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The impact of Toll-like receptor activation on neuroinflammation and neurodegeneration in the central nervous system

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Summary

The innate immune system is able to detect and respond to a diverse range of pathogen- and host-derived molecules, such as cell wall components, nucleic acids and heat shock proteins. These exogenous and endogenous molecules are recognized by the Toll-like receptors (TLRs), a family of 13 highly conserved pattern-recognition receptors. TLRs are present in the central nervous system (CNS), predominantly expressed in microglia, the major immune cells of the brain. Increasing evidence suggests that, along with microglia-mediated neuroinflammation, TLRs play a crucial role in CNS pathologies such as infection, stroke and classical neurodegenerative diseases.

This thesis describes the consequences of TLR activation in the CNS. Highly specific ligands for TLR2, TLR4, TLR7 and TLR9, were used to address the TLR's individual impact on neuroinflammation and –degeneration. In addition, combined TLR activation was assessed, as it can be assumed that in both physiological and pathological states of the CNS, more than one TLR ligand is present and multiple TLRs are activated.

In vitro, activation of TLR2, TLR4, TLR7 and TLR9 with their specific exogenous ligands resulted in the microglia-mediated release of inflammatory molecules. Moreover, combined TLR stimulation of TLR4 with TLR2 and TLR4 with TLR9 resulted in an increased release of TNF-α, IL-6, IL-10 and nitric oxide compared to activation of the respective single TLR *in vitro*. In contrast, additional activation of TLR7 suppressed the inflammatory response mediated by TLR2, TLR4, or TLR9, indicating that TLRs individually modulate the immune response. Further, neuronal cell injury occurred in response to activation of each TLR, whereas only the combination of TLR4 with TLR2 resulted in significantly more neuronal cell death compared to activation of each TLR alone. Remarkably, when replacing the exogenous ligand of TLR4 by its endogenous ligand heat shock protein 60 (HSP60), a stress-related protein, combined activation of TLR4 with TLR7 resulted in an increased loss of neurons compared to single TLR activation, indicating diverging events subsequent of TLR4 activation.

In vivo, the impact of TLR activation in the murine brain was evaluated after intrathecal injection of the respective exogenous ligands for TLR2, TLR4, TLR7 and TLR9. Real-time PCR analysis revealed that activation of each TLR leads to a distinct change in the mRNA expression pattern of TLR1-9 in the murine brain. Furthermore, immunohistological analysis of brain sections revealed that activation of TLR2, TLR7 and TLR9 causes axonal and neuronal cell loss in the cerebral cortex. This was in part accompanied by a neuroinflammatory response as indicated by microglia displaying an activated morphology, by the influx of leukocytes into the cerebrospinal fluid and by elevated levels of TNF-α and IL-1β mRNA in brain homogenates.

Further, the effect of TLR4 activation by its endogenous ligand HSP60 was evaluated

in vivo. Intrathecal injection of HSP60 caused neuronal cell death and axonal injury, as well as demyelination and loss of oligodendrocytes in the cerebral cortex of wild-type mice. These deleterious effects were dependent on the TLR4-pathway as mice lacking TLR4 were protected. However, in contrast to the exogenous ligand of TLR4, lipopolysaccharide, intrathecal HSP60 did not induce a measurable inflammatory response in the murine brain. An additional challenge with HSP60 did not amplify TLR2- and TLR7-induced CNS inflammation, and loss of axons and neurons. To determine whether HSP60 is present in the CNS in a physiological context, brain sections of mice were subjected to immunohistological analysis. Endogenous HSP60 was found to be predominantly expressed in neurons. Moreover, elevated levels of HSP60 were detected in the cerebrospinal fluid of mice subjected to a middle cerebral artery occlusion, the mouse-model of ischemic stroke, compared to sham-operated mice, indicating a release of HSP60 during brain injury and a mechanism by which TLR4 is exposed to HSP60 in CNS pathologies.

Taken together, these findings demonstrate that sole and combined activation of TLRs modify the pattern and extent of TLR expression, of inflammation and of neuronal injury in the CNS, a mechanism by which the intrinsic innate immunity might co-determine the diversity of CNS diseases. Further, as demonstrated with the endogenous TLR4 ligand HSP60, the release of TLR ligands during CNS cell damage, a common event in many forms of brain disease, bi-directionally links CNS injury to neurodegeneration and demyelination *in vivo*.

Zusammenfassung

Das angeborene Immunsystem ist in der Lage auf unterschiedliche pathogen- und wirtsspezifische Moleküle, wie Zellwandkomponenten, Nukleinsäuren und Hitze Schock Proteine zu reagieren. Diese exogenen und endogenen Moleküle werden durch die Toll-like Rezeptoren (TLR), eine Familie aus 13 Rezeptoren, welche hoch-konservierte Muster erkennen, detektiert. TLR sind im Zentralen Nerven System (ZNS) vorhanden und werden überwiegend von Mikroglia, den zentralen Immunzellen des ZNS exprimiert. Es gibt zunehmend Hinweise dafür, dass neben der Mikroglia-vermittelten Neuroinflammation, TLR eine wichtige Rolle in ZNS Erkrankungen, wie Infektionen, Schlaganfall und klassischen neurodegenerativen Erkrankungen spielen.

Diese Arbeit beschreibt die Auswirkungen von TLR Aktivierung im ZNS. Hoch-spezifische Liganden für TLR2, TLR4, TLR7 und TLR9 wurden verwendet, um den Einfluss einzelner TLR auf neuroinflammatorische und -degenerative Prozesse zu untersuchen. Da anzunehmen ist, dass sowohl im gesunden als auch im erkranktem ZNS mehr als nur ein TLR Ligand anwesend sind und somit mehrere TLR aktiviert werden, wurde zusätzlich untersucht, welche Auswirkungen die Aktivierung mehrere TLR hat.

Die Aktivierung von TLR2, TLR4, TLR7 und TLR9 mit ihren spezifischen exogenen Liganden resultierte in einer Mikroglia-vermittelten Freisetzung von inflammatorischen Molekülen *in vitro*. Darüber hinaus führte die kombinierte Aktivierung von TLR4 mit TLR2 und TLR4 mit TLR9 zu einer vermehrten Sezernierung von TNF-α, IL-6, IL-10 und Stickstoffmonoxid im Vergleich zur Aktivierung der jeweiligen einzelnen TLR. Im Gegensatz dazu unterdrückte die gleichzeitige Aktivierung von TLR7 die TLR2-, TLR4- und TLR9-induzierte inflammatorische Antwort, ein Hinweis dafür, dass TLR die Immunantwort individuell beeinflussen. Desweiteren führte eine Aktivierung der einzelnen TLR zu neuronalem Zelltod *in vitro*, der durch eine gemeinsame Aktivierung von TLR4 mit TLR2 signifikant erhöht war. Als jedoch der exogene Ligand von TLR4 durch den endogenen Liganden *Heat Shock Protein 60* (HSP60) ersetzt wurde, führte im Vergleich zur Stimulation der jeweiligen einzelnen TLR die gemeinsame Aktivierung von TLR4 und TLR7 zu einem vermehrten Verlust von Neuronen. Dies deutet darauf hin, dass der exogene und endogene Ligand von TLR4 unterschiedliche Signalkaskaden induzieren.

In vivo wurde die Auswirkung einer TLR Aktivierung im ZNS nach der intrathekalen Injektion der jeweiligen Liganden von TLR2, TLR4, TLR7 und TLR9 in Mäuse untersucht. Eine Analyse mittels quantitativer PCR zeigte auf, dass die Aktivierung der einzelnen TLR zu unterschiedlichen mRNA Expressionsmustern von TLR1-9 im Mausgehirn führte. Eine immunohistologische Analyse von Gehirnschnitten ergab, dass die Aktivierung von TLR2, TLR7 und TLR9 mit einem Verlust von Neurone und Axone im zerebralen Kortex einhergeht. Dieser Verlust wurde zum Teil von einer neuroinflammatorischen Antwort begleitet,

gekennzeichnet durch Mikroglia mit einer aktivierten Morphologie, die Einwanderung von Leukozyten in die zerebrospinale Flüssigkeit und eine Zunahme an TNF-α and IL-1β mRNA in Gehirnhomogenaten.

Desweiteren wurde der Einfluss einer TLR4 Aktivierung durch den endogenen Liganden HSP60 *in vivo* untersucht. Die intrathekale Injektion von HSP60 hatte eine Schädigung der Axone, einen Verlust von Neuronen und Oligodendrozyten, sowie eine Demyelinisierung im zerebralen Kortex zur Folge. Diese zerstörerischen Effekte waren von der TLR4-vermittelten Signalkaskade abhängig, da TLR4 defiziente Mäuse geschützt waren. Jedoch im Gegensatz zu dem exogenen TLR4 Liganden, Lipopolysaccharid, verursachte HSP60 keine messbare inflammatorische Antwort, wie z.B. die Aktivierung von Mikroglia oder Sezernierung von Zytokinen. Interesanterweise, verstärkte eine zusätzliche Injektion von HSP60 die TLR2- beziehungsweise TLR7-induzierte inflammatorische Antwort und den Verlust von Neuronen und Axonen im ZNS nicht. Um feststellen zu können, ob HSP60 unter physiologischen Bedingungen im ZNS vorhanden ist, wurden Gehirnschnitte von Mäusen immunohistologisch untersucht. Endogenes HSP60 wurde überwiegend in Neuronen gefunden. Desweiteren wurde, im Vergleich zu Kontroll-Mäuse, erhöhte Mengen an HSP60 in der zerebralospinalen Flüssigkeit von Mäusen detektiert, bei denen ein ischemischer Schlaganfall durch den Verschluss der mittleren zerebralen Arterie hervorgerufen wurde. Dies ist ein Hinweis dafür, dass HSP60 während einer Gehirnverletzung freigesetzt wird und zeigt ein Mechanismus auf durch den TLR4 in Erkrankungen des ZNS HSP60 ausgesetzt wird.

Zusammenfassend demonstrieren diese Ergebnisse, dass die alleinige und gemeinsame Aktivierung von TLR das Muster und Ausmaß von TLR-Expression, Inflammation und Schädigung im ZNS beeinflussen. Ein Mechanismus durch den womöglich das angeborene Immunsystem im ZNS die unterschiedlichen Ausprägungen von ZNS Erkrankungen mitbestimmt. Außerdem wurde anhand des endogenen TLR Liganden HSP60 demonstriert, dass die Freisetzung von TLR Liganden durch eine Schädigung von ZNS Zellen, eine Begleiterscheinung vieler ZNS Erkrankungen, wechselseitig ZNS Schädigung mit Neurodegeneration und Demyelinisierung koppelt.

List of Abbreviations

Aβ	Amyloid beta
AD	Alzheimer's Disease
AP-1	Activator protein 1
APC	Adenomatous Polyposis Coli
CCL	Chemokine (C-C motif) Ligand
CNS	Central Nervous System
CpG	Cytosine-phosphate-guanosine
CSF	Cerebrospinal Fluid
CT	Cycle Threshold
CXCL	Chemokine (C-X-C motif) Ligand
d	days
DAMP	Danger-associated Molecular Pattern
DMEM	Dulbecco's modified Eagle's Medium
DNA	Desoxyribonucleic Acid
dsRNA	Double stranded RNA
EAE	Experimental Autoimmune Encephalomyelitis
ELISA	Enzyme-linked Immunosorbent Assay
FACS	Fluorescence activated Cell Sorter
FCS	Fetal Calf Serum
g	Gravitational acceleration
GFAP	Glial Fibrillary Acidic Protein
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
h	hour
HBSS	Hank's buffered Salt Solution
HSP60	Heat Shock Protein 60
Iba1	Ionized Calcium Adaptor binding Molecule 1
IFN	Interferon
IL	Interleukin
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon Regulatory Factor
kDA	Kilo Dalton
LPS	Lipopolysaccharide
MBP	Myelin Basic Protein
MCAo	Middle Cerebral Artery occlusion
min	Minute
mRNA	Messenger RNA

MyD88	Myeloid differentiation primary response 88
NeuN	Neuronal Nuclei
NF	Neurofilament
NF-κB	Nuclear Transforming Factor kappa B
NO	Nitric Oxide
PAMP	Pathogen-associated Molecular Pattern
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
rpm	Revolutions per minute
RT	Room temperature
SA	Serum Albumin
SD	Standard Deviation
SEM	Standard error of the mean
ssRNA	Single stranded RNA
TGF-β	Tranforming Growth Factor beta
TIR	Toll/Interleukin-1 receptor
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
TRAF6	TNF receptor-associated factor
TRIF	TIR domain-containing adaptor inducing interferon-beta
Tris	Trisaminomethane
TUNEL	Terminal desoxyribosyl-transferase mediated dUTP nick end labeling
WT	Wild-type

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Introduction

Neuroinflammation is potentially destructive as it involves release of cytotoxic molecules. The central nervous system (CNS) is a tissue with limited regenerative capacity and severe damage can be a threat for essential body functions and even survival. Thus restriction of neuroinflammation is crucial. The idea that the CNS is largely ignored by the immune system was formed when experiments showed its foreign graft acceptance (Medawar, 1948). The presence of the blood-brain barrier and blood-cerebrospinal fluid barrier with restricted entry of antibodies and peripheral immune cells, as well as the absence of conventional lymphatic drainage, of major histocompatibility complex expression and of resident professional antigen presenting cells, supported the idea of the CNS being an immune privileged site (Arck et al., 2008). Meanwhile, a large body of evidence exists that the brain is not immunologically inert, but dynamically interacts with the immune system within a complex bi-directional relationship. Immunocompetent cells, such as astrocytes and microglia reside within the CNS (Ransohoff and Brown, 2012) and even under non-pathological conditions leukocytes can traffic into the CNS (Hickey, 1999). Further, the brain is actively kept in an immunosuppressed state by high concentrations of anti-inflammatory molecules such as the cytokines TGF-β and IL-10 and the T cell toxic gangliosides (Ransohoff and Brown, 2012). In addition, inflammatory responses can be inhibited or attenuated by a myriad of soluble factors secreted by neurons themselves (Chavarría and Cárdenas, 2013). However, pro-inflammatory processes can be elicited in the CNS. Although inflammation makes way for repair processes and restoration of homeostasis after infection and injury, it is of considerable debate what tips the balance leading to excessive or chronic inflammation, and how inflammation is involved in autoimmune and neurodegenerative diseases of the CNS. Numerous studies point to Toll-like receptors as pivotal contributors.

