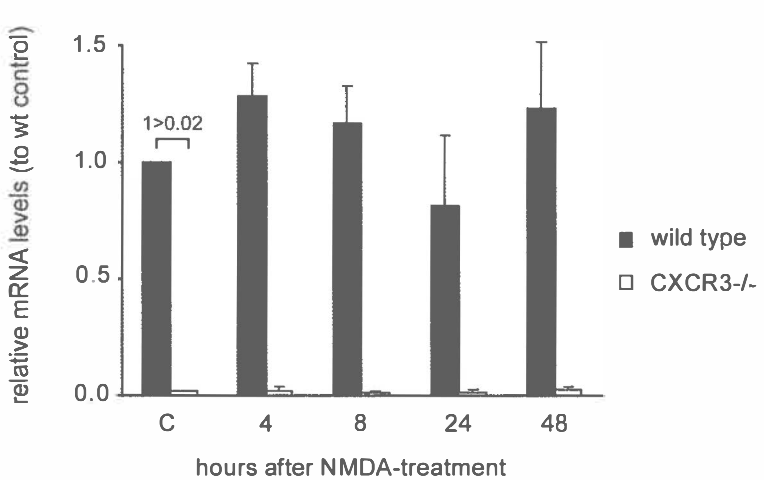


Figure 4.4. CCL21 mRNA expression levels are downregulated in CXCR3$^{-/-}$ OHSCs. Graph represents the relative mRNA expression levels of CCL21, as determined by QPCR analysis, in wild type (closed bars) and CXCR3$^{-/-}$ slice cultures (open bars) before (control, c) and 4, 8, 24, 48 hours after excitotoxicity-induction with 50 µM NMDA. CCL21 mRNA was detected in both control wild type- and CXCR3$^{-/-}$ slice cultures. Relative levels of CCL21 mRNA did not change in response to NMDA treatment at any time-point measured. However, relative CCL21 mRNA levels in CXCR3$^{-/-}$ slice cultures were 50 times lower when compared to wild type conditions (1<0.02 respectively), which remained constant at all time-points measured after NMDA-treatment. Relative mRNA expression levels were determined as the expression of CCL21 normalized to β-actin and related to non-treated control conditions. Data are a summary of four separate experiments. Bars indicate mean ± SEM.

DISCUSSION

CCL21-signaling in the CNS is associated with a neurotoxic response

Among all chemokines expressed in the CNS, CCL21 displays a unique feature as it is exclusively expressed in damaged neurons (Ambrosini et al., 2005; Biber et al., 2001; Columba-Cabezas et al., 2003; de Jong et al., 2005; Zhao et al., 2007). Moreover, CCL21 has been reported to activate glia cells *in vitro* and *in vivo*, in particular microglia and astrocytes, and it is therefore suggested that CCL21 could function as a neuronal stress signal that activates and/or attracts surrounding glia cells (Biber et al., 2001; de Jong et al., 2005; Dijkstra et al., 2004; Rappert et al., 2002; Van Weering et al., 2009; Zhao et al., 2007). Despite this knowledge, little is yet known about the consequences of CCL21 expression in the injured CNS.

Here, we have investigated the impact of CCL21-signaling in a model for excitotoxicity-induced neuronal cell death using mouse hippocampal slice cultures. Our data demonstrate that, in the absence of a peripheral immune response, CCL21 negatively affects neuronal survival under excitotoxic conditions, suggesting an endogenous, neurotoxic role for CCL21 in the hippocampus. Accordingly, we show that hippocampal slice cultures derived from *plt* mice are less susceptible to a neurotoxic insult, as the levels of neuronal cell death in the CA regions in response to NMDA-induced excitotoxicity were markedly lower when compared to wild-type conditions. Furthermore, we could rescue this phenotype by exposure of *plt*-derived slice cultures to 10^{-8} M recombinant CCL21, which significantly enhanced neuronal cell death in response to NMDA-treatment. No effects were observed in response to 10^{-8} M recombinant CCL19. These observations are in line with previous findings, where it was demonstrated that transgenic over-expression of CCL21 in the CNS of intact mice (MBPCCL21 mice) resulted in reactive gliosis and severe neuropathology, while mice transgenic for CCL19 (MBPCCL19 mice) did not show any apparent physiological abnormalities (Chen et al., 2002). Moreover, these results suggest that expression of CCL21 within the CNS is associated with a neurotoxic response, which strongly corroborates our findings.

Involvement of CXCR3

Although the actions of CCL21 in the periphery are well described and have been shown to be induced through its corresponding receptor CCR7 (Forster et al., 1999; Forster et al., 2008; Lira, 2005), there is strong evidence that CCL21-induced signaling in the CNS

is mediated through another receptor (Biber et al., 2001; de Jong et al., 2005; Dijkstra et al., 2004; Rappert et al., 2002). Several lines of evidence point towards CXCR3 as a likely candidate for CCL21-induced effects in the CNS (Biber et al., 2001; Chen et al., 2002; Dijkstra et al., 2004; Jenh et al., 1999; Rappert et al., 2002; Soto et al., 1998; Van Weering et al., 2009). In line with this, it has been demonstrated *in vitro* that CCL21-induced activation of both microglia and astrocytes is not mediated through CCR7, but depends on the presence of CXCR3 (Biber et al., 2001; Dijkstra et al., 2004; Rappert et al., 2002; Weering et al., 2009). Our results corroborate these findings. First of all, we did not detect CCR7 mRNA expression in our slice cultures, while CXCR3 mRNA was readily detected both under control- and NMDA-treated conditions. Secondly, slice cultures derived from CXCR3$^{-/-}$ showed a similar decrease in NMDA-induced neuronal cell death as in *plt*-derived slice cultures when compared to wild type conditions. Moreover, whereas in *plt*-derived slice cultures a clear increase in NMDA-induced neuronal cell death was observed in the presence of recombinant CCL21, exposure of CXCR3$^{-/-}$ slice cultures to CCL21 had no such effect. Taken together, our results suggest that the neurotoxic effect of CCL21 in CNS-excitotoxicity is not mediated through CCR7, but through another receptor, most likely through CXCR3. Additional studies in *plt*-derived slice cultures with selective CXCR3 antagonists could prove this assumption.

Involvement of microglia

Various lines of evidence show that CCL21 activates glia cells, in particular microglia (Biber et al., 2001; Dijkstra et al., 2004; Rappert et al., 2002; Zhao et al., 2007). Moreover, recent evidence suggests that CCL21-induced activation of microglia in the CNS might also be involved in the modulation of pain sensation. Injection of recombinant CCL21 in intact mice directly into the ventral posterolateral nucleus of the thalamus resulted in transient activation of local microglia and induced pain-related behaviour (Zhao et al., 2007). This effect could be blocked by co-injection with minocycline, which is known to inhibit microglia activation, suggesting that CCL21-induced pain behaviour is dependent on the activation of microglia (Zhao et al., 2007).

Although direct evidence is lacking in our paradigm, it is likely that the neurotoxic effect of CCL21 in our slice cultures is mediated through microglia too. In line with this, several publications have shown that suppression of microglia function in excitotoxicity might be beneficial for the survival of neurons in this process (Hailer, 2008). Blocking of CCL21-signaling in the injured CNS might therefore be of therapeutic value.

In summary, we propose that CCL21 is a neuron-derived factor that is part of a signaling-cascade (most likely through CXCR3-mediated activity of microglia) leading to increased neuronal cell death in excitotoxicity.

Remaining questions

Several questions remain open in this study. First of all, it is yet unclear why we did not observe a significant induction of CCL21 mRNA in our slice cultures after NDMA-treatment. Here, only a slight increase (p=0.147) was found at 4 hours after NMDA-treatment. Since neuronal CCL21 expression *in vivo* is only demonstrated in injury models, the basic levels of CCL21 mRNA expression in our system may be due to cellular stress and thus may reflect the culture situation (de Jong et al., 2005). Therefore, the CCL21 mRNA expression levels present in control slice cultures might be sufficient for the degree of neuronal damage in response to NMDA-treatment. In this way, a further increase in CCL21 expression might not reach significant levels. The second question concerns the difference in CCL21 mRNA expression levels between wild type- and $CXCR3^{-/-}$ slice cultures, indicating a role of CXCR3 in the regulation of CCL21 mRNA expression. How neuronal CCL21 expression is regulated on the molecular level and whether CXCR3 signaling is required here is at the moment not clear. Further experiments are needed to address this question.

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CHAPTER 5

CCL21-induced calcium transients and proliferation in primary mouse astrocytes: CXCR3-dependent and independent responses

Hilmar R.J. van Weering *(1)*; Arthur P.H. de Jong *(1,2)*; Alexander H. de Haas *(1,4)*; Knut P.H. Biber *(1)*; Hendrikus W.G.M. Boddeke *(1)*

(1) Department of Neuroscience, Section Medical Physiology, University Medical Center Groningen (UMCG), Rijksuniversiteit Groningen (RUG), Groningen, The Netherlands. *(2)* Current affiliation: Department of Functional Genomics, Center for Neurogenomics and Cognitive Research (CNCR), VU university Amsterdam, Amsterdam, The Netherlands. *(3)* Current affiliation: Department of Pathology and Laboratory Medicine, University Medical Center Groningen (UMCG), Rijksuniversiteit Groningen (RUG), Groningen, The Netherlands

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ABSTRACT

CCL21 is a homeostatic chemokine that is expressed constitutively in secondary lymph nodes and attracts immune cells via chemokine receptor CCR7. In the brain however, CCL21 is inducibly expressed in damaged neurons both *in vitro* and *in vivo* and has been shown to activate microglia *in vitro*, albeit not through CCR7 but through chemokine receptor CXCR3. Therefore, a role for CCL21 in CXCR3-mediated neuron-microglia signalling has been proposed. It is well established that human and mouse astrocytes, like microglia, express CXCR3. However, effects of CCL21 on astrocytes have not been investigated yet. In this study, we have examined the effects of CCL21 on calcium transients and proliferation in primary mouse astrocytes. We show that similar to CXCR3-ligand CXCL10, CCL21 (10^{-9} M and 10^{-8} M) induced calcium transients in astrocytes, which were mediated through CXCR3. However, in response to high concentrations of CCL21 (10^{-7} M) calcium transients persisted in CXCR3-deficient astrocytes, whereas CXCL10 did not have any effect in these cells. Furthermore, prolonged exposure to CXCL10 or CCL21 promoted proliferation of wild type astrocytes. Although CXCL10-induced proliferation was absent in CXCR3-deficient astrocytes, CCL21-induced proliferation of these cells did not significantly differ from wild type conditions. It is therefore suggested that primary mouse astrocytes express an additional (chemokine-) receptor, which is activated at high CCL21 concentrations.