1.1 The intrinsic immune system of the CNS

A vast population of glial cells exist in the CNS, easily outnumbering neurons, indicating how much support neurons need to process and transmit information. Glial cells are specialized CNS cells that ensure the structural and physiological integrity of the CNS, by contributing to developmental processes, neuronal metabolism and function, maintenance and repair of the CNS, as well as by exerting immunological functions to name but a few of their functions. Particularly astrocytes establish numerous supportive and functional connections with their highly ramified processes to adjacent cells such as neurons and blood vessels (Brambilla et al., 2013). Astrocytes are important regulators of

CNS homeostasis, as they provide neurons with nutrients, regulate the permeability of the blood-brain barrier and control levels of extracellular neurotransmitters and ions (Brambilla et al., 2013). Further, it has become clear that they facilitate all aspects of CNS performance and even participate in neuronal communication (Brambilla et al., 2013). Moreover, astrocytes are involved in sustaining the anti-inflammatory microenvironment of the CNS (Gimsa et al., 2013). Although, in pathological conditions astrocytes can change their morphological and biochemical phenotype displaying "reactive astrocytosis". In this state astrocytes secrete pro-inflammatory mediators and if necessary proliferate and accumulate to form a glial scar (Gimsa et al., 2013). However, the overall picture of astrocytes suggests that they harbor mechanisms to limit damage by releasing factors with anti-inflammatory, neuroprotective and growth promoting properties (Gimsa et al., 2013). Although astrocytes and even neurons participate in CNS inflammation (Jensen et al., 2013; Chavarría and Cárdenas, 2013), microglia–a population of innate immune cells in the CNS- are regarded as the main effector cells in immune surveillance of the CNS (Kettenmann et al., 2011) and will be introduced in the following.

1.1.1 Microglia - resident immune sentinels of the CNS

Microglia are resident macrophages and are distributed throughout the CNS. Microglia invade the CNS during development and therefore do not arise from neuroectoderm-derived neural progenitor cells as neurons, oligodendrocytes and astrocytes do (Levison et al., 2003), but they also are not born from hematopoietic progenitors as the majority of immune cells. Recently, it was reported that microglia are derived from primitive myeloid precursors in the yolk sac, prior to the development of the bone marrow-derived hematopoietic system (Alliot et al., 1999; Ginhoux et al., 2010). These primitive myeloid precursors populate the CNS as tissue specific macrophages, which are well adapted and participate in CNS development and homeostasis (Ginhoux et al., 2010). Depending on the brain region 0.5-16.6% of the CNS cells are microglia (Mittelbronn et al., 2001). Microglia are capable of self-renewal independent of hematopoietic progenitors (Ginhoux et al., 2010). On the other hand, blood-borne monocytes/macrophages can repopulate the CNS after depletion of microglia and mimic their function to a certain extent (Varvel et al., 2012) pointing to the importance of constant presence of macrophages in the brain.

In the healthy brain, parenchymal microglia exhibit a ramified morphology visible as fine processes with secondary and tertiary branching, radially arranged around a small somata. This apparent quiescent state is caused by direct cell-cell contact as well as immunosuppressive molecules in their environment such as IL-10, TGF-β and fractaline, produced by microglia themselves and neighboring cells (Kettenmann et al., 2011). However, the microglia are not inactive or silent. They continuously monitor their microenvironment

and adjacent cells by palpation with their motile processes (Nimmerjahn et al., 2005). Since microglia are constantly alert they can rapidly react to disturbances and are therefore the predominant cell type in initiating an immune response in the CNS (Streit et al., 1988). Thereby they swiftly adopt diverse functional phenotypes and an amoeboid morphology, a state commonly termed as "activated". Depending on the stimulus confronted with they migrate to the infected area or lesion, with local proliferation and accumulation, up-regulate cell surface markers, produce various inflammatory molecules as well as growth factors and/or increase phagocytic activity. Benefits of microglial activation are elimination of pathogens or removal of cell debris, restoration of homeostasis and initiation of repair processes in the CNS (Czeh et al., 2011). However, exaggerated or proceeding activation of microglia and their excessive release of free radicals is thought to contribute to progressive damage of the CNS (Block et al., 2007). The impact of activated microglial, as key contributors to neuroinflammation, may depend on the pathological context in which they are activated and the cues they are provided with as outlined below.

1.1.2 Neuroinflammation – a pivotal factor in CNS pathologies

Neurons require a strictly regulated environment to function properly. They are highly sensitive to excessive amounts of the major excitatory transmitter glutamate (Takeuchi et al., 2006) or of inflammation-related factors, such as TNF-α and reactive oxygen and nitrogen species (Lyman et al., 2014), which are released by activated microglia. Depending on the brain area affected, loss of neurons and their projections can result in severe neurological deficits and disabilities, which cannot be compensated or only to a certain degree. As CNS neurons show poor regenerative capacities due to sub-optimal repopulation by resident neuronal precursors (Björklund and Lindvall, 2000), molecular inhibitors of axonal growth (Fournier and Strittmatter, 2002) and brain tissue scaring (Kawano et al., 2012), neurodegeneration, the progressive, irreversible loss of neurons, is detrimental. Oligodendrocytes, which form the myelin sheath, thereby accelerating neural conduction and providing long-term integrity of neuronal axons in the CNS (Fünfschilling et al., 2012), are likewise affected by the hostile environment generated during an immune response. Oligodendrocytes can be replaced by precursor cells (Crawford et al., 2013); however, the initial loss or dysfunction of oligodendrocytes and their myelin (demyelination) impairs axonal function and contributes to neurodegeneration (Lee et al., 2012). Therefore, inflammatory conditions and release of mediators with potentially cytotoxic properties can be detrimental in the CNS.

Invasion of bacteria, viruses or parasites into the brain can be appreciated as a situation, which requires an inflammatory response. Upon pathogen-recognition microglia release pro-inflammatory factors, such as TNF-α, IL-1β, IL-6 (Olson and Miller, 2004) and recruit peripheral immune cells with chemokines such as CCL2 and CCL5 (Rock et al., 2004).

In addition, in defense against bacteria, microglia release free radicals during a respiratory burst (Gerber and Nau, 2010). Activation of the immune response is primarily beneficial, since it leads to elimination of pathogens, but an excessive generation of pro-inflammatory cytokines and reactive oxygen and nitrogen species elicit neurotoxicity (Block et al., 2007; Banati et al., 1993). For example, TNF-α, a central pro-inflammatory cytokine, can fuel the immune response by inducing the generation of further cytokines, such as IL-1 and IL-6, and the synthesis of nitric oxide (Aggarwal and Natarajan, 1996). Further, TNF-α can stimulate excessive glutamate release resulting in exitoneurotoxicity (Takeuchi et al., 2006). Moreover, transgenic mice overexpressing TNF-α in the CNS developed a spontaneous inflammatory demyelinating disease (Probert et al., 1995). Another crucial molecule is nitric oxide. Nitric oxide (NO) is a messenger molecule in the CNS (Calabrese et al., 2007); however since it is membrane permeant, cells cannot regulate its local concentration. Inappropriate formation during inflammation can cause neuronal cell overstimulation. Moreover, after conversion into the potent oxidant peroxynitrite it can react with lipids, proteins and nucleic acid thereby causing severe cell damage (Dawson and Dawson, 1996). It has been demonstrated that excessive release of NO by activated microglia causes neuronal cell death (Chao et al., 1992; Lehnardt et al., 2006). Thus, in addition to the toxicity of the pathogen itself, neuroinflammation can contribute to a great deal to CNS damage (Weber and Tuomanen, 2007; Gerber and Nau, 2010).

Besides during infection, activated microglia and inflammatory processes are found likewise in several other CNS insults and disorders such as traumatic injury, stroke and Alzheimer' disease (Kreutzberg, 1996). The factors triggering onset of Alzheimer's disease (AD), a neurodegenerative CNS disorder causing prevailing dementia, are largely unidentified. However, besides its pathological hallmarks of extensive amyloid beta protein (Aβ) deposits, neurofibrillary tau-tangles and considerable neuronal atrophy in certain brain regions, increased levels of cytokines, chemokines, scavenger receptors and oxidative damage are found within the brain (Landreth and Reed-Geaghan, 2009; Akiyama et al., 2000). Activated microglia and elevated cytokine levels are closely related to Aβ plaques (Bolmont et al., 2008). The activated microglia, which extend and wrap their processes around Aβ plaques, are considered to contribute to the progressive neuronal injury in AD by release of neurotoxic products (Bolmont et al., 2008) in addition to neurotoxicity of Aβ itself (Hickman et al., 2008). On the other hand, microglia are capable of clearing Aβ by phagocytosis; however, Aβ-induced chronic inflammation likely results in malfunction of microglia thereby promoting accumulation of Aβ and progression of AD (Hickman et al., 2008). Thus, chronic inflammation has emerged as one cause of persistent neuronal cell damage in neurodegenerative diseases (Block et al., 2007; Lull and Block, 2010). Approaches for treating AD therefore focus on supporting microglia-mediated Aβ removal, but without inducing neurotoxic inflammation (Weiner and Frenkel,

2006).

Neuroinflammation is also a considerable element in CNS injury caused by trauma or an ischemic event. Traumatic CNS injury can be caused by mechanical forces (e.g. compression, contusion, laceration), swelling or bleeding of brain tissue as a result of an accident or surgery. In experimental models such as entorhinal cortex lesion, facial nerve crush or traumatic brain injury, an accumulation of activated microglia is evident around the lesion and neuroninflammation is established as a key mechanism promoting a continuous spread of tissue damage (Kumar and Loane, 2012). Another form of brain injury is caused by an ischemic event, the acute reduction of the blood supply in a given brain region, leading to oxygen and glucose shortage. As a primary consequence neurons, with their high metabolic rate, rapidly suffer and most likely die. Thereby acute and post-ischemic inflammation is triggered, which is associated with exacerbation of brain damage (Macrez et al., 2011). In the serum and cerebralspinal fluid of stroke patients elevated levels of pro-inflammatory cytokines have been detected and increased IL-6 is associated with early clinical deterioration (Vila et al., 2000). The underlying mechanisms of ischemia-induced sterile inflammation are multifaceted; however the intrinsic innate immune system is regarded as an important component (Wang et al., 2011).

Inflammation is indispensable in the response to pathogen invasion and tissue injury, by clearing pathogens and cell debris, promoting restoration of homeostasis and paving the way for repair processes (Chavarria and Alcocer-Varela, 2004). Immune responses are commonly self-limiting, but continuous stimulation or failure in termination may result in uncontrolled inflammation and production of neurotoxic factors, which amplify the underlying disease states (Glass et al., 2010), resulting in collateral damage. In neurodegenerative diseases inflammation-mediated neurotoxicity is proposed to be a consequence of dysregulation and overactivation of microglia (Block et al., 2007). How microglia, as the intrinsic innate immune cells of the CNS, sense danger in infectious and sterile CNS pathologies and initiate an inflammatory response will be illustrated in the following.