INTRODUCTION

Chemokines are small chemotactic cytokines of approximately 8-14 kDa and are classified into four distinct groups (CXC, CC, XC and CX3C) based on the position of their conserved cysteine residues in the N-terminus (Bacon et al., 2002; Murphy et al., 2000). Chemokines are produced locally and exert their actions on seven-transmembrane, G-protein coupled chemokine receptors (Allen et al., 2007; Murphy et al., 2000). In general, activation of chemokine receptor-induced signaling pathways results in an increase in intracellular calcium and is in most cases pertussis toxin sensitive, suggesting that most of these receptors are G_i-protein linked (Murphy, 1996). In recent years approximately twenty chemokine receptors and over forty chemokines have been identified (Bacon et al., 2002; Murphy et al., 2000; Murphy, 2002).
CXC-, CC-, XC- and CX3C-chemokines predominantly induce their actions through their respective CXC-, CC-, XC- and CX3C receptors. One exception is chemokine C-C motif ligand 21 (CCL21, formerly known as TCA-4, Secondary Lymphoid-organ Chemokine (SLC), Exodus-2, 6CKine), which not only binds its primary receptor CCR7, but also functionally binds to CXCR3, a member of the CXC-chemokine receptor family (Soto et al., 1998). In the periphery, CCL21 is constitutively expressed in secondary lymphoid organs and controls the homing of mature dendritic cells and of naive T- and B-cells (Dieu et al., 1998; Sallusto et al., 1998; Yanagihara et al., 1998; Stein et al., 2000). In the CNS however, CCL21 is rapidly expressed by endangered neurons during an ischemic insult or spinal cord injury (Biber et al., 2001; Zhao et al., 2007). CCL21 expression was detected in damaged neurons but not in other brain-resident cells (Biber et al., 2001). In addition, CCL21 is inducibly expressed in neuronal cultures and neurons in hippocampal slice cultures after exposure to high levels of glutamate (de Jong et al., 2005). Interestingly, it has been demonstrated that CCL21 induces activation of human and mouse microglia as measured by chemotaxis, calcium signaling and induction of Cl^- currents (Biber et al., 2001; Dijkstra et al., 2004; Rappert et al., 2002). Moreover, the activation of mouse microglia by CCL21 was shown to be dependent on the presence of CXCR3, but not CCR7 (Rappert et al., 2002), as microglia derived from CCR7-deficient mice migrated towards CCL21, while microglia derived from CXCR3-deficient mice did not. In line with this evidence it has been proposed that CCL21/CXCR3 functions as an alternative communication system between damaged neurons and surrounding microglia

during the development of various diseases in the CNS (Biber et al., 2001; de Jong et al., 2005; Rappert et al., 2002).

Next to microglia, astrocytes are an integrative component of the neuron-glia support system both under healthy and pathological conditions. Astrocytes not only have been shown to be involved in neuronal information processing and synaptic transmission (Volterra and Meldolesi, 2005), but also seem to be important mediators in the activation of microglia under pathological conditions (Davalos et al., 2005). Interestingly, astrocytes express various chemokine receptors (Ambrosini and Aloisi, 2004) and respond to a variety of chemokines such as CXCL10, CXCL12, CCL2 and CX3CL1 *in vitro* (Andjelkovic et al., 2002; Bajetto et al., 2001; Biber et al., 2002; Maciejewski-Lenoir et al., 1999; Rezaie et al., 2002). Therefore it would be of interest to determine whether astrocytes, like microglia, have the capacity to respond to CCL21.

Although the presence of CCR7 has not been detected on astrocytes (Biber et al., 2002; Columba-Cabezas et al., 2003), it has been shown that both human and mouse astrocytes functionally express CXCR3 protein (Biber et al., 2002; Flynn et al., 2003). Astrocytes clearly respond to the CXCR3-ligand CXCL10, as measured by calcium transients, chemotaxis and cell proliferation (Biber et al., 2002; Flynn et al., 2003). Whereas the response of microglia to CCL21 is known and depends on the presence of CXCR3, possible effects of CCL21 on astrocytes have not yet been investigated. In this study, we have examined the effects of CCL21 and CXCL10 on primary mouse astrocytes by means of single-cell calcium imaging and proliferation and determined whether these effects were mediated through CXCR3 or another receptor. Additionally, we characterized the calcium responses to both chemokines with respect to calcium mobilization and involvement of G_i-proteins.

EXPERIMENTAL PROCEDURES

Animals

All experiments have been approved by the Dutch animal experimental committee. C57BL/6J (Harlan) and CXCR3$^{-/-}$ mice with C57BL/6J background were housed and handled in accordance with the guidelines of the central animal laboratory (CDL) facility of Groningen, the Netherlands. The CXCR3$^{-/-}$ mice were kindly provided by B. Lu and C. Gerard (Children's Hospital, Harvard Medical School, Boston, MA).

Materials

Cell culture media and supplements were all obtained from GIBCO® (Invitrogen Corporation; Breda, The Netherlands), unless mentioned otherwise. Recombinant murine CCL21 and CXCL10 were obtained from PeproTech® (London, United Kingdom). Pertussis toxin and thapsigargin were purchased from Sigma-Aldrich (Bornhem, Belgium).

Primary mouse astrocyte cultures

For primary mouse astrocyte cultures brains from postnatal C57BL/6J and $CXCR3^{-/-}$ mice (< 1 day) were rapidly isolated and, after removal of the meninges, the cortices were dissected in ice-cold medium-A (Hank's Balanced Salt Solution (HBSS) supplemented with 0.5% glucose and 1.5 mM HEPES). Cortices were carefully dissociated by trituration in medium-A using a 1 ml pipette and subsequent trypsinisation for 20 minutes at 37°C in medium-A, supplemented with 0.25% trypsin and 1000 U/ml Dnase I (Roche). After a trypsin inhibition step with medium-A, containing 0.1 mg/ml trypsin inhibitor (Sigma-Aldrich), 20% fetal calf serum (FCS) and 1000 U/ml Dnase I, the cells were centrifuged for 10 minutes at 150 RCF. Single cell suspensions were seeded in 75 cm^2 culture flasks (Greiner), containing 10 ml Dulbecco's Modified Eagle's Medium (DMEM) with 10% FCS, 1% L-Glutamine, 1% sodium pyruvate, 100 µg/ml streptomycin and 100 U/ml penicillin. These mixed glial cell cultures were kept in a humidified incubator (5% CO_2) at 37°C. Culture medium was refreshed one day after the isolation. After 7-10 days *in vitro*, the mixed glial cultures were placed overnight on a cell-shaker (240 rpm) at 37°C and the following day the non-adherent glial cells were removed. Next, astrocytic mono-layers were carefully separated from the underlying adherent microglia by a mild trypsinisation step (5 ml HBSS with 0.05% trypsin-EDTA) and plated in new 75 cm^2 culture flasks. In consecutive weeks, overnight shaking and trypsinisation steps were repeated until pure astrocyte cultures (>98%) were obtained. The astrocyte cultures were passaged every week by mild trypsinisation and culture medium was refreshed one day after passaging.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

For RNA analysis, astrocyte cultures were lysed in guanidinium isothiocyanate/ monopthiolglycerol buffer (both from Sigma) and total RNA was extracted and precipitated with a one-chloroform-phenol step (Chomczynski and Sacchi, 1987). 1 µg of

total RNA was transcribed into cDNA, as described previously (Biber et al., 1997). Subsequently, 2 µl of cDNA was amplified by polymerase chain reaction (PCR) and its products were visualized as bands on a 1.5% agarose gel with ethidiumbromide. The quality of RNA was checked using primers for house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH; accession number **AF10680)**, with the following sequences; forward primer 5`- CATCCTGCACCACCAACTGCTTAG-3` and backward primer 5`-GCCTGCTTCACCACCTT CTTGATG-3` (product size 346 base pairs). Contamination of RNA isolates by genomic DNA was checked for by running the reactions without reverse transcriptase, followed by PCR amplification with GAPDH primers. The following primer sequences for mouse CXCR3 (accession number **AF045146)** were used; forward primer 5`-AGAATCATCCTGGTCTGAGACAA-3` and backward primer 5`-AAGATAGGGCATGGCAGCTA-3` (product size 288 base pairs). Primer sequences used for mouse CCR7 (accession number **L31580)** were; forward primer 5`-ACAGCGGCCTCCAGAAGAACAGCGG-3` and backward primer 5`-TGACGTCATAGGCAATGTTGAGCTG-3` (product size 345 base pairs). The PCR-products have been verified by cloning and subsequent sequencing.

Flow Cytometry

For analysis of chemokine receptor-expression astrocyte cultures were collected by mild trypsinisation, then pelleted by centrifugation for 10 minutes at 150 RCF (4 ^{o}C) and resuspended in ice-cold, sterile phosphate-buffered saline (PBS) at a concentration of 100,000 cells per vial. Next, the astrocytes were stained for phycoerythyrin-coupled monoclonal anti-mouse CXCR3 (R&D, FAB1685P), CCR7 (eBioscience, 12-1971) or concentration-matched isotype IgG2A controls (eBioscience, 12-4321) for 20 minutes at 4 ^{o}C in PBS containing 1% FCS. Cell-size, granularity and fluorescence intensity were analyzed by flow cytometry using a 488-nm laser FACSCalibur (Becton Dickinson), supported by CellQuest software (Becton Dickinson) and the acquired data (minimal 10,000 events per sample) was analyzed using WinMDI version 2.9 (Joseph Trotter).

Intracellular calcium measurements

For single-cell calcium imaging, astrocytes were cultured on glass cover-slips (30 mm Ø) for at least two days in culture medium, as described above. Next, the cover-slips were rinsed shortly in standard loading buffer, containing 150 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES/NaOH and 10 mM glucose (pH ~ 7.35) and loaded

with 5 µM Fura-2(AM) (Teflabs.com) together with 0.4% pluronic© F-127 (Molecular Probes) in 1 ml loading buffer for 30-45 minutes at 37°C. After an additional washing step of 30 minutes in plain loading buffer, the cover-slips were fixed in a perfusion chamber (37°C) and attached to an inverted microscope (Axiovert 35M, Zeiss) equipped with a 12 bit Sensicam CCD camera (PCO) supported by Imaging Workbench 5.0 software (INDEC systems, Inc.). Using a 16x plan-neofluar objective digital images were taken at an emission wavelength of 510 nm using paired exposure at 340 nm and 380 nm excitation wavelengths at a frequency of 1Hz. Changes in intracellular calcium levels were expressed as the ratio of the 340 and 380 nm excitation wavelengths (D340/380) in time (seconds).

During recording experiments, compounds were administered directly to the loading buffer using a pipette. Recombinant chemokines CCL21 and CXCL10 were administered at end-concentrations of 10^{-9} M, 10^{-8} M and 10^{-7} M (in PBS). Administration of 10^{-5} M (end-concentration) ATP served as a positive control and PBS as a negative control. The percentage of chemokine-responsive cells was related to the total number of ATP-responsive cells per experiment. Astrocytes non-responsive to ATP were excluded from the experiments.

For pertussis toxin experiments, astrocytes were pre-incubated with 200 ng/ml pertussis toxin 24 hours before calcium measurements. In addition, experiments were done in calcium-free loading buffer, containing 150 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 0.5 mM EGTA, 2 mM $MgCl_2$, 10 mM HEPES/NaOH and 10 mM glucose (pH ~ 7.35). Cells were loaded with Fura-2(AM) and washed as described above. During recording experiments, standard loading buffer was replaced by calcium-free loading buffer.

For intracellular calcium-depletion experiments, cells were loaded with Fura-2(AM) as described above, followed by incubation with 2 µM thapsigargin in standard loading buffer for 45 minutes. Also here, intracellular calcium measurements were done in calcium-free buffer.

Astrocyte proliferation assay

Astrocyte proliferation in response to CXCL10 and CCL21 was assessed by conversion of 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT) into formazan. Freshly prepared astrocyte cultures were plated in 24-wells plates at a density of 10,000 cells per well in standard culture medium (10% FCS). After attachment (3 to 4 hours) the cells were rinsed once in PBS and incubated with serum-free culture medium (0% FCS),

supplemented with either CCL21 or CXCL10 at concentrations of 10^{-9} M and 10^{-8} M for 72 hours at 37°C. Medium was refreshed every 24 hours. After 72 hours, MTT (Sigma) was added to the medium at an end-concentration of 0.5 mg/ml for an additional 4 hours. Subsequently, the medium was carefully aspirated and the formazan crystal residues were solved in 700 µl DMSO. The optical density was measured by spectrophotometry at a wavelength of 570 nm with background subtraction at 690 nm. Serum-free culture medium served as a negative control for proliferation and standard culture medium with 10% FCS served as a positive control.

Statistical analysis

Data are represented as the mean ± standard error of the mean (SEM). For calcium imaging experiments, *n* is the total number of cells measured. For proliferation experiments, *n* is the total number of experiments performed. Statistical comparison between groups was performed using the two-tailed Students' *t*-test and *p*-values smaller than 0.05 were considered significant. All statistical tests were performed in SPSS version 14.0.2 (SPSS Inc., Chicago).

RESULTS

Primary mouse astrocytes express CXCR3, but not CCR7

Since CCL21 has been reported to signal through CXCR3 as well as CCR7, we performed RT-PCR analysis for both receptors in primary mouse astrocytes, using spleen tissue as a positive control. Presence of CXCR3 mRNA was detected in both astrocyte cultures and spleen control tissue, as indicated by clear bands at 288 base-pairs (Fig. 5.1A). In contrast, whereas in spleen the presence of CCR7 mRNA (345 base-pairs) was found, this message was absent in the astrocyte cultures (Fig. 5.1A). Subsequently, surface expression of CXCR3 and CCR7 protein was analyzed by flow cytometry. Astrocytes showed substantial surface expression of CXCR3 protein, whereas no expression of CCR7 protein was detected, corroborating the RT-PCR results (Fig. 5.1B and C).

Both CXCL10 and CCL21 induce calcium transients in primary mouse astrocytes

In order to determine whether primary mouse astrocytes respond to CCL21 and whether this response is mediated through CXCR3, single-cell calcium imaging was performed.

CXCR3-ligand CXCL10 was used as a reference, since this chemokine has been shown to induce calcium transients in primary murine astrocytes (Biber et al, 2002).

Here, the percentage of CXCL10-responsive astrocytes was dependent on the concentration as 10^{-9} M CXCL10 induced calcium transients in 23% (out of n=47), 10^{-8} M CXCL10 in 25% (out of n=145) and 10^{-7} M CXCL10 in 68% (out of n=40) of the astrocytes (Fig. 5.2A). The amplitude of the calcium transients induced by CXCL10 was highly variable and did not show any concentration-dependency (p=0.659).

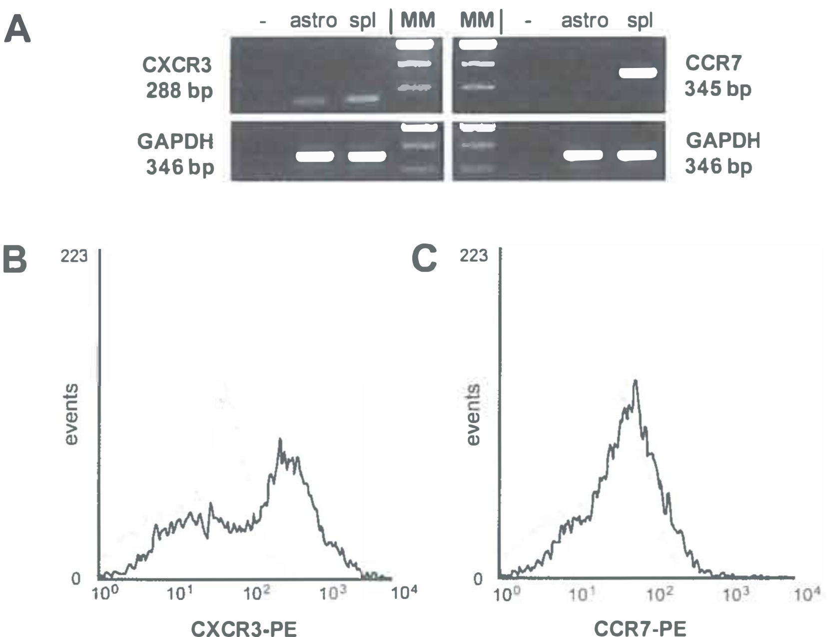


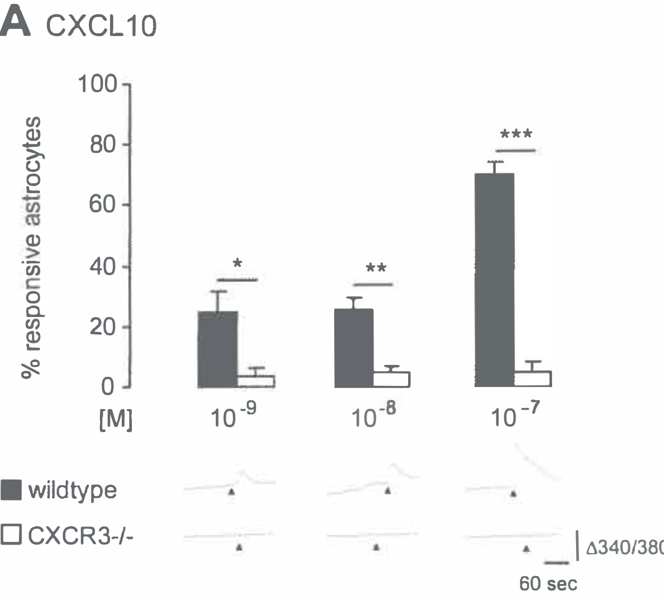
Figure 5.1. Primary mouse astrocytes express CXCR3, but not CCR7. (**A**) RT-PCR analysis revealed CXCR3 mRNA positivity (288 bp) in cultured mouse astrocytes (astro), while CCR7 mRNA was not detected. Spleen tissue (spl), which served as a positive control, was positive for both markers. To check for comparable RNA quality, amplification of the housekeeping gene GAPDH was used. MM; molecular marker, - ; control without reverse transcription. (**B**,**C**) Flow cytometric analysis confirms these findings as CXCR3 protein expression (**B**, solid black line) was found on a subpopulation of astrocytes, while CCR7 protein expression (**C**, solid black line) was not detected. Grey dotted lines represent concentration-matched IgG_{2A} isotype controls. Events were normalized.

Figure 5.2 (right). Both CXCL10 and CCL21 induce calcium transients in primary mouse astrocytes, which are (partly for CCL21) mediated by CXCR3. (A) The percentage of responsive astrocytes in wild type (■) and CXCR3$^{-/-}$ (□) induced by 10^{-9}, 10^{-8} and 10^{-7} M recombinant mouse CXCL10 as calculated to the percentage of ATP-responsive cells (=100%). Bars indicate the mean ± SEM. 10^{-9} M and 10^{-8} M CXCL10 induced calcium transients in 23% (out of *n*=47) and 25% (out of *n*=145) of the astrocytes, respectively, while 10^{-7} M CXCL10 induced responses in 68% (out of *n*=40) of the astrocytes. CXCR3$^{-/-}$ astrocytes showed hardly any responses to the various CXCL10 concentrations. Bottom calcium traces represent typical CXCL10-induced calcium responses in both wild type and CXCR3$^{-/-}$ astrocytes, expressed as the change in the 340/380 nm ratio (Δ340/380) in time (sec). Arrows indicate time-point of chemokine application. The amplitude and duration of the calcium responses were highly variable and showed no concentration-dependency (data not shown). **(B)** The percentage of responsive astrocytes in wild type (■) and CXCR3$^{-/-}$ (□) induced by 10^{-9}, 10^{-8} and 10^{-7} M recombinant mouse CCL21 as calculated to the percentage of ATP-responsive cells (=100%). Similar to CXCL10, 10^{-9} M and 10^{-8} M CCL21 induced calcium transients in 22% (out of *n*=41) and 26% (out of *n*=157) of the astrocytes respectively, while 10^{-7} M CCL21 induced responses in 70% (out of *n*=56) of the astrocytes. Although hardly any responses were detected in CXCR3$^{-/-}$ astrocytes in response to 10^{-9} and 10^{-8} M CCL21, 10^{-7} M CCL21 induced calcium transients in 70% (out of *n*=33) of these cells, suggesting involvement of another (chemokine) receptor. Bottom traces represent typical CCL21-induced calcium responses in both wild type and CXCR3$^{-/-}$ astrocytes, expressed as the change in the 340/380 nm ratio (Δ340/380) in time (sec). Also here, the amplitude and duration of the calcium responses did not show concentration-dependency (data not shown). Arrows indicate time-point of chemokine application. Student's *t*-test, * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Wild type astrocytes clearly responded to CCL21 with an increase in intracellular calcium and also here the number of responsive astrocytes depended on the concentration of CCL21 (Fig. 5.2B). The percentages of CCL21-responsive astrocytes were similar to that of CXCL10 as 10^{-9} M and 10^{-8} M CCL21 induced calcium transients in approximately 22% (out of *n*=41) and 26% (out of *n*=157) of the astrocytes. Also here, application of 10^{-7} M CCL21 gave the highest percentage of responsive astrocytes (70% out of *n*=56). Similar to CXCL10-induced calcium responses, the amplitude of the transient calcium responses induced by CCL21 varied considerably and did not show any concentration-dependency ($p = 0.276$).

Unlike CXCL10, CCL21-induced calcium transients are just partly dependent on CXCR3

In order to determine the involvement of CXCR3, single-cell calcium experiments were performed in astrocytes derived from CXCR3$^{-/-}$ mice. CXCR3$^{-/-}$ astrocytes did not respond to application of either 10^{-9} M (4% out of *n*=53), 10^{-8} M (5% out of *n*=147) or 10^{-7} M (6% out of *n*=35) CXCL10, confirming that CXCL10-induced calcium transients are mediated entirely through CXCR3 in primary mouse astrocytes (Fig. 5.2A).