1.2 Toll-like receptors bring specificity to the innate immune system

Cells of the innate immunity are the first to encounter pathogens and rapidly mount an immune response by secreting inflammatory molecules, attracting lymphocytes and presenting the pathogen's antigens. Notably, innate immune cells are also capable of limiting spread of infection by phagocytosis and release of reactive oxygen species. However, until the 1990ies, innate immune cells were thought to respond in an unspecific manner. Moreover, the mechanism by which they detected pathogens was not known. It was then postulated that a system of "pattern recognition receptors" must exist which allows the innate immune

cells to recognize microbial components (Janeway, 1989) albeit in a different way than via antigen-receptors characteristic for the T and B cells. The Toll-like receptors (TLRs) were identified as these pattern recognition receptors.

1.2.1 The discovery of the Toll-like receptors

In 1985, a protein required for formation of the dorsal-ventral axis in embryonic development of fruit flies was identified, termed Toll (Anderson et al., 1985). The cytoplasmic domain of Drosophila Toll was found to be homologous to that of the human interleukin (IL-) 1 receptor (IL-1R), indicating that signal transduction is similar among both proteins (Gay and Keith, 1991). Likewise, it emerged that the transcription factor dorsal, induced by Toll, is a homologue of NF-κB (Govind and Steward, 1991) which is important for the transcription of genes of inflammatory molecules, such as TNF-α, IL-6 or IL-10, and enzymes, such as iNOS. Due to these parallels among Toll and IL-1R, a possible role of Toll in sensing infection and inducing a innate immune response was addressed (Lemaitre et al., 1996). It was reported that the Toll-mutant of Drosophila infected with *Aspergillus fumigates* died, due to an impaired immune defense. Further, it was found that Toll is responsible for the production of antifungal and antibacterial peptides upon infection (Lemaitre et al., 1996). Hence, a specific receptor complex was identified, which is involved in activation of innate immunity-Drosophila do not exhibit an adaptive immunity-upon infection with a microorganism. Yet, how the pathogen is recognized was not addressed. Soon, the cloning and molecular characterization of five mammalian Toll-like receptors (TLR1-5) was described (Rock et al., 1998). In sum, it was clear that TLRs function as immune receptors in vertebrates, capable of inducing a typical immune response, but it was not evident that the TLRs are pathogen-sensing receptors.

At the same time, lipopolysaccharide (LPS)-induced septic shock was investigated. LPS is a component in the cell wall of gram-negative bacteria, which is released upon breakdown of the bacteria and acts toxic ("endotoxin"). Notably, TNF-α, a pro-inflammatory cytokine induced by NF-κB, was identified as a major soluble factor mediating the lethal effect of LPS in septic shock (Beutler et al., 1985). The mouse strain C3H/HeJ, at that time widely used for experiments on septic shock, is hyporesponsive to LPS (Sultzer, 1968), due to a point mutation in a single autosomal locus (Watson and Riblet, 1974), which was designated *Lps*. This indicated that there was a receptor for LPS, that is, a pathogen-sensing receptor. It took several years until the exact position of *Lps* was found, which revealed that *Lps* encoded one of the Toll-like receptors, that is TLR4 (Poltorak et al., 1998).

The discovery of the TLRs opened up a new field in innate immunity and Bruce A. Beutler and Jules A. Hoffmann were awarded the Nobel Prize in Medicine in 2011.

1.2.2 Toll-like receptors sense pathogens

In the following years 13 TLRs were identified. TLRs 1-9 are conserved among species. There are 10 functional TLRs in human with TLR11 likely not functional due to a stop codon (Zhang et al., 2004) and TLR12 and TLR13 missing. Twelve functional TLRs exist in the mouse, with TLR10 nonfunctional due to a retroviral insertion (Akira et al., 2006). TLRs are located on the cell surface as well as in intracellular vesicles, such as the endoplasmic reticulum, endosomes, lysosomes and endolysosomes. A wide range of pathogen-associated molecular patterns (PAMPs) are recognized by TLRs, as listed below.

The following group of TLRs has been described to mainly recognize outer membrane components of microorganisms. As mentioned above TLR4 recognizes gram-negative bacteria via LPS. In contrast, TLR2 is established as the major receptor for gram-positive bacteria as it senses their cell wall components, such as lipopetides (Aliprantis et al., 1999), peptidoglycan and lipoteichoic acid (Schwandner et al., 1999). However, TLR2 also recognizes a wide range of PAMPS from other pathogens, such as lipoarabinomannan from mycobacteria (Means et al., 1999), zymosan from fungi (Underhill et al., 1999), and hemagglutinin protein from measles virus (Bieback et al., 2002). Further, TLR2 forms heterodimers with TLR1 and TLR6 to discriminate subtle structural differences among lipopeptides. In particular, TLR2/1 recognizes triacylated lipopeptides such as peptidoglycan and Pam3CSK4 (Jin et al., 2007), while TLR2/6 recognizes diacylated lipopeptides and MALP-2 (Takeuchi et al., 2001). TLR5 recognizes bacterial flagellin (Hayashi et al., 2001). TLR11 is thought to recognize uropathogenic bacterial components (Zhang et al., 2004) and, just as TLR12, the profilin-like molecule derived from Toxoplama gondii (Yarovinsky and Sher, 2006; Koblansky et al., 2013). Murine TLR13 recognizes 23S ribosomal RNA from gram-positive and gram-negative bacteria (Oldenburg et al., 2012).

Another group of TLRs was identified to recognize microbial nucleic acids. TLR3 is the receptor for double stranded RNA (dsRNA) (Alexopoulou et al., 2001). TLR7 was originally identified in recognizing imidazoquinoline derivatives such as imiquimod and resiquimod (R-848) (Hemmi et al., 2002) and the guanine analog loxoribine (Akira and Hemmi, 2003). Soon, single stranded RNA (ssRNA) derived from influenza virus (Diebold et al., 2004) and from HIV-1 (Heil et al., 2004), was described as the physiological ligand of mouse TLR7. TLR8 is phylogenetically similar to TLR7 and also recognizes R-848 and viral ssRNA, indicating a redundancy among TLR7 and TLR8 (Jurk et al., 2002; Heil et al., 2004). TLR9 recognizes DNA with unmethylated cystosine-phosphate-guanosine (CpG) motifs, which are frequently present in bacteria and viruses but rare in mammalian cells (Hemmi et al., 2000).

In comparison with the infinite variety of T cell receptors, the set of TLRs is relatively small. However, TLRs can form homo- and heterodimers and utilize co-receptors (e.g.

CD14) and accessory proteins (e.g. MD-2). Thus, as illustrated above, the family of TLRs can recognize a great variety of invariant pathogen-associated molecules, in particular cell wall components, intracellular proteins and nucleic acids. Notably, these structures are required for the survival of the microorganism and therefore escape mutants do not exist. The structure of TLRs is defined by highly conserved genes, and a constitutive expression makes them constantly available for rapid pathogen recognition upon infection.

1.2.3 Extending Toll-like receptor recognition to danger

Immunologists had once adopted the doctrine in which the driving force of the immune system was defined by the discrimination between self and non-self. This fundamental concept has been replaced by the more sophisticated danger theory (Matzinger, 1994). Accordingly, TLRs do not sense only the presence of a pathogen, but react to any type of danger, even signaled by the host itself. Several host-derived (endogenous) TLR-ligands have been identified, termed danger-associated molecular patterns (DAMPs) or alarmins. It has been reported that beta-defensin (Biragyn et al., 2002), high-mobility group box-1 (Tian et al., 2007; Curtin et al., 2009), Heat Shock Proteins (Ohashi et al., 2000; Vabulas et al., 2001; Asea et al., 2002; Kakimura et al., 2002) and components of the extracellular matrix, such as fibronectin (Okamura et al., 2001) and hyaluronan (Jiang et al., 2005), are recognized by TLR4 and/or TLR2. Further, TLR3 detects mRNA (Karikó et al., 2004) and TLR7 recognizes small interfering RNA (Hornung et al., 2005) and also mRNA (Diebold et al., 2004). Recently, our group identified the microRNA let-7b and its family members, which are all abundantly expressed in the brain, as agonists of TLR7 (Lehmann et al., 2012a). In the healthy state these endogenous ligands and the respective TLRs are localized in different cellular compartments and cannot interact physiologically (Yu et al., 2010). However, in pathological conditions, endogenous ligands are liberated from injured and dying cells and/or are actively secreted (Yu et al., 2010).

1.2.4 Toll-like receptor activation initiates an inflammatory response

TLRs are type I transmembrane proteins. Varying numbers of leucine-rich repeats in the extracellular domain of TLRs are responsible for the recognition of the diverse ligands (e.g. lipoproteins, lipids and nucleic acid) (Sasai and Yamamoto, 2013). In addition, the specificity of ligand recognition by TLRs is determined by their homo- or heterodimerization and can include an additional binding of co-receptors and/or accessory proteins. The cytoplasmic domain, homologous to that of interleukin 1 receptor and therefore termed Toll/interleukin(IL)-1 receptor (TIR) domain, is required for the engagement with TIR-containing adaptors molecules (Watters et al., 2007). The main adaptor molecule myeloid differentiation primary response 88 (MyD88) was identified as the downstream media-

tor of nearly all TLRs (Kawai et al., 1999; Takeuchi et al., 2000; Hemmi et al., 2002; Häcker et al., 2000; Hayashi et al., 2001). TLR3 is the only TLR, which does not employ MyD88, but signals via TIR domain-containing adaptor inducing interferon-β (TRIF) (Yamamoto et al., 2002b). Remarkably, TLR4 signals via MyD88 and TRIF (Yamamoto et al., 2003).

Upon ligand binding downstream signaling is initiated as displayed in Fig. 1.1. The complex signaling network results in activation of the transcription factors NF-κB, activator protein 1 (AP-1) and/or interferon regulatory factor (IRF) 3, 5 and 7. Whereas MyD88 mediates an early phase of NF-κB activation, TRIF mediates the production of IFN-β and a late phase of NF-κB activation (Dunne and O'Neill, 2005; Sasai et al., 2005). NF-κB, AP-1 and the IRFs induce the transcription of genes encoding cytokines, chemokines, interferons, enzymes and other molecules essential for mounting and modulating an immune response (Kawai and Akira, 2008). A number of negative regulators (e.g. IRAK-M, MyD88s, TRAF1 and 4) exist, which inhibit binding or dissociation, or increase degradation of protein complexes, thereby terminating the immune response to prevent excessive inflammation (Marsh et al., 2009; Sasai and Yamamoto, 2013).