A CXCL10
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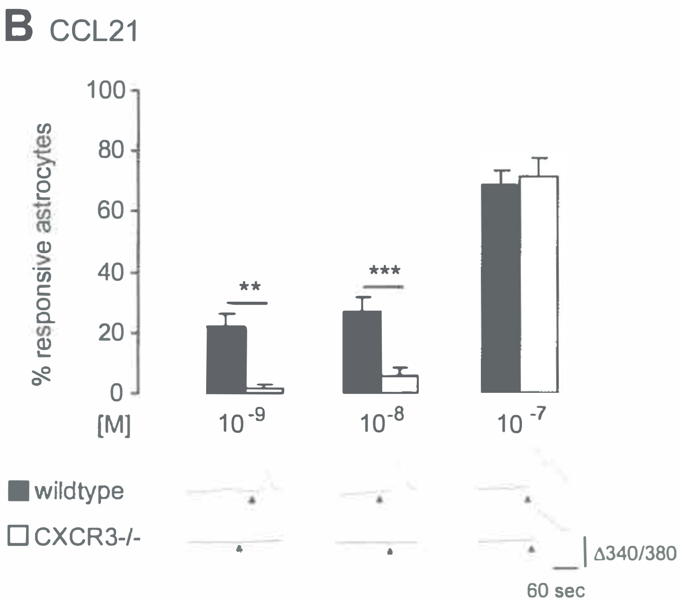
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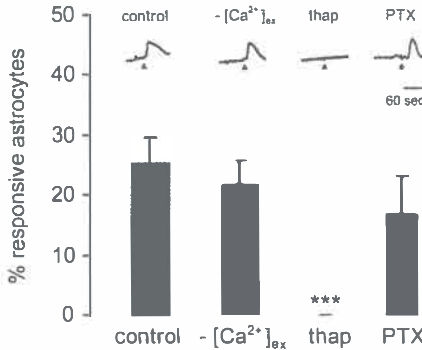
Figure 5.3 (right). CXCL10 and CCL21-induced calcium transients in primary mouse astrocytes originate from intracellular calcium stores and are partly inhibited by pertussis toxin. Effects of calcium-free buffer (-$[Ca^{2+}]_{ex}$), thapsigargin (thap) and pertussis toxin (PTX) on intracellular calcium transients in primary mouse astrocytes induced by 10^{-8} M CXCL10 (**A**), 10^{-8} M CCL21 (**B**) and 10^{-7} M CCL21 (**C**). The percentage of CXCL10-responsive astrocytes (**A**) was not significantly affected when experiments were performed in calcium-free buffer (21% out of n=42; p=0.650). Pre-treatment with 2 µM thapsigargin, blocking calcium mobilization from intracellular stores, completely inhibited the response to 10^{-8} M CXCL10 (0% out of n=40; p<0.001). Pre-treatment with PTX resulted in a slight reduction in the percentage of CXCL10-responsive astrocytes from 25% (out of n=145) to 17% (out of n=79), which was not significant (p=0.277). Top graphs show representative calcium traces, expressed as the change in the 340/380 nm ratio (Δ340/380) in time (sec), showing the effects of calcium-free buffer, thapsigargin and PTX on CXCL10-induced calcium transients (**A**). Similar results were found for 10^{-8} M CCL21 (**B**) as depletion of extracellular calcium had no significant effect on the number of responsive astrocytes (20% out of n=137, p=0.099) nor on the amplitude and duration of the transients. Thapsigargin completely abolished CCL21-induced transients (0% out of n=76, p<0.001). Also here, pre-treatment with pertussis toxin resulted in a slight reduction in the percentage of astrocytes responsive to 10^{-8} M CCL21 from 27% (out of n=157) to 15% (out of n=92), which was not significant (p=0.059). In response to 10^{-7} M CCL21 (**C**), omission of extracellular calcium had no effect on the percentage of responsive wild type (closed bars, 71% out of n=42, p=0.834) and $CXCR3^{-/-}$ (open bars, 72% out of n=43, p=0.960) astrocytes and treatment with thapsigargin fully inhibited these responses in both cell-types (0% out of n=47 and n=53, respectively, p<0.001). Pre-treatment with PTX had no effect on the percentage of wild type (72% out of n=42, p=0.685) and $CXCR3^{-/-}$ astrocytes (65% out of n=37, p=0.554) responsive to 10^{-7} M CCL21. The percentage of chemokine-responsive astrocytes was calculated to the percentage of ATP-responsive cells (= 100%). Bars indicate mean ± SEM. Student's t-test, *** p<0.001, $^{\#}p$=0.059.

Like CXCL10, 10^{-9} M (2% out of n=48) and 10^{-8} M CCL21 (6% out of n=82) failed to induce calcium transients in $CXCR3^{-/-}$ astrocytes. Surprisingly, whereas application of 10^{-7} M CXCL10 had no effect on $CXCR3^{-/-}$ astrocytes, clear responses were detected in response to 10^{-7} M CCL21 with similar percentages (70% out of n=33) as detected in wild type astrocytes (fig. 5.2B). Interestingly, the latency (time between application and the start of the response) of 10^{-7} M CCL21-induced calcium responses in $CXCR3^{-/-}$ astrocytes (3.1 ± 1.9 sec) was clearly shorter then in astrocytes derived from wild types (23.2 ± 5.3 sec, p<0.001).

Intracellular elements involved in CXCL10- and CCL21- induced calcium transients

To determine whether CXCL10 and CCL21-induced calcium transients originate from intracellular calcium stores or are caused by extracellular calcium influx, measurements were performed in calcium-free buffer while intracellular calcium stores were depleted using thapsigargin, or in calcium-free buffer alone.



A CXCL10 (10-8M)
% responsive astrocytes
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30
20
10
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control
- [Ca2+]ex
thap
PTX
60 sec
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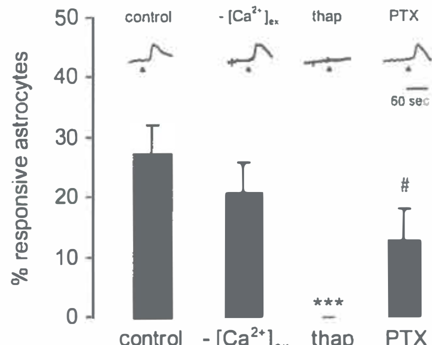

B CCL21 (10-8M)
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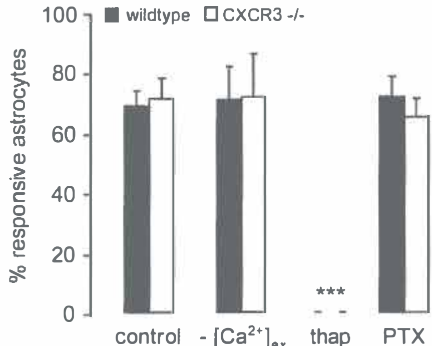


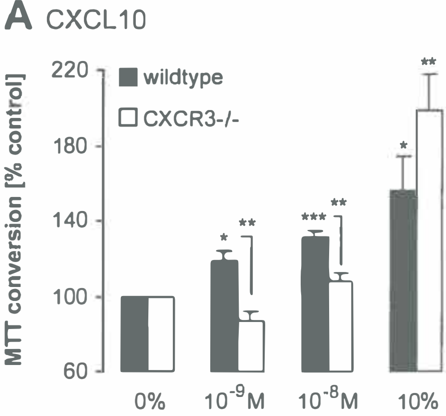
C CCL21 (10-7M)
wildtype
CXCR3 -/-
% responsive astrocytes
100
80
60
40
20
0
control
- [Ca2+]ex
thap
PTX
***

Thapsigargin inhibits sarco/endoplasmic reticulum Ca^{2+} ATPases (SERCA), resulting in an incapability of a cell to pump calcium into the sarco/endoplasmic reticulum which causes these stores to become depleted. Omission of extracellular calcium did not significantly affect the number of astrocytes responding to 10^{-8} M CXCL10 (21% out of n=42, p=0.650; Fig. 5.3A). The calcium responses to CXCL10 in calcium-free buffer had similar amplitudes and kinetics as observed in control experiments. Pre-treatment with thapsigargin (2 µM) completely abolished the response to 10^{-8} M CXCL10 (0% out of n=40, Fig. 5.3A). These results show that CXCL10-induced calcium responses in astrocytes derive from intracellular calcium stores.

To determine involvement of G_i- and G_o-coupled proteins in CXCL10-induced calcium transients, astrocytes were pre-treated with the G_i- and G_o-inactivating agent pertussis toxin. Treatment with pertussis toxin reduced the number of responsive astrocytes induced by 10^{-8} M CXCL10 from 25% (out of n=145) to 17% (out of n=79), although this reduction was not significant (p=0.277; Fig. 5.3A), suggesting involvement of other G-proteins than G_i and G_o.

Like in response to CXCL10, the percentage of astrocytes responding to 10^{-8} M CCL21 (Fig. 5.3B) was not significantly affected after omission of extracellular calcium (20% out of n=137, p=0.099; Fig. 5.3B) and the average response amplitude (Δ0.18 ± 0.03) was similar to that observed in control experiments (Δ0.25 ± 0.03). Also here responses to 10^{-8} M CCL21 were completely abolished after pre-treatment with 2 µM thapsigargin (0% out of n=76; Fig. 5.3B). Pre-treatment with pertussis toxin reduced the number of responsive astrocytes induced by 10^{-8} M CCL21 from 27% (out of n=157) to 15% (out of n=92), although also here this reduction was not significant (p=0.059; Fig. 5.3B). Further analysis showed that treatment with pertussis toxin did not significantly affect the amplitude, latency or duration of CCL21-induced calcium responses.

As a high (10^{-7} M) CCL21-concentration induced calcium transients in both wild type and $CXCR3^{-/-}$ astrocytes, we determined also here the effects of pertussis toxin, extracellular calcium omission and thapsigargin on these responses. Like the calcium transients induced by 10^{-8} M CCL21 in wild type astrocytes, 10^{-7} M CCL21 induced calcium release from intracellular stores in both wild type and $CXCR3^{-/-}$ astrocytes (Fig. 5.3C). Omission of extracellular calcium did not influence the percentages of responsive cells in wild type (71% out of n=42, p=0.833) or $CXCR3^{-/-}$ astrocytes (72% out of n=43, p=0.960) and treatment with thapsigargin fully blocked these responses in both cell-types (0% out of



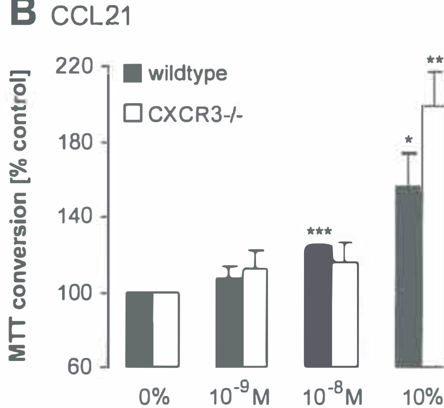


Figure 5.4. Both CXCL10 and CCL21 promote proliferation in primary mouse astrocytes, albeit through different receptors. Effects of CXCL10 (A) and CCL21 (B) on wild type and CXCR3$^{-/-}$ astrocyte proliferation as measured by MTT conversion. Prolonged exposure (72 hrs) to 10^{-9} M CXCL10 (p=0.016) and 10^{-8} M CXCL10 (p<0.001) promoted proliferation in wild type astrocytes (A, black bars), while this effect was significantly reduced in CXCR3$^{-/-}$ astrocytes (A, open bars with p=0.007 for 10^{-9} M and p=0.005 for 10^{-8} M CXCL10 compared to wild type conditions). Prolonged exposure to 10^{-8} M CCL21 (p<0.001) also induced proliferation of wild type astrocytes (B, black bars), while 10^{-9} M CCL21 showed no significant effects (p=0.271) on these cells. However, unlike CXCL10, CCL21-induced proliferation in wild type astrocytes did not significantly differ from the effects observed in CXCR3$^{-/-}$ astrocytes, suggesting involvement of another receptor than CXCR3. Culture medium without FCS (0%) served as a negative control (=100%) for proliferation and medium with 10% FCS served as a positive control. Exposure to 10% FCS induced equal amounts of proliferation in wild type and CXCR3$^{-/-}$ astrocytes (p=0.149). Bars indicate mean ± SEM. Data are a summary of four individual experiments performed in quadruplets. Student's t-test, * p<0.05; ** p<0.01; *** p<0.001.

n=47 and n=53, respectively, $p<0.001$). In addition, pre-treatment with pertussis toxin prior to application of 10^{-7} M CCL21 had no significant effect on the percentages of responsive wild type (72% out of $n=42$, $p=0.685$) and CXCR3$^{-/-}$ astrocytes (65% out of $n=37$, $p=0.554$), corroborating our findings in response to 10^{-8} M CCL21.

Both CXCL10 and CCL21 promote primary mouse astrocyte proliferation, albeit through different receptors

Finally, we determined the effects of CXCL10 and CCL21 on astrocyte proliferation. Prolonged exposure (72 hours) to either 10^{-9} M ($p=0.016$) or 10^{-8} M CXCL10 ($p<0.001$) promoted proliferation of wild type astrocytes (Fig. 5.4A). In addition, long-term exposure of both 10^{-9} M ($p=0.096$) and 10^{-8} M CXCL10 ($p=0.124$) had no effect on CXCR3$^{-/-}$ astrocyte proliferation and was significantly inhibited compared to wild type conditions (Fig. 5.4A, $p=0.007$ and $p=0.005$ respectively). These results confirm again that CXCL10 mediates its effects through CXCR3 in primary mouse astrocytes.

Interestingly, exposure to 10^{-8} M CCL21 ($p<0.001$), but not 10^{-9} M CCL21 ($p=0.271$), also clearly promoted proliferation of wild type astrocytes with values comparable to that of CXCL10 (Fig. 5.4B). However, in contrast to the effects of CXCL10, we did not find any difference ($p=0.440$) for the effects of 10^{-8} M CCL21 on wild type- and CXCR3$^{-/-}$ astrocytes (Fig. 5.4B), suggesting also here that another receptor than CXCR3 might be involved.

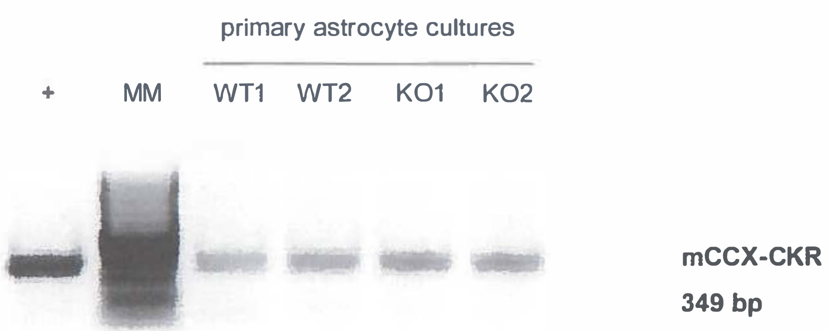
DISCUSSION

In this study we have addressed the effects of chemokine CCL21 (and CXCL10) on intracellular calcium mobilization and proliferation of primary mouse astrocytes, and determined to what extent CXCR3 and CCR7 were involved.

Here, pronounced expression of CXCR3 both at mRNA and protein level was found in primary mouse astrocyte cultures, whereas no expression of CCR7 mRNA or protein could be detected in these cells. These results are in line with previous findings in both human and mouse astrocytes *in vitro* (Biber et al., 2002; Croitoru-Lamoury et al., 2003; Flynn et al., 2003). Moreover, expression of CXCR3, but not CCR7, has been demonstrated in human and mouse astrocytes *in vivo* (Simpson et al., 2000; Goldberg et al., 2001; Van der Meer et al., 2001; Columba-Cabezas et al., 2003; Omari et al., 2005;

Tanuma et al., 2006), indicating that the chemokine receptor expression pattern observed in this study resembles the one observed in astrocytes *in vivo*.
In order to determine CXCR3 functionality in our primary astrocyte cultures, CXCL10 was used as a reference chemokine. Previous work in our lab showed that mouse astrocytes respond to CXCL10 with intracellular calcium mobilization (Biber et al., 2002). Here, the astrocytes responded to CXCL10 with intracellular calcium transients in a concentration-dependent manner. In addition, we confirmed that this CXCL10-response was mediated through CXCR3, as astrocytes derived from CXCR3$^{-/-}$ did not respond to this ligand.
Secondly, we showed that CXCL10 promoted proliferation of primary mouse astrocytes in a CXCR3-dependent manner. This phenomenon of CXCL10-enhanced proliferation has been described previously in human astrocytes (Flynn et al., 2003), where it was shown that similar concentrations (5 ng/ml) of recombinant human CXCL10 promoted astrocyte growth. Interestingly, chemokine-induced proliferation of astrocytes *in vitro* has been described for other chemokines such as CCL2, CCL3 and CXCL12 (Bajetto et al., 2001; Rezaie et al., 2002). Although it is currently unknown whether chemokines have the capacity to induce/enhance astrocyte proliferation *in vivo*, it is tempting to speculate that chemokines could be involved in the regulation of astrogliosis, a process which is characterized by astrocyte proliferation in response to CNS injury.
Similar results were found for CCL21 demonstrating not only that this chemokine acts on astrocytes, but confirming also that CCL21, like CXCL10, mediates its action at least partly through CXCR3 in these cells. Experiments with primary cultured astrocytes derived from CXCR3$^{-/-}$ mice clearly showed no calcium transients in response to lower concentrations of CCL21 (10^{-9}M and 10^{-8}M) suggesting that CXCR3 is the primary receptor for this ligand. However, in contrast to CXCL10, a high concentration of CCL21 (10^{-7}M) induced clear calcium transients in CXCR3$^{-/-}$ astrocytes, with similar percentages of responding cells as observed in wild types. Moreover, we showed that prolonged CCL21 stimulation promoted astrocyte proliferation, albeit that this response seemed not to depend on the presence of CXCR3 as we observed no significant differences between CXCR3$^{-/-}$ and wild type astrocytes.
Taken together, these responses to CCL21 in CXCR3$^{-/-}$ astrocytes, in absence of CCR7, suggest the involvement of another receptor apart from CXCR3 and CCR7. Given the unknown binding properties of CCL21 to most of the other chemokine receptors it is unknown at the moment which alternative (chemokine-) receptor might be involved here.

Currently, the only chemokine-receptor like protein that is known to bind CCL21 with high affinity is CCX-CKR (Gosling et al., 2000; Townson and Nibbs, 2002). Interestingly, RT-PCR analysis showed that both wild type and CXCR3$^{-/-}$ astrocytes express CCX-CKR mRNA (supplementary figure 5.5). However, so far no signalling properties have been published for this protein and CCX-CKR is discussed to function as a chemokine scavenger (Comerford et al., 2006; Comerford and Nibbs, 2005; Haraldsen and Rot, 2006). Whether high CCL21-concentrations activate CCX-CKR and induce calcium transients or proliferation through this chemokine receptor-like protein in mouse astrocytes remains to be established. Alternatively, a chemokine receptor heterodimer-system with other (non)-chemokine receptors may be involved in the CCL21-induced effects seen in both CXCR3$^{-/-}$ and wild type astrocytes. Examples of altered pharmacology of chemokine receptors after heterodimerization with either chemokine- or non-chemokine receptors have been described (Chakera et al., 2008; El-Asmar et al., 2005; Mellado et al., 2001; Pello et al., 2008; Sohy et al., 2007).



Supplementary figure 5.5. Primary mouse astrocytes express CCX-CKR. RT-PCR analysis revealed CCX-CKR mRNA expression (349 bp) in wild type astrocytes (WT1, WT2) and CXCR3$^{-/-}$ astrocytes (KO1, KO2). Primers used for CCX-CKR (accession number **AY072938**): forward 5'-AGGACCAAGACCGATGTGTA-3', backward 5'-GAAAGATGGGAGTGCACCTA-3'. +; positive control (mouse heart tissue), MM; molecular marker, WT; wild type, KO; CXCR3$^{-/-}$, -; control without reverse transcription.

Finally, involvement of a not yet characterised chemokine receptor could explain the response to CCL21 in astrocytes derived from CXCR3$^{-/-}$ mice.
Further analysis of the CXCL10- and CCL21-evoked calcium transients in astrocytes shows that the primary source of the calcium response to CXCL10 and CCL21 comes, like in microglia, from the intracellular calcium stores. This is further corroborated by application of thapsigargin, which fully blocked calcium responses to CXCL10 and CCL21. However, differences in CXCL10- and CCL21-induced calcium responses between astrocytes and microglia are evident. One difference between astrocytes and microglia concerns the concentration-dependency of the parameters involved. In case of astrocytes the number of cells responding to CXCL10 and CCL21 showed concentration-dependency, whereas in microglia the concentration-dependency involved the amplitude of the transient calcium responses (Biber et al., 2001). Most likely, the concentration-dependency of the amplitude of the calcium response in astrocytes is very steep, resembling an all-or-none response. Secondly, it seems that CCL21 induces recruitment of other sets of G-proteins in astrocytes than in microglia. Treatment of astrocytes with the G_i- and G_o-inactivating agent pertussis toxin did not significantly affect the transient calcium response to CXCL10 and CCL21, whereas in human microglia the response to CCL21 could be fully blocked by pre-treatment with pertussis toxin (Dijkstra et al., 2004).
Whether or not CCL21 directly binds to CXCR3 in an expression system is a matter of debate (Soto et al., 1998; Jenh et al., 1999). However, in both human and mouse microglia CXCR3 is the primary receptor for CCL21-induced migration and calcium transients (Biber et al., 2001; Dijkstra et al., 2004; Rappert et al., 2002). Microglia derived from CXCR3-deficient mice did not migrate towards a CCL21-gradient and cross-desensitization experiments between CCL21 and CXCL10 in both human and mouse microglia suggest that these chemokines activate the same chemokine receptor, namely CXCR3 (Biber et al., 2001; Dijkstra et al., 2004; Rappert et al., 2002). It has therefore been suggested that the pharmacology of CXCR3-signalling depends on the cellular background in which CXCR3 is expressed (Dijkstra et al., 2004) and the data presented here in astrocytes further corroborate this assumption.

In conclusion, we report for the first time that CCL21 induces calcium responses and proliferation in primary mouse astrocytes with similar dynamics as seen in response to CXCR3-ligand CXCL10. However, in absence of CXCR3, high concentrations of CCL21 still induce calcium transients, and proliferation of CXCR3$^{-/-}$ astrocytes in response to

prolonged CCL21 exposure did not differ from wild type conditions, suggesting involvement of another or additional (chemokine-) receptor. Furthermore, these CCL21-induced responses in mouse astrocytes seem to differ from microglia in dynamics and G-protein- and receptor involvement, suggesting that CCL21 has specific separate functions in these two glia cell-types. The precise role of neuronal CCL21 expression *in vivo* remains to be determined, although it is clear that both microglia and astrocytes have the capacity to respond to CCL21 *in vitro*, suggesting that this chemokine plays a role in the communication between neurons and surrounding glia cells under pathological conditions.