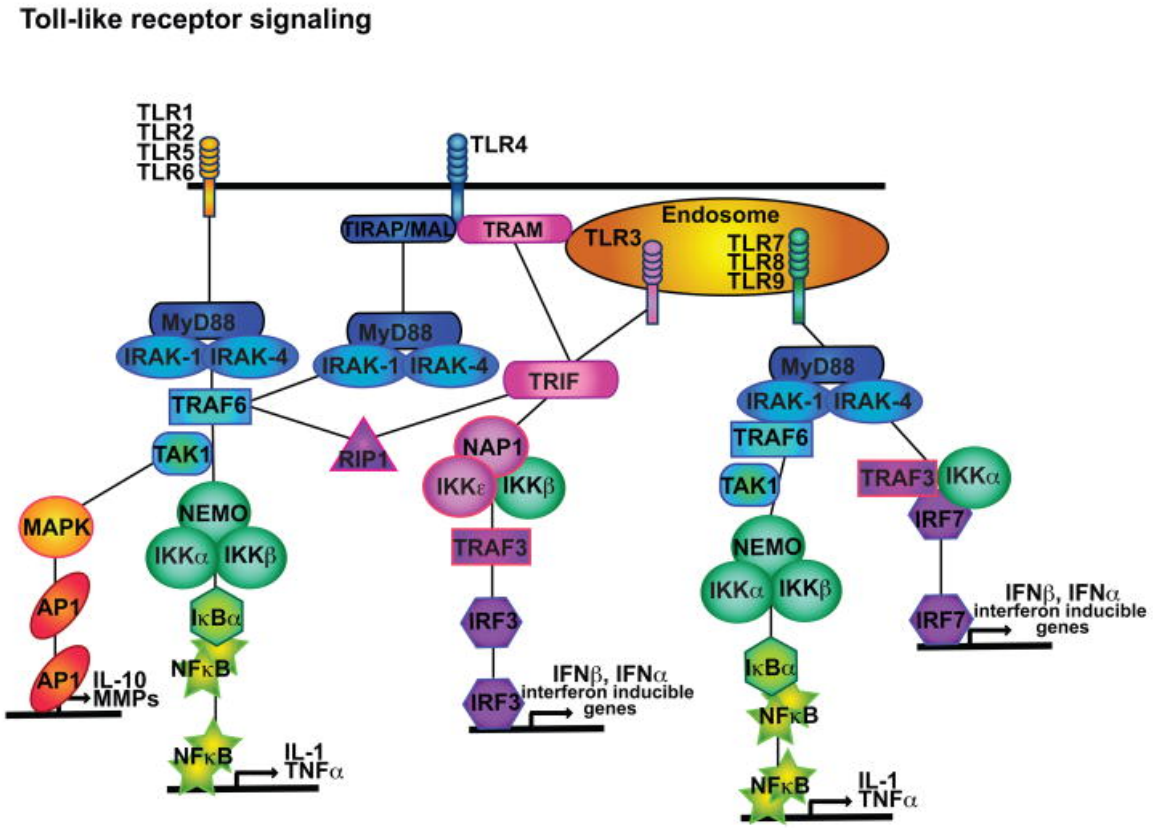

Figure 1.1: TLR signaling cascade. Upon ligand binding TLRs dimerize and undergo conformational changes that lead to the induction of a complex cascade of intracellular signaling events resulting in activation of transcription factors and production of inflammatory molecules. Except TLR3, each TLR initiates a signal via MyD88 - either directly (TLR5, 7, 8, 9 and 11) or via the bridging adaptor TIRAP (TLR1, 2, 4 and 6) - which engages members of the IRAK family (IRAK1, 2, 4) to start a process of auto- and cross-phosphorylation among the IRAK proteins. IRAK1 (and possibly IRAK-2) dissociate from the receptor complex and subsequently associate with Traf6, which activates TAK1, which in turn activates the IKK complex and MAPKKs. The IKK complex (IKKα, IKKβ and regulatory subunit IKKγ/NEMO) phosphorylates IκB proteins, which are ubiquitinated and degraded proteosomally, allowing subsequent translocation of transcription factor NF-κB to the nucleus. Transcription factor AP-1 is activated through phosphorylation by members of the MAPK family. Together NF-κB and AP-1 induce generation of inflammatory cytokines and chemokines. When recruited to TLR7 and TLR9 MyD88 and the phosphorylated IRAK proteins can bind TRAF3 in addition to TRAF6. Activation of TRAF3 results in phosphorylation, dimerization and nuclear localization of the transcription factors IRF3, 5 and 7. This pathway leads to production of Type I interferon (IFN-α/β) in addition to the activation of NF-κB and AP-1. The receptor TLR3 is unique since it signals via TRIF, TBK1 and IKKepsilon, which activate IRF3, the transcription factor of Type I interferons. In addition TRIF can signal via TRAf6, RIP-1 and TAK1 which results in late phase NF-κB and AP-1 translocation and cytokine production. TLR4 is the only one able to recruit MyD88 via TIRAP, or TRIF via TRAM inducing both pathways. Illustration adapted from (Marsh et al., 2009).

1.3 Toll-like receptors in the CNS

Initially, studies on the function of TLRs concentrated on the innate immune cells of the peripheral immune system. However, since inflammation does take place in the CNS and involves microglia, the intrinsic innate immune cells, efforts were initiated to characterize the role of TLRs in the CNS, some of which are outlined below.

1.3.1 Toll-like receptor expression in the CNS

Several *in vitro* and *in vivo* studies have reported on the presence of mRNA and protein of TLRs and their related signaling proteins in the CNS of human and mice. Moreover, it has become clear that TLR expression is dynamically regulated during brain development (Kaul et al., 2012) and pathological conditions of the CNS, such as stroke, infection, CNS autoimmunity and neurodegenerative disorders (Konat et al., 2006; Landreth and Reed-Geaghan, 2009; Downes and Crack, 2010; Marta, 2009). Further, evidence is emerging that the CNS cells exhibit their own cell type specific set of TLRs and adaptor molecules, allowing a distinct cell type specific response to TLR stimuli as is the case for peripheral immune cells (Kawai and Akira, 2010).

Microglia express the wide repertoire of TLRs 1-9 (Jack et al., 2005; Olson and Miller, 2004; Kielian et al., 2002; Lehnardt et al., 2006; Dalpke et al., 2002). Stimulation of purified microglia with TLR ligands results in an inflammatory response with the secretion of cytokines, chemokines and reactive oxygen and nitrogen species such as TNF-α, IL-1β, IL-6, IL-10, IL-12, IL-18, IFN-α, IFN-β, CCL2, CCL3, CCL5 and NO (Olson and Miller, 2004; Bsibsi et al., 2002). Furthermore, proliferation and phagocytosis are induced (Redlich et al., 2013).

Expression of TLRs 1-9 has also been reported on astrocytes, however in low levels except for a striking preference for high levels of TLR3 (Jack et al., 2005; Farina et al., 2005; Bsibsi et al., 2006). Upon TLR3 stimulation, human astrocytes showed a marked induction of large array of transcripts for anti-inflammatory and neuroprotective mediators, whereas response to TLR4 activation was minimal (Bsibsi et al., 2006). In addition, in comparison to microglia, astrocytes were slower in upregulating TLRs and immunostimulatory mediators, pointing to a role in a subsequent repair response (Bsibsi et al., 2006). On the other hand, astrocytes challenged with the gram-positive bacterium *S. aureus* or its cell wall product peptidoglycan produced pro-inflammatory mediators including IL-1β, TNF-α and CCL2 in a TLR2-dependent manner (Esen et al., 2004), indicating that astrocytes likewise participate in TLR-mediated pro-inflammatory immune responses.

Reports on TLR expression and function in oligodendrocytes are limited. Howsoever, TLR2 and TLR3 are present whereas TLR4 is not (Bsibsi et al., 2002; Lehnardt et al., 2002). Activation of TLR2 on cultured oligodendrocytes promotes differentiation, survival

and myelination, whereas TLR3 is a potent inducer of apoptosis (Bsibsi et al., 2012).

Strikingly, even neurons exhibit TLRs, in particular TLR3, TLR7 and TLR8 (Cameron et al., 2007; Lehmann et al., 2012b; Ma et al., 2006). Activation of any of these nucleic acid-sensing TLRs causes detrimental effects, such as growth cone collapse, inhibition of neurite outgrowth and extension, and apoptosis. Importantly, in all these studies TLR signaling was found to be independent of NF-κB, the classical terminal effector in microglial signaling and inflammation. Thus, in neurons TLRs rather promote death than induce an inflammatory cascade as it is known for innate immune cells.

In summary, CNS cells, predominantly microglia, express TLRs and activation of TLRs can trigger pro-inflammatory as well as anti-inflammatory reactions and cell-autonomous cell death.

1.3.2 Toll-like receptors are involved in CNS pathologies

As TLRs are pathogen-recognition receptors, it is self-evident to determine the role of TLRs during CNS infection. Studies on injection of the gram-positive bacteria *S. pneumonaie* into the CNS of mice revealed that TLR2$^{-/-}$ mice (the major receptor of this class of bacteria) were more susceptible to infection than wild-type mice. TLR2$^{-/-}$ mice developed more severe clinical symptoms, displayed a higher bacterial load in the CNS, a massive leukocyte infiltration and an increased blood-brain barrier permeability (Echchannaoui et al., 2002; Koedel et al., 2003). These studies point to the necessity of TLRs in the CNS to combat infection. Notably, intrathecal injection of the TLR2 ligand Pam3CSK4 can also induce signs of meningitis, such as influx of leukocytes, elevated regional cerebral blood flow and intracranial pressure, which are absent in TLR2$^{-/-}$ mice (Hoffmann et al., 2007). Similarily, intracisternal injection the TLR9 ligand CpG ODN can mimic symptoms of meningitis with elevated numbers of leukocytes and levels of TNF-α in the cerebrospinal fluid (Deng et al., 2001). These studies demonstrate how potent TLRs are in inducing neuroninflammation *in vivo*.

As outlined earlier inflammation and activation of microglia is a near universal incidence in CNS pathologies, even in those where the presence of pathogens is not evident. Host-derived molecules released from injured cells or accumulation of abnormal proteins such as Aβ are foreign in the extracellular compartment. TLRs are capable of detecting these endogenous molecules thereby eliciting the production of cytokines and chemokines (Henderson et al., 2010; Jana et al., 2008). This is of particular interest as inflammation is involved in secondary tissue damage following CNS injury. Therefore, the contribution of TLRs in non-infectious CNS insults, such as stroke, trauma and Alzheimer's disease, has been evaluated in numerous studies as outlined below.

Mice with a genetic or functional absence of TLR2 (Lehnardt et al., 2007a; Ziegler et

al., 2007) or TLR4 (Cao et al., 2007; Caso et al., 2007; Tang et al., 2007; Kilic et al., 2008; Hyakkoku et al., 2010) subjected to experimental stroke display smaller infarct volumes than their wild-type counterparts. Several aspects indicate that attenuation of ischemia-induced brain injury is due to the lack of a microglial TLR-mediated pro-inflammatory response (Kilic et al., 2008; Caso et al., 2007; Hyakkoku et al., 2010; Lehnardt et al., 2007). Hence, as pathogens are likely not present short after an ischemic event, these studies point to endogenous ligands causing TLR-induced inflammation and exacerbation of tissue injury. Accordingly, levels of endogenous TLR ligands such as fibronectin, HSP60, HMGB-1 were found to be elevated in the cerebrospinal fluid and brain of mice, as well as in the serum of stroke patients, and correlated with parameters of inflammation and disease severity (Brea et al., 2011; Yang et al., 2011). Thus, TLRs appear to be involved in the amelioration of CNS tissue damage following ischemia (Wang et al., 2011).

Similar to the studies on the role of TLRs in stroke, studies on traumatic CNS injury suggest that TLRs contribute to inflammation-mediated secondary brain damage. Experimental nerve transection or contusion injury led to elevated levels of TLR2 and/or TLR4 in the lesion-site and associated areas, predominantly visible in activated microglia (Babcock et al., 2006; Zhang et al., 2012b; Chen et al., 2008). On the other hand, in the injured brains of TLR2$^{-/-}$ mice, glial cells displayed a less activated morphology, levels of distinct inflammatory mediators were reduced and the recruitment of leukocytes was delayed compared to those of wild-type mice (Babcock et al., 2006; Park et al., 2008). These results suggest an important role of TLR2-signaling in the early response to brain injury. Moreover, the decreased expression of inflammatory cytokines (TNF, IL-1β, IL-6) was associated with less neurological deficits in the TLR2$^{-/-}$ mice (Yu and Zha, 2012), showing which impact TLR2 activation can have on the outcome of injury. Similar brain injury-mediated mechanisms were also found to be dependent on TLR4 (Ahmad et al., 2013). However the potential endogenous activators of TLR2 and TLR4 remained unidentified.

An imbalance of Aβ production and clearance influences the pathogenesis of Alzheimer's disease and increasing evidence implicates that neuroninflammation, mediated by microglia and TLRs, is a critical factor (Landreth and Reed-Geaghan, 2009). Strikingly, AD-mice deficient for functional TLR4 exhibit a higher Aβ load (Tahara et al., 2006). Analysis of brain lysates revealed a TLR4-dependent elevation of IL-1β, IL-10, IL-17 and TNF-α, which likely originates from Aβ-stimulated microglia as shown in cell culture (Jin et al., 2008), pointing to a requirement of inflammation for Aβ removal. Moreover, *in vitro* studies revealed that Aβ uptake by microglia can be boosted by activation of TLR2, TLR4 or TLR9, demonstrating that TLRs are involved in clearance of Aβ deposits by microglia (Chen et al., 2006; Tahara et al., 2006). On the other hand, it has been demonstrated that TLR4 mediates Aβ-induced microglial neurotoxicity (Walter et al., 2007). Thus, by modulating neuroninflammation TLRs are involved in Alzheimer's disease pathogenesis (Landreth and Reed-

Geaghan, 2009).

Taken together, neuroinflammation is a crucial underlying mechanism of CNS diseases and disorders and TLRs, the key innate immune receptors, are pivotal contributors. Notably, the studies on the role of TLRs in CNS pathologies indicate on the one hand, that during the formation and course of the disease a single TLR can play a crucial role, and on the other that different TLRs can mediate similar outcomes. Thus, a remarkable point is the redundancy among TLRs. This is given by the conserved TIR domain present in cytoplasmic portion of all TLRs and the intracellular adaptor molecules (Watters et al., 2007), which allow the induction of similar signaling pathways downstream of TLR engagement. However, as outlined earlier, the variety of receptors-dimers, TLR-specific adaptor molecules and cell-specific set of signaling molecules confer a certain degree of specificity in the overall redundancy of TLRs (Netea et al., 2004). In addition, it must be kept in mind, that there are numerous TLR agonists present in pathogens and host cells, which can engage the different TLRs. Whereas this permits the detection of a pathogen or injured cell by any single TLR, it is conceivable that these diverse ligands trigger multiple TLRs in a combined manner.

1.4 Aim of the study

The intrinsic innate immunity of the CNS, as represented by microglia and TLRs, detects invading pathogens and damaged tissue and initiates an inflammatory cascade to encounter these forms of danger. Whereas promoting restoration of homeostasis is intended, molecules generated during the inflammatory event are potentially neurotoxic. Accumulation of these molecules during an exaggerated inflammatory response, causing loss of neurons and oligodendrocytes, is deleterious in the CNS, a vital organ with a limited regenerative capacity. Toll-like receptors have proven to be pivotal contributors in several CNS pathologies and the underlying neuroninflammatory response.

This study aims at a comprehensive evaluation of the consequences of specific TLR activation in the CNS *in vitro* and *in vivo*. Highly specific agonists allow a selective activation of TLRs to address what functional relevance single as well as combined TLR activation has on neuroinflammation and neuronal cell survival.

Materials and Methods

2.1 Materials

Table 2.1: Consumables.

Consumables	Supplier
Cell Strainer 70 µm Nylon	BD Falcon
Circular Cover Slips (1 mm)	Marienfelde Superior
Low Profile Microtome Blades	Leica
MicroAmp Fast Optical 96-well Reaction Plates (0.1 ml)	Applied Biosystems
MicroAmp Fast Optical Adhesive Film	Applied Biosystems
Nunc F96 MaxiSorb	Thermo Fisher Scientific Inc.
Super Frost Ultra Plus® glass slides	Thermo Scientific
MaxTract Tubes	Qiagen
Syringes	BD

Table 2.2: Reagents.

Reagents	Supplier
2% Lidocain	Astra Zeneca
2.5% Trypsin	Gibco
B-27 supplement	Gibco
BCA Protein Assay Kit	Pierce
cOmplete ULTRA Tablets, mini	Roche Diagnostics GmbH
Dapi (4',6-Diamidin-2'-phenylindoldihydrochlorid)	Sigma
DMEM [+]4.5 g/l Glucose, L-Glutamine, Pyruvate	Gibco
DNaseI (1 mg/ml)	Roche
DTT	Promega
Fetal calf serum (FCS)	Gibco
Griess' reagent for nitrite	Fluka
HBSS (+ $MgCl_2$/$CaCl_2$) and (w/o $MgCl_2$/$CaCl_2$)	Gibco
Isotonic saline 50 ml (0.9% sodiumchlorid)	Fresenius Kabi Deutschland GmbH
Ketamin-Actavis 50mg/ml	Actavis
Leibovitz's L-15	Gibco
L-Glutamine 200 mM	Gibco

Reagents	Supplier
M-MLV Reverse Transcriptase	Promega
Neurobasal™ Medium	Gibco
Normal goat serum	Vector Laboratories Inc.
Nose and eye ointment	Bayer
Penicillin/Streptomycin	Gibco
Poly-D-lysine hydrobromide	Sigma-Aldrich
RNasin® Ribonuclease Inhibitor	Promega
Rompun® 2%	Bayer
RQ1 RNase-Free DNase	Promega
RT2 SYBR Green ROX™ qPCR Mastermix	Qiagen
Shandon Immu-Mount™	Thermo Scientific
Tissue freezing medium®	Jung
TMB (3,3'5,5'-Tetramethylbenzidine dihydrochloride)	Sigma
TRIzol® reagent	Invitrogen
UltraPure™ Phenol:Chloroform:Isoamyl Alcohol (25:24:1 v/v)	Invitrogen

Table 2.3: Kits.

Kit System	Supplier
BD OptEIA™ Set Mouse TNF (mono/mono)	BD
BD OptEIA™ Set Mouse IL-1β	BD
FlowCytomix	Bender MedSystems
RT^2 qPCR Primer Assays	SA Biosciences Corporation (a Qiagen company)
BCA Protein Assay Reagent	Pierce
In Situ Cell Death Detection Kit, TMR red	Roche
ApopTag® Plus Fluorescein In Situ Apoptosis Detection Kit S7111	Millipore
ECL Western Blotting Detection Reagent	GE Healthcare Lifesciences

Table 2.4: TLR Ligands.

TLR Ligand	Receptor	Supplier
CpG thioat-stabalized oligonucleotide "1668" (5' TCCATgACgTTCCTgATgCT 3')	TLR9	TIB MolBiol

TLR Ligand	Receptor	Supplier
Hsp60 (low endotoxin, human, recombinant) dissolved in sterile PBS	TLR4	Enzo Life Sciences Stressgen (ADI-ESP-540)
Imiquimod	TLR7	InvivoGen (tlrl-imq)
Loxoribine (Guanosine analog)	TLR7	InvivoGen (tlrl-lox)
LPS from *E. coli*, Serotyoe 0111:B4	TLR4	Enzo Life Sciences (581-002-M005)
Pam3Cys-SK4 x 3HCl	TLR2	EMC microcollection GmbH (L2000)
Recombinant human serum albumin dissolved in sterile PBS	control	Humanzyme (HZ-3002)

Table 2.5: RT^2 qPCR Primer Assays.

Gene Symbol	Gene Name	UniGene no.
CCL2	Chemokine (C-C motif) ligand 2 (MCP-1)	Mm.290320
CCL5	Chemokine (C-C motif) ligand 5 (RANTES)	Mm.284248
IFN-γ	Interferon gamma	Mm.240327
TGF-β	Transforming growth factor, beta 1	Mm.248380
IL-23	Interleukin 23	Mm.125482
Cox-2	Prostaglandin-endoperoxide synthase 2	Mm.292547
Gls	Glutaminase	Mm.440465
IDO	Indoleamine 2,3-dioxygenase 1	Mm.392
IL-10	Interleukin 10	Mm.874
IL-1β	Interleukin 1 beta	Mm.222830
IL-6	Interleukin 6	Mm.1019
iNOS	Inducibale nitric oxide synthase	Mm.2893
IRAK1	Interleukin-1 receptor-associated kinase 1	Mm.38241
IRAK4	Interleukin-1 receptor-associated kinase 4	Mm.422858
MyD88	Myeloid differentiation primary response gene 88	Mm.213003
NF-κB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105	Mm.256765
TLR1	Toll-like receptor 1	Mm.273024
TLR2	Toll-like receptor 2	Mm.87596
TLR3	Toll-like receptor 3	Mm.33874
TLR4	Toll-like receptor 4	Mm.38049
TLR5	Toll-like receptor 5	Mm.116894
TLR6	Toll-like receptor 6	Mm.42146

Gene Symbol	Gene Name	UniGene no.
TLR7	Toll-like receptor 7	Mm.489377
TLR8	Toll-like receptor 8	Mm.196676
TLR9	Toll-like receptor 9	Mm.44889
Tnf	Tumor necrosis factor (alpha)	Mm.1293
TRAF6	Tnf receptor-associated factor 6	Mm.292729
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Mm.304088

Table 2.6: Antibodies.

Antibody	Antigene	Manufacturer	Dilution
Ms-anti-NeuN	Neuronal nuclei (MAB377)	Millipore	1:300
Ms-anti-NF	Neurofilament 200kDa, clone RT97 (MAB5262)	Millipore	1:300
Anti-IB4	Isolectin binding protein 4 (I21411)	Invitrogen	1:1000
Rb-anti-Iba1	Ionized calcium binding adaptor molecule 1 (019-19741)	Wako	1:500
Rat-anti-CD11b	Cluster of differentiation molecule 11B (14-0112)	eBioscience	1:100
Rb-anti-GFAP	Glial fibrillary acidic protein	Dako	1:500
Rb-anti-Caspase-3	active Caspase-3 (557035)	BD Pharmingen	1:100
Anti-APC	adenomatous polyposis coli	Merck Millipore	1:100
Anti-MBP	myelin basic protein	Merck Millipore	1:100
Anti-HSP60	Heat schock protein 60 (ADI-SPA_807)	Enzo Life Sciences	1:100

Table 2.7: Equipment.

Instrument	Manufacturer
7500 Fast Real-Time PCR System	Applied Biosystems
APT.line® CB CO2 Incubator	Binder
balance CP225D	Sartorius AG
balance Navigator™	Ohaus® Corporation
Binocular GZ6 and cold-light source KL 1500 LCD	Leica Camera AG and Schott AG
Binocular Stemi DV4	Zeiss
Biomate 3 UV-Vis Spectrophotometer (software Visionlite)	Thermo Scientific
cryostat CM1900	Leica

Instrument	Manufacturer
Elix-5 Water Purification System tap water deionisized and filtered for analytical purposes (Milipore type II)	Merck Millipore
Epson-Twain 5 scanner	Epson
horizontal shaker	Edmund Bühler GmbH
instruments	Fine science tools
Megafuge 1.0R	Heraeus Instruments
microscop BX51 (software Magnafire, camera)	Olympus
microscop IX 70 (software Magnafire, camera)	Olympus
microscop X50	Olympus
Milli-Q Synthesis A10 System Elix-5 Water Purification System with ultra pure water for molecular biological purposes (Milipore type I)	Merck Millipore
Opsys MW™ Microplate Washer	Dynex Technologies
peristaltic pump BVP ISM444B (+Model 7015-20)	Ismatec® IDEX Health and Science GmbH
pH meter SevenEasy with InLab® Routine Pro	Mettler-Toledo GmbH
Plate reader	
sonifier Sonopuls GM70 with SH70G probe	Bandelin Electronic
Thermomixer Comfort	Eppendorf
Ultra-Turrax® T 25 basic (S25N-8G dispersing tool)	IKA®-Werke

Table 2.8: Buffers, Media and Solutions.

Name	Formula
4% paraformaldehyde (PFA)	4% paraformaldehyde was dissolved in 0.1 M PB at approx. 57°C and filtered
4x loading buffer	0.1 M Tris (pH 6.8); 10% Glycerin; 4% SDS; 4% β-mercaptoethanol; bromphenolblue in Millipore type I water
Ammonium persulfate (APS)	10% APS in H2O
Anode I buffer	300 mM Tris; 10% methanol; pH 10.4
Anode II buffer	25 mM Tris; 10% methanol; pH 10.4
Block solution	PBS + 5% FCS + 0.1% Triton X-100
Brain lysis buffer	50 mM Tris complemented with cOmplete ULTRA protease inhibitor short before use
Electrophoresis buffer	0.25 M Tris; 1.92 M Glycin; 1% SDS; pH 8.3
Cathode buffer	40 mM Glycin; 25 mM Trisbase; 10% methanol; pH 9.4

Name	Formula
ELISA Stop Solution	1% SDS
ELISA Substrate solution	5.14 ml 0.2 M Na_2HPO_4*$2H_2O$ + 4.86 ml 0.1 M $C_6H_8O_7$*H_2O + 1 tab. TMB + 2 µl H_2O_2
Ketamin-Cocktail	Ketamin 100 mg/kg BW and Xylazin 16 mg/kg BW in 0.9% NaCl solution
Microglia medium	DMEM + 1% Pen/Strep + 10% FCS
Neuronal medium	Neurobasalmedium + 1% L-Glutamine + 1% Pen/Strep + 2% B-27; freshly prepared
Phenylmethanesulfonylfluoride (PMSF)	10 mM in isopropanol
Phosphate buffer (PB) pH 7.4	0.1 M Na_2HPO_4; 0.1 M NaH_2PO_4
Phosphate buffered saline (PBS) pH 7.4	140 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4*$7H_2O$; 1.8 mM KH_2PO_4
Poly-D-lysine (PDL)	20 µg/ml poly-D-lysine; sterile filtered
Resolving gel (12%)	3.25 ml Millipore type I water; 1.9 ml resolving gel buffer; 2.25 ml 40% acrylamid; 75 µl 10%SDS; 37.5 µl 10% APS; 15 µl TEMED
Resolving gel buffer	1.5 M Tris (pH 8.8)
SDS lysis buffer	20 mM Tris (pH 7.4); 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 2.5 mM Na4P2O7; 1 mM Na3VO4; 1 µg/ml Leupeptin; 3 µg/ml Aprotinin; 1 mM Glycerolphosphat; 1% SDS in Millipore type I water
Stacking gel	4.5 ml Millipore type I water; 1.9 ml stacking gel buffer; 810 µl 40% acrylamid; 75 µl 10%SDS; 57 µl 10% APS; 19 µl TEMED
Stacking gel buffer	0.5 M Tris (pH 6.8)
Sucrose solution	10%, 20% or 30% D-sucrose in 0.1 M PB
Tris buffered saline (TBS)	0.5 M Tris; 1.5 M $NaCl_2$; pH 7.4
TBST	Tris buffered saline + 0.05% Tween20

2.2 Mice

Wild type mice (C57BL/6J) were obtained from the Forschungseinrichtung für experimentelle Medizin Berlin (FEM). TLR2 knock out ($TLR2^{-/-}$), $TLR2/4^{-/-}$, $TLR7^{-/-}$, $TLR9^{-/-}$ and $MyD88^{-/-}$ mice were generated in the lab of and generously provided by Dr. Shizuo Akira (Osaka Universtiy, Department of Host Defense, Osaka, Japan). $TLR4^{-/-}$ were gen-

erated by back-crossing TLR2/4 $^{-/-}$ with C57BL/6J. Genotyping was performed according to the protocols of the Akira Lab (http://hostdefense.ifrec.osaka-u.ac.jp/ja/other/index.html). All mice were maintained according to the guidelines of the committee for animal care in the animal facilities of the FEM in a specific pathogen free (SPF) environment.