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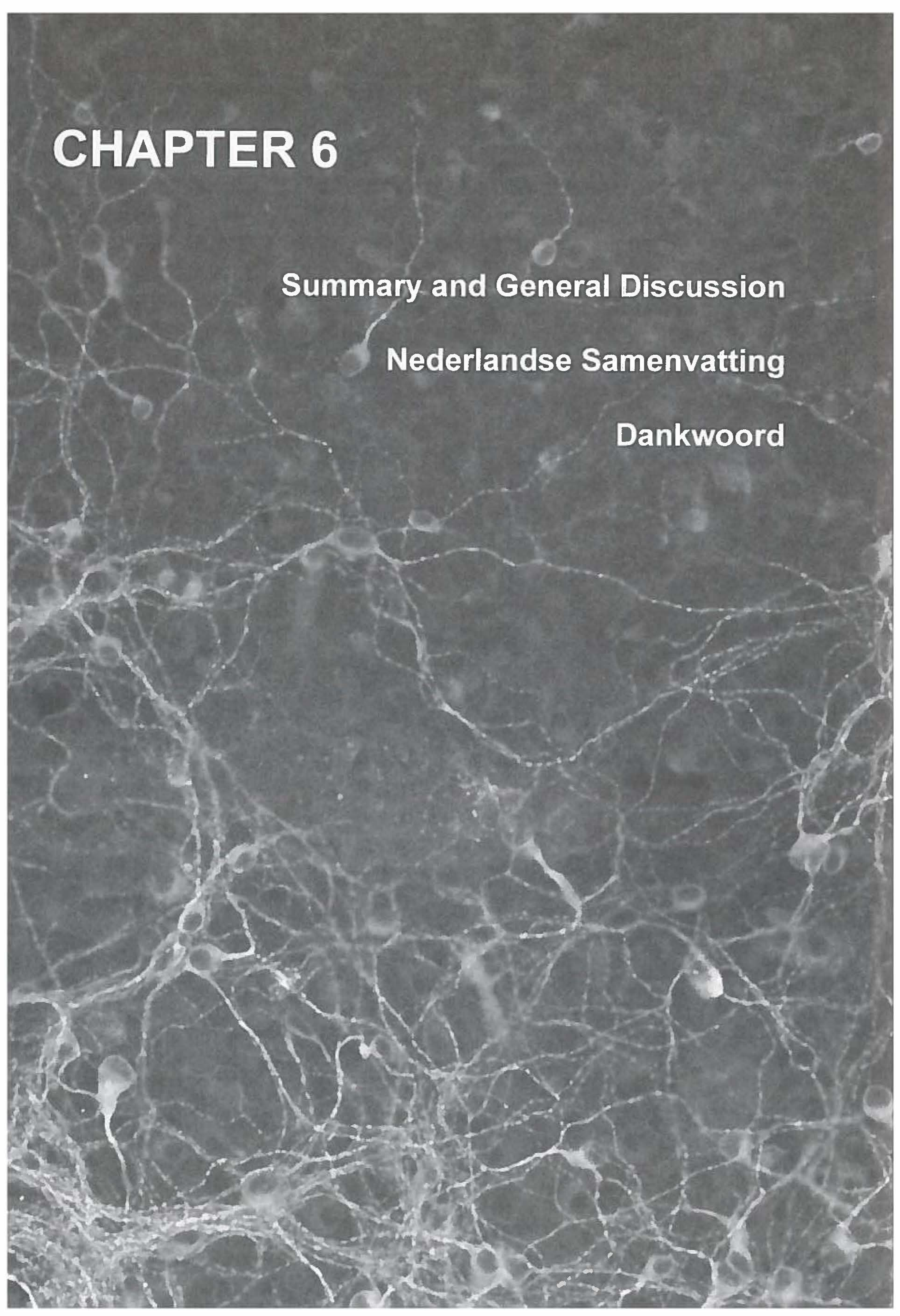

CHAPTER 6

Summary and General Discussion

Nederlandse Samenvatting

Dankwoord

SUMMARY & GENERAL DISCUSSION

Glia cells rapidly respond to neuronal injury

Next to their neuronal support functions in the healthy CNS, glia cells (in particular microglia and astrocytes) are intimately involved in the initiation and regulation of neuroinflammatory responses within the CNS. Any type of neuronal injury leads to rapid activation of these cells. Tight regulation of these glia responses is a prerequisite for neuronal survival under these conditions and therefore requires intimate interactions between neurons and glia cells through cell-cell contact and expression of soluble factors. However, glia reactions in the injured CNS are not inevitably neurosupportive as excessive or chronic activation may lead to the development of neurodegenerative responses. Identification of these mechanisms will give important insight in the role of glia cells in acute CNS injury and in the development of chronic CNS disease.

Microglia activation in excitotoxicity: beneficial or detrimental?

Excitotoxicity is a process associated with many CNS pathologies and is characterized by a progressive loss of neurons as a result of persistent activation of glutamate receptors, in particular NMDA receptors, by excessive levels of extracellular glutamate. Although the classical concept of excitotoxicity-induced neuronal cell death has been considered for a long time as a result of endogenous processes, recent data suggest a prominent role for microglia in this process. However, based on several contradictory observations it is still unclear whether microglia promote neuronal survival or exacerbate the original extent of neuronal damage in this paradigm. In **Chapter 2** we have addressed the role of microglia in an *ex vivo* model for NMDA-induced excitotoxicity in mouse hippocampal slice cultures. In this paradigm, treatment with 10-50 µM NMDA resulted in region-selective neuronal cell death in the hippocampal regions CA1, CA3 and DG, with CA1 neurons being most vulnerable to NMDA-treatment, followed by CA3 and DG neurons, respectively. Our data demonstrate that hippocampal microglia display a profound *neuroprotective* role in this paradigm as specific ablation of microglia prior to NMDA-treatment resulted in severely enhanced neuronal cell death in the hippocampal regions CA3 and DG. Moreover, our data suggest that morphological activation of microglia is not a prerequisite for their neuroprotective function. At lower concentrations of NMDA (10 µM) we did not observe neuronal cell death in the CA3 and DG regions and microglia in these regions retained their ramified phenotype. From a classical point

of view one would assume that these microglia are not active. However, neuronal loss was profound in these neuronal regions in the absence of ramified microglia when exposed to 10 µM NMDA. Therefore, it is proposed that ramified microglia should not be regarded as solely inactive "resting" cells, but as active contributors in the protection of neurons under excitotoxic conditions.

Our results are in line with other reports describing neuroprotective properties for microglia upon excitotoxic or ischemic injury (Imai et al., 2007; Kitamura et al., 2004; Lalancette-Hebert et al., 2007; Turrin and Rivest, 2006) and it is therefore tempting to suggest that microglia activation in excitotoxicity-related disorders is primarily a beneficial process. However, this assumption may be presumptuous for the following reasons. Several lines of evidence have shown that inhibition of microglia activation by immunosuppression is *beneficial* after acute CNS damage (discussed in (Hailer, 2008)). In all cases treatment with immunosuppressive agents resulted in improved neuronal survival and function, which coincided with a decrease in microglia activation. Although most of these immunosuppressants have shown to directly inhibit the pro-inflammatory profile of isolated microglia (e.g. the secretion of pro-inflammatory cytokines and NO), there is an equal amount of evidence that these agents directly affect neurons and/or astrocytes as well. Therefore, a direct link between the beneficial effects of immunosuppression and the observed downregulation of microglia function in acute CNS injury remains to be elucidated. Nonetheless, it is likely that the basal protective function of microglia might be overshadowed by their pro-inflammatory profile in response to excitotoxic conditions (Hailer, 2008; Hanisch and Kettenmann, 2007; Ransohoff and Perry, 2009). It is therefore of importance to elucidate at what time-point microglia turn from "neuroprotective" into "neurotoxic" cells in this paradigm and which factors may be implicated here.
Chapter 3 and **Chapter 4** of this thesis provide evidence that the expression of chemokines, in particular of *CXCL10* and *CCL21*, within the CNS affects neuronal damage under excitotoxic conditions, possibly through modulation of glia function.

CXCL10
Expression of the chemokine CXCL10 in the CNS has been associated with both acute and chronic CNS pathologies. Depending on the type of injury, *in vivo* expression of CXCL10 has been found predominantly in reactive astrocytes surrounding primary

lesions (Balashov et al., 1999; Omari et al., 2005; Simpson et al., 2000; Tanuma et al., 2006; Wang et al., 1998; Xia et al., 2000), and to a lesser extent in neurons (Rappert et al., 2004; Wang et al., 1998). Interestingly, expression of its corresponding receptor CXCR3 has been demonstrated in microglia (Biber et al., 2001; Biber et al., 2002a; Dijkstra et al., 2004; Rappert et al., 2002) and in astrocytes (Goldberg et al., 2001; Omari et al., 2005; Simpson et al., 2000; Tanuma et al., 2006). These findings suggest that CXCL10 and CXCR3 might provide a signaling system between neurons and glial cells under pathological conditions. In line with this suggestion, it has been demonstrated *in vitro* that CXCL10 induces CXCR3-mediated migration of microglia (Biber et al., 2001; Biber et al., 2002a; Rappert et al., 2002) and proliferation in primary astrocyte cultures (Flynn et al., 2003). However, the potential role for CXCL10/CXCR3 signaling between neurons and/or glia cells under pathological conditions is far from understood. In **Chapter 3** we have addressed this concept by examining the effect of CXCL10/CXCR3-signaling in hippocampal slice cultures in response to NMDA-induced excitotoxicity. Here, we show that NMDA-induced neuronal cell death coincided with a rapid upregulation (within 4 hours) of CXCL10 expression in astrocytes. To examine the effect of CXCL10 expression in our paradigm, we compared the levels of NMDA-induced neuronal cell death in wild type (control) slice cultures with those in slice cultures derived from CXCL10$^{-/-}$ and CXCR3$^{-/-}$ mice. In the absence of CXCL10 (CXCL10$^{-/-}$), neurons of the CA1 and CA3 regions displayed a decreased vulnerability to (10-25 µM) NMDA-treatment, while the opposite effect was observed in the DG region when exposed to high (50 µM) NMDA-concentrations. These findings not only support a role for CXCL10 in excitotoxicity, but also show that CXCL10 expression differentially affects CA and DG neurons in this paradigm. In addition, we could demonstrate that the phenotype observed in CXCL10$^{-/-}$-derived slice cultures was identical to that observed in CXCR3$^{-/-}$-derived slice cultures, indicating that CXCL10 mediates its effects through CXCR3. We confirmed these findings by demonstrating that exposure to exogenous CXCL10 (10^{-8} M) directly after NMDA-treatment "rescued" the phenotype observed in CXCL10$^{-/-}$-derived slice cultures, but not in slice cultures derived from CXCR3$^{-/-}$-mice.

Finally, we demonstrated that microglia in slice cultures expressed CXCR3 and that ablation of these cells prior to NMDA-treatment fully abrogated the differences in neuronal cell death observed between wildtype- and CXCR3$^{-/-}$-derived slice cultures. These findings suggest a prominent role for microglial CXCR3 in the degeneration of

neurons under excitotoxic conditions, which is in agreement with earlier studies from our group (Rappert et al., 2004).

Taken together, the results presented in **Chapter 3** identify a region-specific role for CXCL10/CXCR3 signaling in the communication between damaged neurons and/or glia in hippocampus excitotoxicity. Why CXCL10/CXCR3 signaling under excitotoxic conditions is detrimental in the CA regions of the hippocampus and protective in the DG region remains to be elucidated. This dual effect of CXCL10/CXCR3 signaling may depend on regional differences in CXCR3 expression. For example, heterogeneity in CXCR3 expression has been demonstrated previously in distinct microglia subpopulations (Li et al., 2006), and it would therefore be of interest to determine whether the expression profile of CXCR3 in hippocampal microglia (and other hippocampal cell-types) in the various regions vary. Another possibility for the dual role of CXCL10 in the hippocampus may be regional differences in the response of microglia to CXCR3-stimulation or regional differences in co-expression of local factors that, together with CXCL10, direct microglia to either a pro-inflammatory or a neuroprotective phenotype. Additional experiments are necessary to address these questions.