2.3 *In vitro* experiments

Primary cultures were prepared under a laminar hood with sterile instruments. Further procedures were carried out under a clean bench. Cultures were incubated at 37°C and 5% CO_2 in a humid atmosphere.

2.3.1 Primary cortical microglial cell cultures

T-75 flasks were treated with 7-10 ml of PDL at RT for at least overnight. Before platting cells, flasks were washed 3 times with sterile PBS. Pups at postnatal stage 0-4 were killed by decapitation. In a petri dish with ice-cold Leibovitz's L-15 buffer the cranium was lifted off and the cortices isolated from the scull with forceps. After removing the hippocampus and the meninges the tissue was coarsely minced with a scalpel and collected in L-15 buffer. The buffer was discarded and 3 ml 2.5% trypsin were added to enzymaticaly dissociate the tissue while lying in an incubator at 37°C. After 25-30 min 3 ml FCS were added to minimize tissue digestion. The fluffy tissue was treated with DNase I at a final concentration of 20 µg/ml for 30 sec to digest DNA released from damaged cells. Tissue was carefully dissociated by resuspending with a 10 ml cell culture pipette (maximum 10 times). Following centrifugation at 1200 x g for 5 min the pellet was resuspended in 10 ml pre-warmed microglia medium and passed through a 70 µm cell strainer to remove remaining tissue pieces and debris. The filtrate was plated with approx. 4 cortices per flask in a volume of 10 ml. After 24 h the medium was replaced by 12 ml fresh pre-warmed medium. At 6-7DIV 1-2 ml fresh medium were added. Microglia were shaken between DIV8-12 (and again between DIV15-19) for 30 min on an orbital shaker at 200-250 rpm. The supernatant was centrifuged at 1200 x g for 5 min. Leaving approx. 5 ml medium in the tube the pellet was resuspended with another 5 ml fresh microglia medium. Cells were counted diluted in the vital stain trypan blue (commonly 1:2) in a Neubauer hemocytometer. An usual yield of 0.5-1x10^6 microglia per flask was plated at 30x10^3 cells/96-Well in 200 µl (analysis of inflammatory molecules in supernatant) or added to DIV3-neurons at 60-75x10^3 cells/24-Well in 500 µl microglia medium (see 2.3.3).

2.3.2 Primary cortical neuronal cell cultures

For immunohistochemical purposes circular cover slips were washed 2 h in 70% ethanol, then 3 min in 100% ethanol, air-dried and heat sterilized at 200°C for 2 h. Placed in 24-well plates, they were treated with 500 µl PDL at 37°C for 2 h or at RT for maximum 2 days. Before platting cells, wells were washed 3 times with sterile PBS. Female mice at gestational stage 17 were killed by cervical dislocation. The abdomen was disinfected with 70% ethanol (v/v) and the uterus removed. In a petri dish with ice-cold HBSS buffer (w/o $MgCl_2$/$CaCl_2$) the embryos were removed from the amniotic sac and decapitated. In fresh buffer the cranium was lifted off and the cortices isolated from the scull with forceps. After removing the hippocampus and the meninges the tissue was collected in HBSS (w/o $MgCl_2$/$CaCl_2$) and washed 3 times. With 3.5 ml buffer remaining, 0.5 ml 2.5% trypsin were added to disintegrated the tissue at 37°C in a water bath. After 20-25 min 3 ml FCS were added to the fluffy tissue to minimize tissue digestion, followed by 3 careful washes with HBSS (+ $MgCl_2$/$CaCl_2$). DNase I was added at a final concentration of 20 µg/ml for 30 sec to digest chromosomal DNA released from lysed cells thereby reducing viscosity, followed by 3 washes with pre-warmed neuronal medium. Tissue was dissociated in 2 ml by carefully resuspending with a 10 ml cell culture pipette (maximum 10 times). The suspension was centrifuged at 30 x g for 5 min to pellet leftovers of tissue, and microglia and astrocytes, then the supernatant was transferred into a new tube and cells pelleted at 1200 x g for 5 min. The pellet was carefully resuspended in 10 ml neuronal medium and cells were counted diluted in the vital stain trypan blue (usually 1:4) in a Neubauer hemocytometer. A common yield of 20-40x10^6 cells per 8 embryos was plated at 500x10^3cells/24-Well in 500 µl (neuronal death assay). After 24 h half the medium was replaced with fresh pre-warmed medium. Purity of cultures was 96% after plating as verified by immunohistological staining (NeuN, Ib4, GFAP).

2.3.3 Co-culture

Co-cultures of primary neurons with primary microglia were obtained by adding approx. 60-75x10^3 microglia per 24-Well to DIV3 neurons. Before adding the 500 µl microglia suspension (120-150x10^3 cells/ml in microglia medium) neuronal medium was completely removed from the neurons.

2.3.4 Experimental set-ups

Neuronal and co-cultures

Experiments with pure neuronal cultures were performed at DIV4. Experiments with co-cultures were started after microglia were allowed to settle for 24 h (neurons were then

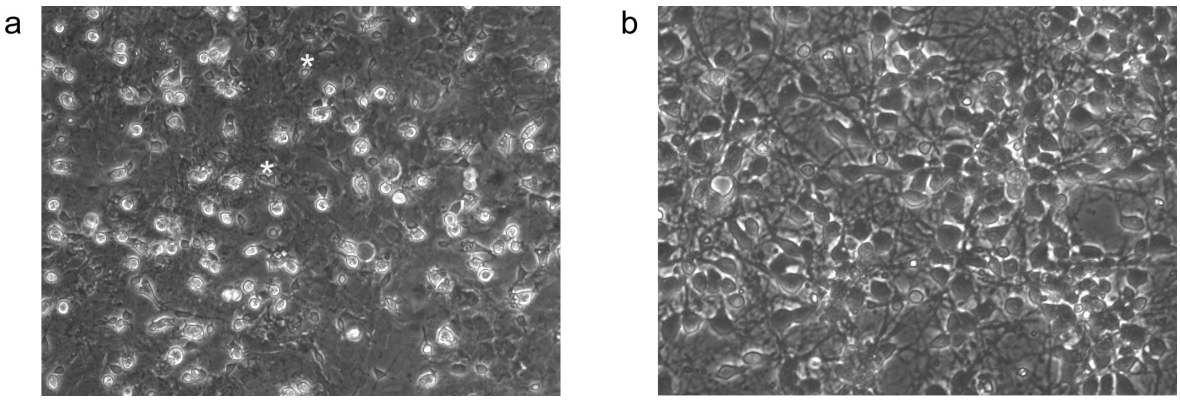

Figure 2.1: Mixed CNS culture and neuronal culture. a Mixed CNS culture 8 days after preparation (DIV8). Astrocytes have established a confluent layer (grey irregular background). Microglia cells are visible as resting adherent cells (white *) and as white loosely attached luminescent spheres, which are collected with the supernatant. **b** Cortical neuron culture at DIV3. Neurons have established a network of neurites and axons.

DIV4). For neuronal death assays groups were randomly distributed over the plate in duplicate. After the indicated time points cells were washed 3 times with PBS and fixed with 4% PFA for 10-15 min for subsequent immunohistological staining.

Microglial cultures

Experiments were always performed 24 h after platting. Stimulation of microglia for detection of inflammatory molecules was performed in duplicate. Microglia require their conditioned medium (established during the 24 h after platting). Therefore ligands were pre-mixed in microglia medium at twofold concentration and for each well 100 µl medium were replaced by 100 µl ligand solution thereby obtaining the final concentration of the ligands. For each condition (ligand, time point) one well was used (hence, no successive removal of supernatant from the same well). Supernatants were collected at the indicated time-points and stored at -20°C until analysis by ELISA, multiple analyte or nitrite oxide detection.

2.4 *In vivo* experiments

Experimental procedures were performed in compliance with institutional and state authorities.

2.4.1 Intrathecal injection

The protocol is according to (Lehmann et al., 2012a). Intrathecal injection into the spinal canal allows easy application of TLR ligands to the CNS avoiding the risk of mechanically damaging the brain. The ligands are flushed into the brain via the cerebrospinal fluid (CSF).

Mice were anesthetized with 10 µl/g body weight Ketamin-Cocktail by intraperitoneal injection. Anaesthesia was verified by absence of nociceptive reflex. Eyes were kept moist with an eye ointment. In prone position the skin was incised at hip level to expose the lumbar spine. Using the side of arcuate forceps the gap between lumbal vertebrae 2 and 3 was identified by palpation. The 28-gauge needle of a 50 µl Hamilton syringe with the opening facing cranial was allowed to sink in vertically between the vertebrae until it hit the ventral vertebrae. Correct positioning could be verified by moving the syringe left and right causing the hips to sway. The injection of 40 µl ligand solution was executed discontinuously with 1 µl every 2 sec and a short pause before withdrawing the canula. 2% Lidocain gel was applied for local analgesia and the skin was closed with dermal clips. Mice were kept in an incubator until fully awake and obtained free access to water and food. Commonly, paraparesis and sickness behavior were not observed.

2.4.2 Liquor extraction

To quantify leucocyte infiltration as an indicator for inflammation CNS liquor was extracted (Hoffmann et al., 2007). Mice were anesthetized as mentioned above at the indicated time point post intrathecal injection. The scalp was cut open longitudinally to expose the nape and the scull. The muscles were cut along the midline and at the base of the scull (T-cut) with a Nachstar-scissor to expose the cisterna magna. This was punctured with a 28-gauge butterfly canula and the cerebrospinal fluid (2-20 µl) withdrawn. Mice were then decapitated to retrieve the brain. CSF leukocytes were counted in a Fuchs-Rosenthal hemocytometer.

2.4.3 Transcardial perfusion

Brain tissue was fixed by transcardial perfusion of the mice. Therefore, mice were anesthetized as described above 72 h post intrathecal injection. The heart was exposed from ventral. Transcardial perfusion was carried out by inserting the blunt G26 canula of the perfusing system into the left heart ventricle and opening the right atrium with an incision. Using a peristaltic pump approx. 50 ml 0.9% saline solution were used to rinse out the blood followed by 50 ml 4% PFA for fixation (both solutions were prepared a day ahead and used at RT). After decapitation brains were removed from the skull and immersed in 4% PFA over night.

2.4.4 Cryopreservation and sectioning of brain tissue

Perfusion fixed and post-fixed brains were subjected to a sucrose series of 10% for 12-24 h, 20% for 24 h and 30% at 4°C (completely infiltrated brains sink to the bottom of the tube) until freezing. Deep-freezing was carried out in 2-methylbutane on dry-ice for 3-5 min after dabbing off the sucrose solution. The brains were stored at -80°C until sectioning. Brains

were cut in 14 µm thick coronal sections of five defined levels (Fig. 2.2) on a cryostat at -18°C and thaw-mounted on Super Frost Ultra Plus® slides. After air-drying they were stored at -80°C until subjected to immunohistostaining.

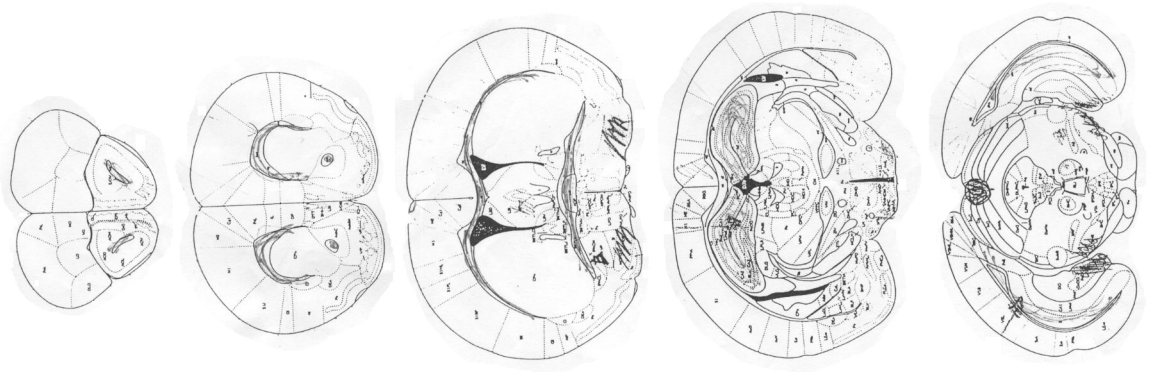

Figure 2.2: The five brain levels as mounted on glass slides. Levels were declared 1 to 5 (left to right). Relative position are as following interaural 6.60 mm; 5.3 mm; 3.94 mm; 1.86 mm; -0.08 mm

2.4.5 Brain preparation for protein and mRNA analysis

Mice were deeply anesthetized 14 h after intrathecal injection and liquor obtained (see 2.4.2). After decapitation the brain (with the bulbus olfactorius and cerebellum) was removed from the skull, the hemispheres separated along the midline with a scalpel and each hemisphere shock frozen in liquid nitrogen and stored at -80°C until further processing.

2.4.6 Experimental set-ups

Experiments were commonly performed with 3-4 mice per group and in 2-3 independent repeats.

Evaluation of neurodegeneration and -inflammation

For analysis of neuronal death (NeuN), loss of fibers (NF) and morphology of microglia (Iba1 and CD11b) and astrocytes (GFAP) 40 µl of the indicated ligands were injected intrathecally and mice sacrificed after 3 days. LPS is a very potent endotoxin (causing septic shock), therefore a maximum amount of 10 µg was chosen; 40 µg Pam3CSK4 according to (Hoffmann et al., 2007); 136 µg Loxoribine according to a personal communication with Sabrina Hoche, née Lehmann; a maximum amount of 10 µg CpG ODN according to (Tauber et al., 2009), which applied 1 µg/d for 4 weeks. The maximum possible amount of HSP60 (40 µg) was used.

Analysis of inflammatory factors and mRNA expression

For analysis of inflammation (leucocyte infiltration, multiple analyte detection, ELISA and nitrite oxide) and mRNA expression 40 µl of the indicated ligands were injected intrathecally and mice were sacrificed after 12 h or after 6 h, 12 h and 72 h, as indicated. LPS is a very potent endotoxin (causing septic shock), therefore a maximum amount of 10 µg was chosen; the amounts of Pam3CSK4, Loxoribine and CpG ODN used, were adjusted to that of LPS for comparison of the results.

Double injection

To analyze the impact of TLR stimulation with an exogenous ligand followed by TLR stimulation with an endogenous ligand subsequent intrathecal injections were performed. Exogenous ligands (40 µl) were injected 1 day before injection of the endogenous ligand HSP60 (40 µl). Mice were sacrificed after another 3 days.

2.5 Analysis of inflammatory molecules

2.5.1 ELISA

TNF and IL-1β concentrations in cell culture supernatants or in brain tissue lysate were measured by enzyme-linked immunosorbent assay (ELISA) using the BD OptEIA™ Set Mouse TNF (mono/mono) (range: 15.6-1000 pg/ml) and BD OptEIA™ Set Mouse IL-1β (range: 31.3-2000 pg/ml) according to the manufacturer's manual. Substrate solution was prepared as described in 1.5. Culture supernatants were analyzed undiluted, tissue lysates were diluted to 1 mg/ml in Assay Diluent. Standards and samples were analyzed in duplicate.

2.5.2 Multiple analyte detection

Multiple analyte detection was performed using FlowCytomix. The immunoassay is a bead-based method to detect the concentrations of up to 20 analytes in one sample using a flow cytometer. The mouse/rat basic kit was used with mouse simplex kits and mouse Th1/Th2 10plex sample kit. Undiluted microglial culture supernatants and brain tissue lysates diluted to 15 mg/ml with lysis buffer were analyzed in singular, standards in duplicate.

2.5.3 Nitric oxide detection

The content of nitric oxide (NO) in culture supernatants or brain tissue lysates was analyzed indirectly by assaying the stable end product nitrite (NO2-) with the Griess reaction. In a microtiter plate 50 µl Griess reagent were added to 50 µl sample. After 10 min at RT

colorimetric reaction was detected at 540 nm and the concentrations calculated from an eight point standard curve (1.5 µM-100 µM NaNO3) dissolved in medium or lysis buffer as appropriate. Standards and samples were assayed in duplicate.

2.6 Analysis of mRNA expression

All materials were RNase and DNase free and tips with filters were used.

2.6.1 Extraction of total RNA from brain tissue

One brain hemisphere was homogenized in 1ml TRIzol® with an Ultra-Turrax® at 21500 rpm for 30 sec. The homogenate was centrifuged at 1200 x g and 4°C for 15 min. The pink colored supernatant was transferred to a new tube with 200 µl chloroform and vortexed for 3-5 sec. After centrifugation for 1 min the top aqueous phase was transferred into a new tube. The total RNA was precipitated by adding 500 µl ice cold isopropanol followed by vortexing for 3-5 sec and centrifugation for 10 min. The pellet was washed with 1 ml 75% ethanol (v/v) and dissolved in 20 µl nuclease-free water after discarding the ethanol and air-drying for 5-10 min. To remove DNA the 20 µl total RNA were mixed with 15 µl DNase master mix (per reaction: 5 µl nuclease-free water, 3.5 µl RQ1 RNase-free DNase 10x reaction buffer, 3.5 µl 40 U/mul RNA-sin and 3 µl 1 U/mul RQ1 RNase-free DNase) and incubated at 37°C for 20 min. On ice 165 µl Millipore type I water and 200 µl UltraPure™ phenol:chloroform:isoamyl alcohol were added followed by 3-5 sec of vortexing. After centrifugation at 12000 x g and 4°C for 5 min the top aqueous phase was mixed for 3-5 sec with 200 µl ice cold chloroform in a MaxTract tube and centrifuged for 5 min. The MaxTract tube contains resin, which allows clear separation and recovery of the uppermost RNA containing aqueous phase. This was supplemented with 6 µl 10 mM ammonium acetate and 550 µl 96% ethanol (v/v) in a new tube. After vortexing, RNA was precipitated at -80°C for 1 h and pelleted by centrifugation for 30 min. The pellet was washed with 75% ethanol (v/v) and centrifuged for another 30 min. After discarding the ethanol and air-drying for 5-10 min the RNA was dissolved in 25 µl nuclease-free water and stored at -80°C.

2.6.2 Determination of total RNA concentration

Concentration and purity of total RNA was determined by photometric detection at $\lambda A = 260$ nm. Typically a 1:10 dilution of brain lysate in Millipore type I water was performed and a concentration of 8.8 mg/ml ± 3.5 and a purity of 1.9 ± 0.08 (ratio of RNA to Protein [A_{260nm}/A_{280nm}]) was measured.

2.6.3 cDNA synthesis

For synthesis of cDNA random hexamers were used. A volume containing 40 µg total RNA was adjusted to 15 µl with nuclease-free water and 10.5 µl master mix (per reaction: 5 µl 5x MMLV buffer, 1.5 µl 100 µM random hexamers, 1.5 µl 2.5 mM dNTPs, 0.5 µl 0.1 M DTT, 0.5 µl 40 U/µl RNA-sin and 1.5 µl 200 U/µl MMLV-RT) was added. The RT^- control was obtained by substituting the enzyme by the same volume water. After incubating at 21°C for 5 min and subsequently at 37°C for 1 h the reaction was stopped by denaturing the enzyme at 95°C for 5 min. The cDNA was stored at -20°C.

2.6.4 real-time PCR

SYBR® Green-based quantitative real-time PCR was performed with the RT^2 qPCR Primer Assays according to the manufacturer's manual. The two-step cycling program: 1 cycle (95°C 15 min), 40 cycles (95°C 15 sec; 60°C 1 min) was followed by a dissociation curve program: 1cycle (95°C 15 sec; 60°C 1 min; 95°C 15 sec) to verify the presence of only one PCR product. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as a housekeeping gene due to the fact, that its expression was not influenced by the treatments. For both gene of interest (GOI) and house keeping gene (HKG) the samples, RT^- control and none template control were analyzed in duplicate. The samples were rerun when the cycle threshold (CT) difference between the duplicates was greater 0.5. For statistics ΔCT values ($\Delta CT = CT_{GOI}\text{-}CT_{HKG}$) were log2-transformed according to (Rieu and Powers, 2009). Fold exchange ($2^{-(ddCT)}$) of treatment group to control group was calculated using the median of each group setting the control group to 1.

2.7 Analysis of protein

2.7.1 Protein extraction from primary cultures

Cultures rinsed with ice-cold PBS were placed on cold packs and 100-200 µl SDS lysis buffer (supplemented with PMSF at a final concentration of 1 mM short before use) was added. Lysate was collected with a cell scraper and transferred into a 1.5 ml tube. After sonification for 3 sec and incubation on ice for 30 min the lysate was centrifuged at 13000 x g and 4°C for 20 min. The supernatant was stored at -20°C.

2.7.2 Protein extraction from brain tissue

Protein was isolated from one brain hemisphere on ice by adding 1 ml ice cold brain lysis buffer and homogenising with an Ultra-Turrax® at 17500 rpm for 5 sec with subsequent

sonification for 3 sec. After centrifugation at 10000 rpm and 4°C for 15 min the supernatant was transferred into a new tube on ice and stored at -20°C.

2.7.3 Determination of protein concentration

Concentration of total protein was determined with the BCA Protein Assay Reagent Kit (BSA standard, range: 25-1500 pg/ml) according to the manufacturer's manual using the microplate procedure. A 1:50 dilution of brain lysates in lysis buffer or 1:10 dilution of cell lyate in Millipore type I water was quantified in duplicate.

2.7.4 Western blot analysis

SDS PAGE

Before loading samples were boiled with 4x loading buffer for 5 min at 95°C and were spun down to collect condensate from the tube lid. Gels were run at 70 V until samples reached the border of the resolving gel and then at 140 V as long as required.

Blotting

Blotting was performed with a three buffer system in a semi-dry blotter. Gels were equilibrated in cathode buffer for 15 min. The PDVF membrane was activated/hydrated in methanol for 15 sec, washed in Millipore type I water for 2 min and left in anode II until use. Filter papers were soaked in anode I and cathode buffer, respectively. The blot was set up as following: Anode, filter paper (anode I), membrane (anode II), gel (cathode), filter paper (cathode), cathode. Transfer was commonly at 15 V for 60 min.

Detection

After blotting membranes were washed in TBST for 15 min and blocked with the indicated blocking buffer at RT for 1 h. Primary antibodies were applied in blocking buffer for 1 h at RT or overnight at 4°C. Membranes were washed 3 times for 10 min and incubated with the horseradish peroxidase linked-secondary antibody in blocking buffer for 1 h at RT. Before detection membranes were washed 3 times 10 min each. Periods of washing and incubating were carried out on an orbital shaker at 50 rpm.

Detection of luminescence was performed with ECL Western Blotting Detection Reagent according to the manufacturer's manual and visualized on film.

Stripping and re-probing

To detect β-actin, antibodies were stripped from the membranes with stripping buffer for 10 min at RT while gently agitating und washed with TBST for 5 min. Re-probing was

performed as described above.

2.8 Immunohistology

The applied dilutions of primary and secondary antibodies are found in Tab 2.6. Staining, assessment/evaluation, photographing (except representative photographs) and quantification of all sections were performed in a blindfolded manner.

2.8.1 Quantification of neuronal death *in vitro*

Fixed cultures were stained over night with primary antibody in block solution at 4°C. After washing with PBS three times, secondary antibody was added in block solution for 1-2 h at RT. Nuclei were stained with Dapi for 2-3 min and after washing 3-4 times the coverslips were placed on glass slides with Immumount. The mean amount of NeuN-positive cells per cover slip was quantified by counting at least 6 microscopic fields at 600 x magnification. Fields were counted, when NeuN-positive cells could be distinguished from one another and appeared to be evenly distributed across the field. The mean of the cover slip duplicates was used for calculation. Control was set to 100% to account for biological variation between experiments.

2.8.2 TUNEL assay *in vitro*

Staining of apoptotic cells by Terminal desoxyribosyl-transferase mediated dUTP nick end labeling was performed with In Situ Cell Death Detection Kit, TMR red according to the manufacturer's handbook and nuclei were stained with Dapi for 2-3 min.

2.8.3 Quantification and evaluation of neuronal death and inflammation *in vivo*

Sections were air-dried for 10-15 min at RT and post-fixed with 4% PFA for 10 min. After washing 3 times in a cuvette with PBS for 5 min each, sections were pre-incubated with block solution for 1 h at RT in a wet chamber. Primary antibodies diluted in block solution were applied overnight at 4°C. Negative controls were obtained by omitting the primary antibody. After washing 3 times secondary antibodies diluted in block solution were applied for 1-2 h at RT. Nuclei were stained by incubating with Dapi for 2-3 min at RT. After washing 3-4 times sections were coversliped with Immumount.