CCL21

Another prominent candidate that might influence microglia function under excitotoxic conditions is the chemokine CCL21. The expression of CCL21 *in vivo* has been exclusively demonstrated in ischemic cortical neurons in the close vicinity ("penumbra" zone) of the ischemic lesion core and in dorsal horn neurons after spinal cord injury (Biber et al., 2001; Zhao et al., 2007). In addition, neuronal expression of CCL21 has been observed *in vitro* in hippocampal slice cultures and in cortical neuron cultures when exposed to toxic levels of glutamate, showing that this chemokine is also expressed under excitotoxic conditions (Biber et al., 2002b; de Jong et al., 2005). Previously, we have shown *in vitro* that CCL21 activates microglia as measured by directed migration, induction of calcium transients and changes in electrophysiological properties (Biber et al., 2001; Dijkstra et al., 2004; Rappert et al., 2002). Moreover, it was demonstrated that activation of microglia by CCL21 was not mediated through its cognate receptor CCR7, but depended on the presence of CXCR3 (Biber et al., 2001; Dijkstra et al., 2004; Rappert et al., 2002). Taken together, it was suggested that the expression of CCL21 by neurons might provide a signal that activates/recruits CXCR3-positive microglia under pathological conditions. However, the effect of this proposed communication pathway

between injured neurons and microglia remains elusive. In **Chapter 4** we provide evidence that the presence of CCL21 in hippocampal slice cultures exacerbates neuronal damage under excitotoxic conditions. Accordingly, it was demonstrated that neuronal cell death in response to NMDA-treatment was attenuated in the CA regions of *plt* (CCL21/CCL19-deficient)-derived hippocampal slice cultures when compared to wild type conditions, while application of exogenous CCL21 (10^{-8} M) directly after NMDA-treatment aggravated neuronal cell death in these slice cultures in all three hippocampal regions. In addition, we provide evidence that this "neurotoxic" effect of CCL21 under excitotoxic conditions depends on the presence of CXCR3, and not CCR7.
As we have shown previously that acutely isolated microglia from hippocampal slice cultures express CXCR3 (see Chapter 3) and that CCL21 activates microglia *in vitro* in a CXCR3-dependent manner, it is suggested that these cells might be implicated in the neurotoxic effect of CCL21-signaling we observed in our paradigm. Interestingly, a recent study has demonstrated that injection of CCL21 directly into the ventral posterolateral nucleus of the thalamus resulted in transient activation of local microglia and induced pain-related behavior (Zhao et al., 2007). This CCL21-induced pain behavior could be blocked by co-administration of minocycline, which is known to inhibit microglia function. In line with our own findings, it is therefore likely that CCL21 triggers a response in microglia that is associated with a pro-inflammatory phenotype.
Taken together, we propose that CCL21 is a neuron-derived factor that is part of a signaling-cascade (most likely through CXCR3-mediated activity of microglia) that leads to neuronal cell death under excitotoxic conditions. Therefore, it would be of interest to determine whether specific blocking of CCL21 *in vivo* might be beneficial in animal models for excitotoxicity-related CNS pathologies.

Two ligands, one receptor, distinct signaling events

Summarizing **Chapter 3** and **Chapter 4**, we demonstrated that the expression of CXCL10 and CCL21 in the hippocampus both affected the degree of neuronal cell death in response to NMDA-induced excitotoxicity. Although we showed that the effects of these two ligands were mediated through the same receptor, namely CXCR3, various observations suggest that their expression and mode of action are not redundant but part of distinct signaling cascades. First of all, we demonstrated in our paradigm that CXCL10 was expressed in astrocytes, while CCL21 expression under excitotoxic conditions has been detected exclusively in damaged neurons (Biber et al., 2001; de Jong et al., 2005).

Secondly, if CXCL10 and CCL21 were part of a redundant signaling system, the effects of abrogation of one ligand in our paradigm would have been compensated for by the other ligand, an event we did not observe in our studies. The third observation, and maybe the most evident one, is that the absence of CXCL10- (CXCL10$^{-/-}$) or CCL21-expression (*plt*) in our paradigm resulted in different effects on the survival of CA and DG neurons after treatment with NMDA. Some overlap in effects may exist as NMDA-induced neuronal cell death in the CA regions was attenuated in the absence of either of these two ligands, however, their effects on neuronal cell death in the DG region were opposing. The observation that CXCL10 and CCL21 may have different effects in the CNS has been corroborated recently *in vivo* as transgenic over-expression of CCL21 in the CNS of intact mice resulted in the development of severe neuropathologies and premature death (Chen et al., 2002), while transgenic over-expression of CXCL10 in the CNS did not result in evident neuropathological signs under healthy conditions (Boztug et al., 2002). Although in these studies the involvement of CXCR3 in their paradigm was not investigated, it is likely that CXCL10 and CCL21 induce different signaling events through the same receptor. This assumption is also reflected in the fact that the pharmacology of CXCR3-signaling depends on the cellular background in which CXCR3 is expressed (Dijkstra et al., 2004). Accordingly, it has been demonstrated *in vitro* that CXCL10 induced CXCR3-mediated migration in cultured microglia (Biber et al., 2002a; Rappert et al., 2002), proliferation in astrocytes (Flynn et al., 2003) and changes in excitatory activity in neuronal cultures (Nelson and Gruol, 2004). Similarly, CCL21 induced migration and electrophysical changes in cultured microglia (Biber et al., 2001; Dijkstra et al., 2004; Rappert et al., 2002), while this ligand induced proliferation in astrocytes (see Chapter 5). In line with these findings we provide evidence in **Chapter 5** that the effects of CCL21 (and CXCL10) in cultured astrocytes may differ from those in microglia in dynamics, G-protein- and (partly) receptor involvement, emphasizing that indeed the pharmacology of CXCR3 signaling depends on the cell-type in which it is expressed.

CXCL10- and CCL21-induced responses in primary astrocytes: differences in receptor dependency

Finally, we have demonstrated in **Chapter 5** that CXCL10 and CCL21 induce calcium transients and proliferation in primary mouse astrocytes, suggesting that these cells might be targets for CXCL10 and/or CCL21 under pathological conditions. Although the

effects of CXCL10 on astrocytes have been demonstrated previously (Biber et al., 2002a; Flynn et al., 2003), the involvement of its receptor CXCR3 needed to be confirmed. Pronounced expression of CXCR3 was found both at an mRNA and protein level. In addition, we demonstrated that, in contrast to wild type astrocytes, CXCL10 had no effect on astrocytes derived from CXCR3$^{-/-}$ mice in terms of intracellular calcium mobilization and proliferation, confirming the involvement of this receptor in these processes.

Similar results were observed in response to CCL21 demonstrating not only that this chemokine acts on astrocytes, but confirming also that CCL21 mediates its effects through CXCR3 in these cells (CCR7 expression was not detected in these cells). Indeed, lower concentrations of CCL21 (10^{-9} -10^{-8} M) failed to induce calcium transients in astrocytes derived from CXCR3$^{-/-}$ mice. However, exposure to higher concentrations of CCL21 (10^{-7} M) clearly induced calcium transients in CXCR3$^{-/-}$ astrocytes. Moreover, we demonstrated that CCL21 promoted astrocyte proliferation, albeit that this response did not depend on the presence of CXCR3 as we observed no significant differences between wild type and CXCR3$^{-/-}$ astrocytes in this respect.

Taken together, the response of CXCR3$^{-/-}$ astrocytes to CCL21, in the absence of CCR7, suggests the involvement of another (chemokine-) receptor apart from CXCR3 and CCR7. The only other candidate that is known to bind CCL21 with high affinity is the chemokine receptor-like protein CCX-CKR (Gosling et al., 2000; Townson and Nibbs, 2002). Indeed, we did observe CCX-CKR mRNA expression in both wild type- and CXCR3$^{-/-}$ astrocytes. However, so far no signaling properties have been described for this protein and CCX-CKR is currently discussed to function as a scavenger protein for various chemokines (Comerford and Nibbs, 2005; Comerford et al., 2006; Haraldsen and Rot, 2006). Whether this chemokine receptor-like protein is involved in the CCL21-induced effects in wild type- and CXCR3$^{-/-}$ astrocytes remains to be established. Alternatively, a chemokine receptor heterodimer-system with other (non)-chemokine receptors may be involved here. Various examples of altered pharmacology after chemokine receptor-heterodimerization with other chemokine- or non-chemokine receptors have been described (Chakera et al., 2008; El-Asmar et al., 2005; Mellado et al., 2001; Pello et al., 2008; Sohy et al., 2007). Finally, involvement of a not yet characterized chemokine receptor might explain the response to CCL21 in CXCR3$^{-/-}$ astrocytes.

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NEDERLANDSE SAMENVATTING

De primaire functionele eenheid van het centrale zenuwstelsel (CZS) is de zenuwcel, oftewel het neuron. Neuronen verzorgen de communicatie tussen de verschillende hersengebieden en tussen het CZS en de rest van het lichaam via elektrochemische signaaloverdracht. Deze vorm van neuronale communicatie (*neurotransmissie*) maakt complexe processen zoals het aansturen van bewegingen, het vermogen tot waarnemen (zicht, reuk, smaak, gehoor en tast), bewustzijn, leren, denken en herinneren mogelijk. Neuronen zijn zeer kwetsbare cellen en om goed te functioneren worden deze cellen ondersteund door gespecialiseerde steuncellen, ook wel glia cellen (uit het Grieks, γλια = lijm) genoemd. Deze glia cellen worden onderverdeeld in drie groepen: oligodendrocyten, astrocyten en microglia, en hebben elk een specifieke functie in het CZS. Oligodendrocyten omwinden neuronale uitlopers (*axonen*) met myeline en versnellen zo de elektrische geleiding van neuronale signalen. Astrocyten hebben vele functies, maar zijn met name betrokken bij de handhaving van ion- en water-homeostase, de regulatie van neurotransmitterconcentraties in het CZS, het verschaffen van voedingsstoffen aan neuronen en het verzorgen van de afbraak en afvoer van afvalstoffen naar de bloedcirculatie. Daarnaast spelen astrocyten een belangrijke rol bij het herstel van neuronen na schade in het CZS. Microglia daarentegen worden beschouwd als de immuuncompetente cellen van het CZS. Microglia manifesteren zich in het "gezonde" CZS als kleine cellen met vertakte uitlopers, waarmee ze continu hun directe omgeving aftasten. Stoffen die normaal niet aanwezig zijn of in verhoogde mate tot expressie worden gebracht in het CZS kunnen herkend worden door middel van specifieke receptoren aan het celoppervlak. Voorbeelden van stoffen die herkend kunnen worden door microglia zijn niet alleen "lichaamsvreemde" stoffen zoals bacteriële- en virale componenten, maar ook celresten, bloedcomponenten en stressfactoren afkomstig van naburige cellen. Wanneer er sprake is van schade in het CZS trekken microglia (in bijna alle gevallen) hun uitlopers in en transformeren ze van vertakte cellen in ronde "geactiveerde" macrofaagcellen. Door deze transformatie zijn microglia in staat te migreren naar het schadegebied (*chemotaxis*) en, eenmaal aangekomen, afgestorven cellen en celresten op te nemen (*fagocytose*) en af te breken om zo het herstel na weefselschade te bevorderen. Daarnaast kunnen geactiveerde microglia zich vermeerderen door celdeling (*proliferatie*).

Naast het hebben van een ondersteunende functie is tevens gebleken dat glia cellen (met name astrocyten en microglia) een belangrijke rol spelen bij de initiatie en regulatie van ontstekingsprocessen in het CZS. Elke vorm van weefselschade of verstoring leidt dan ook tot de activering van deze cellen. Deze activering manifesteert zich in de accumulatie van geactiveerde microglia rondom- en in het directe schadegebied en kan zich uiten in littekenweefselvorming door lokale astrocyten. Daarnaast geven astrocyten en microglia stoffen af die het lokale schadeproces kunnen versnellen of vertragen. Omdat neuronen zeer kwetsbaar zijn, is het van belang dat de activering van glia cellen zeer gecontroleerd verloopt. Zowel in het gezonde- als het beschadigde CZS vindt dan ook een continue communicatie plaats tussen neuronen en glia cellen via directe cel-cel interacties en door afgifte van signaalstoffen over en weer. Langdurige verstoringen in deze interacties (b.v. wanneer er sprake is van grote schade of een infectie) kan resulteren in overmatige, chronische of inadequate activering van glia cellen wat weer kan leiden tot een verergering van het originele schadeproces. Het is daarom van belang om beter inzicht te krijgen in het proces van neuron-glia communicatie onder zowel fysiologische als neuropathologische omstandigheden. Met name de rol van microglia in dit proces is interessant, omdat in theorie zowel de neurotoxische als de neuroprotectieve eigenschappen van deze cellen een therapeutisch aangrijpingspunt kunnen zijn voor acute of progressieve hersenaandoeningen.

Het doel van het onderzoek beschreven in dit proefschrift was **1.** om meer inzicht te krijgen in de rol van microglia tijdens neuronale schade en **2.** stoffen te identificeren die mogelijk een rol spelen in de communicatie tussen neuronen en geactiveerde glia cellen tijdens neuronale schade.

Microglia en excitotoxiciteit

Excitotoxiciteit speelt een rol in zowel acute- als chronische hersenaandoeningen en is een proces waarbij te hoge extracellulaire concentraties van de neurotransmitter glutamaat (veroorzaakt door een afname in beschikbare energie in neuronen) leidt tot overprikkeling van neuronen met als gevolg dat deze cellen afsterven. Eerder werd verondersteld dat dit proces van neuronale celdood geheel werd bepaald door intracellulaire cascades in het neuron zelf. In de laatste jaren is echter duidelijk geworden dat glia cellen in hoge mate bijdragen aan dit proces. De rol van microglia in excitotoxiciteit is echter nog onduidelijk, omdat uit onderzoek is gebleken dat, afhankelijk

van de methode, onderdrukking van geactiveerde microglia tijdens excitotoxiciteit tot zowel een versterking als een afzwakking van het schadeproces (= neuronale sterfte) kan leiden. In **Hoofdstuk 2** hebben we de rol van microglia in het proces van excitotoxiciteit-gemedieerde neurodegeneratie nader onderzocht. Om het proces van excitotoxiciteit *ex vivo* na te bootsen hebben we gebruik gemaakt van hippocampale slice cultures (kleine hersenplakjes) afkomstig van muizen en deze vervolgens behandeld met de glutamaat receptor agonist N-methyl-D-aspartaat (NMDA). Behandeling met NMDA resulteert specifiek in het afsterven van neuronen in deze slice cultures.

Onze resultaten laten zien dat na behandeling met NMDA zowel activering als accumulatie van microglia alleen plaatsvindt in gebieden waar neuronale celdood optreedt, terwijl microglia in ongeschonden gebieden hun vertakte vorm behouden. Vervolgens hebben we laten zien dat wanneer de gehele microglia populatie in de slice cultures van tevoren wordt verwijderd met behulp van clodronaat (specifiek toxisch voor microglia), het aantal neuronen dat afsterft na behandeling met NMDA zeer sterk toeneemt ten opzichte van de controle situatie. Dit effect van verhoogde neuronale celdood in de afwezigheid van microglia werd ook waargenomen in neuronale gebieden waar eerder geen celdood gedetecteerd was, wat suggereert dat vertakte microglia bijdragen in de bescherming van neuronen onder excitotoxische omstandigheden. Samengevat laten deze resultaten zien dat microglia een basale neuroprotectieve waarde hebben in het proces van excitotoxiciteit.

Het is echter niet ondenkbaar dat bepaalde stoffen die worden afgegeven tijdens weefselschade in het CZS een negatieve invloed kunnen hebben op het activeringsproces van microglia. Men heeft bijvoorbeeld laten zien dat het herstel na neuronale schade kan worden bevorderd wanneer geactiveerde microglia (deels) worden onderdrukt in hun functie. In hoofdstuk 3 en 4 van dit proefschrift laten we zien dat de expressie van bepaalde chemotactische cytokines (*chemokines, zie kader 1*) in het CZS invloed kan hebben op de mate waarin neuronen afsterven, een proces dat mogelijk gemedieerd wordt door microglia.

Kader 1

Chemokines zijn kleine chemotactische cytokines (8-12 kDa) en zijn met name betrokken bij de regulatie van cellulaire migratie in het lichaam. Een kenmerkend voorbeeld is de migratie van perifere immuuncellen van- en naar de lymfeknopen en naar doelorganen. Andere processen waar chemokines bij zijn betrokken zijn o.a. de ontwikkeling, wondheling, cel adhesie, bloedvatvorming en tumorgroei.

Nomenclatuur
De chemokine familie wordt onderverdeeld in vier subfamilies (CCL, CXCL, CX_3CL en CL), gebaseerd op de lokatie en het aantal hoog geconserveerde cysteine residuen in de amino terminus. Chemokines Induceren hun effecten via G-eiwit gekoppelde receptoren. Op dit moment zijn ongeveer 50 chemokines en 20 chemokine receptoren beschreven. De nomenclatuur van de chemokine receptoren volgt dat van de chemokines waar ze preferent aan binden, namelijk CCR, CXCR, XCR en CX_3CR. Let wel, de meeste chemokine receptoren kunnen meerdere chemokines binden, en één chemokine kan meerdere receptoren binden.

Chemokines in het CZS
In de laatste 10-20 jaren is gebleken dat chemokines door endogene cellen van het CZS tot expressie worden gebracht en met name wanneer er sprake is van weefselschade. Initieel werd gedacht dat dit enkel als doel had om perifere immuuncellen aan te trekken tijdens schade. Nu is echter bekend dat ook neuronen en glia cellen chemokine receptoren tot expressie brengen en dat het chemokine-receptor netwerk in het CZS een belangrijke plaats inneemt in de communicatie tussen neuronen en glia cellen zowel onder fysiologische- als onder pathologische condities.

CXCL10 in excitotoxiciteit

Een van de chemokines die mogelijkerwijs een rol speelt in de communicatie tussen glia cellen en/of neuronen tijdens neuronale schade is CXCL10. Verscheidene studies in zowel mensen als muizen hebben aangetoond dat CXCL10 verhoogd tot expressie wordt gebracht in het CZS wanneer er sprake is van acute schade of een hersenziekte. Afhankelijk van het diermodel of het type hersenziekte heeft men laten zien dat zowel neuronen als astrocyten CXCL10 tot expressie kunnen brengen, terwijl de receptor voor CXCL10, te weten CXCR3, met name werd gevonden in microglia en astrocyten. *In vitro* experimenten hebben uitgewezen dat CXCL10 zowel migratie van microglia als proliferatie van astrocyten kan induceren. Een interessant gegeven is dat wanneer men in muizen de expressie van CXCL10 en/of CXCR3 *in vivo* tegengaat of blokkeert met antilichamen dit invloed heeft op het verloop van het ziekteproces in het CZS. Het is

echter tot nu toe niet bekend welke rol neuronen, astrocyten en microglia hebben in dit proces.

In **Hoofdstuk 3** hebben we de rol van CXCL10 in het proces van excitotoxiciteit nader onderzocht. Dit hebben we wederom gedaan in het slice culture model beschreven in hoofdstuk 2. Onze bevindingen laten zien dat wanneer slice cultures behandeld worden met NMDA, astrocyten CXCL10 tot expressie brengen. Met behulp van slice cultures afkomstig van CXCL10- en CXCR3-knockout muizen hebben we vervolgens laten zien dat, in de afwezigheid van het ligand of de receptor, het aantal neuronen dat afsterft na NMDA behandeling in het ene neuronale gebied toeneemt maar in anderen afneemt. Dit effect in slice cultures afkomstig van CXCL10-knockout muizen kon worden tegengegaan door recombinant CXCL10 toe te voegen aan het medium na behandeling met NMDA. Toevoeging van recombinant CXCL10 na NMDA behandeling had geen effect in slice cultures afkomstig van CXCR3-knockout muizen, wat in combinatie met bovengenoemde resultaten laat zien dat het effect van CXCL10 op neuronale celdood gemedieerd is door CXCR3.

Omdat microglia CXCR3 tot expressie brengen, hebben we gekeken naar het effect van microglia depletie (door middel van clodronaat-behandeling) in slice cultures van controle- en CXCR3-knockout muizen en deze met elkaar vergeleken. Onze resultaten laten zien dat in de afwezigheid van microglia de verschillen in neuronale celdood tussen controle en CXCR3-knockout slice cultures verdwenen zijn, wat suggereert dat het effect van CXCL10/CXCR3 signalering op neuronale celdood door microglia gemedieerd wordt. Samengevat laten de resultaten in hoofdstuk 3 zien dat CXCL10 een rol speelt in de communicatie tussen microglia en astrocyten tijdens excitototoxische schade en dat deze vorm van communicatie effect heeft op de mate van neuronale sterfte in dit proces.

CCL21 in excitotoxiciteit

Een ander chemokine dat mogelijk een rol speelt in de regulatie van geactiveerde microglia onder neuropathologische condities is CCL21. Onlangs heeft men in twee verschillende muismodellen voor acute schade in het CZS laten zien dat beschadigde of bedreigde neuronen CCL21 tot expressie brengen. Daarnaast heeft men *in vitro* aangetoond dat neuronen CCL21 tot expressie brengen onder excitotoxische omstandigheden. Deze observaties zijn zeer interessant, omdat men tevens *in vitro* heeft aangetoond dat microglia geactiveerd kunnen worden door CCL21. Ten tweede is aangetoond dat deze activering niet via de conventionele receptor voor CCL21 (CCR7)

verloopt, maar via CXCR3. Op basis van deze data heeft men dan ook voorgesteld dat de expressie van CCL21 door beschadigde of bedreigde neuronen een rol zou kunnen spelen in de activering en recrutering van CXCR3-positieve microglia uit de directe omgeving. In **Hoofdstuk 4** laten we met behulp van hippocampale slice cultures zien dat het aantal neuronen dat afsterft door NMDA behandeling sterk afneemt wanneer CCL21 niet tot expressie wordt gebracht. Dit effect kon worden tenietgedaan door recombinant CCL21 toe te voegen aan het medium na NMDA behandeling. Deze resultaten suggereren dat CCL21 een negatief effect heeft op de overleving van neuronen onder excitotoxische omstandigheden. Daarnaast laten we zien dat het effect van CCL21 in deze slice cultures gemedieerd wordt door CXCR3. Of het negatieve effect van CCL21 op neuronale celdood van directe aard is of dat dit effect via microglia wordt gemedieerd is op dit moment (nog) niet duidelijk.

CCL21-gemedieerde effecten in astrocyten

Zoals eerder genoemd heeft men aangetoond dat behandeling met CCL21 leidt tot de activering en migratie van microglia *in vitro*, een proces dat wordt bepaald door de aanwezigheid van CXCR3 in deze cellen. Een interessant gegeven is dat, naast microglia, astrocyten ook CXCR3 tot expressie brengen. Het is echter niet bekend of astrocyten in staat zijn te reageren op CCL21. In **Hoofdstuk 5** hebben we daarom onderzocht of astrocyten in staat zijn te reageren op CCL21, en zo ja, welke receptor hierbij een rol speelt. Met behulp van live-imaging microscopie en proliferatie assays hebben we *in vitro* kunnen aantonen dat behandeling met CCL21 leidt tot zowel celdeling (*proliferatie*) als het vrijkomen van intracellulair calcium in deze cellen. Daarnaast laten we zien dat, in tegenstelling tot microglia, CCL21-gemedieerde responsen in astrocyten maar deels worden bepaald door de aanwezigheid van CXCR3. Sinds de expressie van CCR7 (= de conventionele receptor voor CCL21) zowel op mRNA- als op eiwit niveau niet werd gevonden in deze cellen, geven onze data aan dat de effecten van CCL21 op astrocyten (deels) gemedieerd worden door een andere receptor dan CXCR3 en CCR7. De identiteit van deze receptor is vooralsnog niet bekend.

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