Neurons were quantified at three given spots per hemisphere in the cerebral cortex (of level 4 sections). Therefore pictures were taken at 600 x magnification and NeuN-positive nuclei (fragments and unfocused cells were ignored) were counted manually with a clicker. The mean per mouse was calculated and is expressed as $NeuN^+$ cells/mm^2 (area

at 600 x magnification equates to 0.063 mm^2). Microglia and astrocytes were quantified at three given spots per hemisphere in the cerebral cortex (of level 4 sections) at 600 x magnification. Therefore Iba-1-positive (microglia) and GFAP-positive (astrocytes) cell bodies were counted manually with a clicker. The mean per mouse was calculated and is expressed as Iba-1^+ cells/mm^2 and $GFAP^+$ cells/mm^2, respectively.

Morphology and appearance of astrocytes (GFAP) and microglia (Iba-1 and CD11b) was evaluated in the cerebral cortex. The criteria were overall amount and size of cell bodies, number of projections and intensity of staining.

Destruction/degeneration of fibers was analyzed by examining NF-positive projections in the cerebral cortex and the corpus callosum at level 4. An intact corpus callosum has a clear triangle bonfire-like structure with radial projections through the cortex towards the brain surface. Intact NF-positive projections in the corpus callosum and cortex display a bright staining, are thin and continuous. Destroyed fibers appear as thick short bundles or punctual and the staining is weaker.

2.8.4 TUNEL assay *in vivo*

Visualisation of apoptotic nuclei by Terminal desoxyribosyl-transferase mediated dUTP nick end labeling was performed with the ApopTag® Plus Fluorescein In Situ Apoptosis Detection Kit S7111 according to the manufacturer's manual and nuclei were stained with Dapi for 2-3 min. Apoptosis induced DNA fragmentation is detected by enymatically labeling the free 3'-OH ends with modified nucleotides. DNase I treated brain sections were used as a positive control and also as a negative control by omitting the enzyme terminal deoxynucleotidyl transferase. TUNEL-positive cells were quantified in the cortex of all five levels at 200 x magnification, while each one was verified at 400 x magnification accounting for morphological criteria (overlay with Dapi and localization in apoptotic bodies). TUNEL-positive cells are expressed as the sum per mouse.

2.9 Statistics

Statistics were calculated using Graphpad Prism version 5.01 for Windows, Graphpad Software, San Diego California USA. The tests used are indicated in the figure legends. Stastical significance is defined as the following: */#/† when $P \leq 0.05$, **/##/†† when $P \leq 0.01$ and ***/###/††† when $P \leq 0.001$.

Results

Thirteen TLRs have been identified in human and mouse. To study the impact of TLR activation in the CNS TLR2, TLR4, TLR7 and TLR9 have been selected for the following reasons. Toll-like receptor 2 recognizes a broad range of pathogen-derived and host-derived molecular patterns. As the first TLR identified, TLR4 and its binding of bacterial LPS is extensively studied, making it the best described and therefore representative TLR. Moreover, as pointed out in the introduction, TLR2 and TLR4 are implicated CNS pathologies, such as stroke, traumatic brain injury and Alzheimer's disease. Whereas TLR2 and TLR4 recognize a broad range of structurally diverse molecules such as lipopolysaccharides, lipopeptides or glycoproteins, TLR7 and TLR9 detect nucleic acids. Thereby, TLR7 recognizes single stranded RNA of viral and host origin, while TLR9 binds DNA with unmethylated CpG-motifs from both bacteria and viruses. The TLR ligands used in this study are specific for each TLR and thereby enable a selective activation. In the following the TLRs were activated with these ligands: TLR2 with Pam3CSK4, TLR4 with lipopolysaccharide (LPS) or Heat Shock Protein 60 (HSP60), TLR7 with Loxoribine or Imiquimod and TLR9 with CpG-Oligodesoxynucleotide (CpG-ODN) 1668. The ligands are differentiated into exogenous (Pam3CSK4, LPS, Loxoribine, Imiquimod and CpG ODN) and endogenous agonists (HSP60).

3.1 Results (I) – The Impact of single and combined Toll-like receptor activation on the CNS *in vitro*

This first part of the result section is focused on the functional relevance of single and combined TLR-stimulation on microglia and neurons *in vitro* and describes the kinetics and pattern of the microglial TLR-induced inflammatory response and the consequence of TLR activation on neuronal survival.

3.1.1 Combined TLR activation with exogenous ligands modulates microglial TNF-α production

Activation of any TLR in immune cells initiates a canonical pathway resulting in NF-κB-mediated secretion of pro-inflammatory molecules (Akira et al., 2006). It is established that upon TLR stimulation microglia readily release the pro-inflammatory and neurotoxic cytokine TNF-α (Ebert et al., 2005; Lehmann et al., 2012b). Therefore, TNF-α levels were determined in microglial cultures as a functional assay to elucidate the reaction of

microglia to single and combined TLR activation *in vitro*. Microglial cells were incubated with the highly specific ligands Pam3CSK4 (TLR2), LPS (TLR4), Loxoribine (TLR7) and CpG ODN (TLR9) in single or pair-wise simultaneous combination, as indicated, for up to 72 h. The concentrations used, comply with established working doses (Takeuchi et al., 2002; Yamamoto et al., 2002a). Supernatants were collected at the indicated time points and TNF-α concentrations were measured by ELISA.

A direct comparison between the TNF-α levels induced by the different TLR agonists alone is not feasible due to different properties and concentrations of each ligand and was not intended. However, each of the four ligands induced the secretion of TNF-α at every time point measured (Fig. 3.1). Accordingly, TNF-α levels in cultures of unstimulated microglia were very low or undetectable. Simultaneous challenge of microglial TLR2 and TLR4 led to a sub additive increase of TNF-α release from 3 h on to 72 h, which was significant to each single TLR stimulation at 24 h (Fig. 3.1a). Compared to single activation, combined stimulation of TLR4 and TLR9 induced significant supra additive levels of TNF-α from 12 h to 72 h, which first dropped by 72 h (Fig. 3.1c). Activation of TLR2 and TLR9 together resulted in a slight increase, which was not significant compared to sole stimulation of TLR2 or TLR9 at any time point measured (Fig. 3.1b). Thus, simultaneous stimulation of the bacteria sensing TLRs TLR2 and TLR4, TLR4 and TLR9 and to a lesser extent TLR2 and TLR9 resulted in prolonged elevated TNF-α secretion in comparison with the stimulation of the respective single receptor (Fig. 3.1a,b,c). In contrast, simultaneous activation of the virus sensing TLR7 significantly suppressed TLR2-, TLR4- and TLR9-induced TNF-α secretion to levels induced by activation of TLR7 itself from 3 h on and up to 24 h (TLR4 and TLR9; Fig. 3.1d,f) or 72 h (TLR2; Fig. 3.1e). Similar results were found using another TLR7 ligand, namely Imiquimod (10 µg/ml; data not shown).

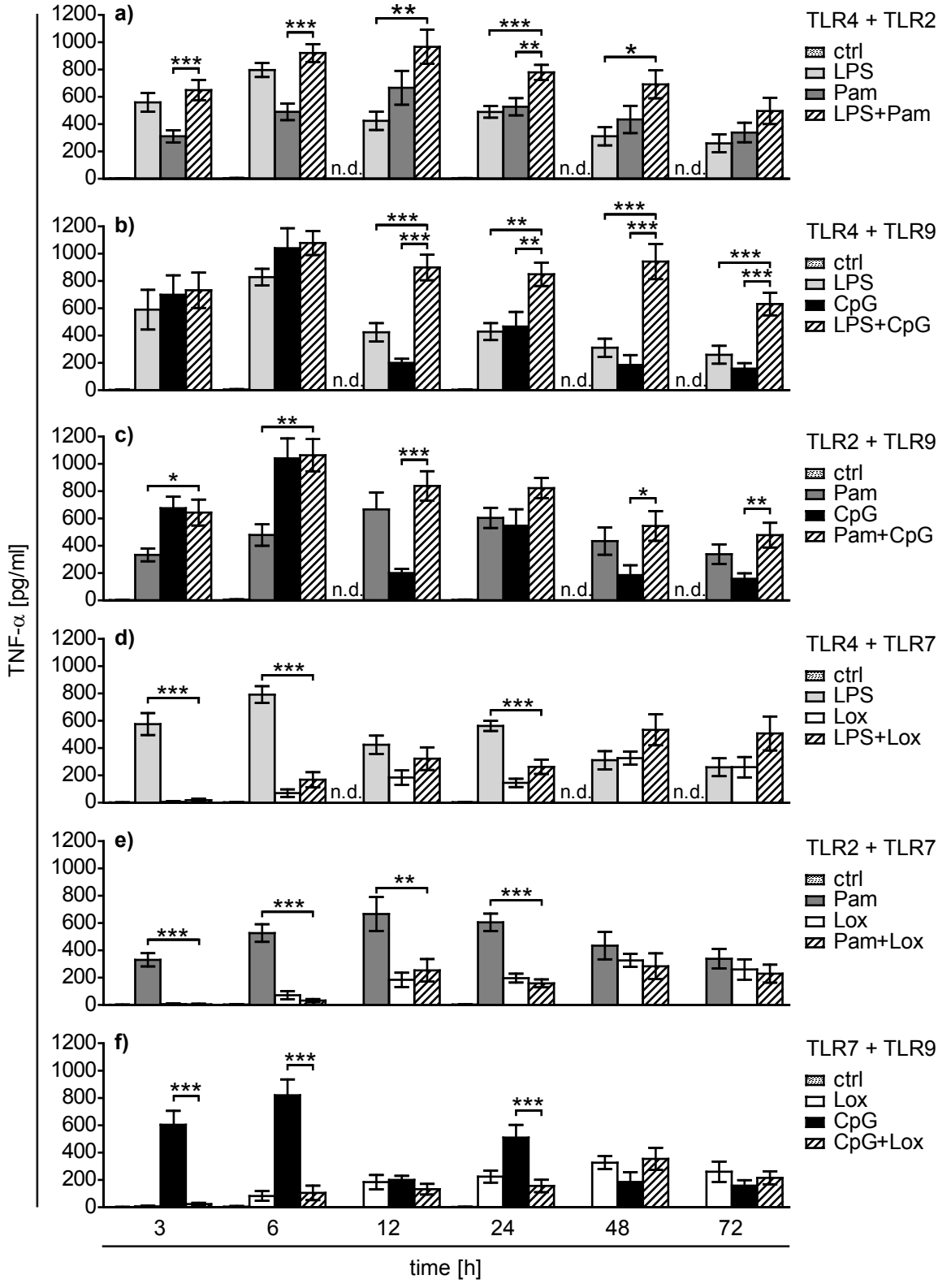

Figure 3.1: Combined activation of distinct TLRs results in an elevated or suppressed pro-inflammatory response from microglia. Microglia were stimulated with TLR specific ligands Pam3CSK4 (Pam, 100 ng/ml), LPS (100 ng/ml), Loxoribine (Lox, 1 mM) and CpG ODN (CpG, 1 μM), with the pairwise stimulations carried out simultaneously. The supernatants were collected at the indicated time points and analysed via TNF-α ELISA; mean ± SEM of n ≥ 5 independent experiments run with duplicates, ANOVA with Bonferroni selected pairs of each individual compound vs. combination; n.d. not detected

3.1.2 Combined TLR activation with exogenous ligands induces the release of a distinct cytokine/chemokine profile from microglia

Upon activation microglia secrete a myriad of immunostimulatory molecules. These molecules enable the microglia to mount and modulate the inflammatory response in part by attracting further immune cells (professional antigen presenting cells and T cells) to the site promoting clearing of pathogen and debris. The effect of combined TLR stimulation on microglial activation, as determined by TNF-α secretion, was most prominent at 24 h (Fig. 3.2a). Therefore, using the same samples, the presence of further cytokines and chemokines was determined by a bead-based assay, allowing the simultaneous detection of multiple analytes by flow cytometry. Except for the anti-inflammatory cytokine IL-10, which was not detected after stimulation of TLR7, similar cytokines and chemokines were induced by stimulation of TLR2, TLR4, TLR7 and TLR9, although to different extents (Fig. 3.2b). The cytokines IL-4, IL-17, IFN-γ, IL-12p70, IL-13, IL-α, GM-CSF and IL-23 were not induced at all by stimulation of any of the TLRs at this time-point (data not shown). Combined simultaneous stimulation of TLR2 and TLR4, and TLR4 and TLR9 led to sub additive levels of IL-6 and significant supra additive levels of IL-10 in comparison to stimulation of each single TLR (Fig. 3.2b 1st, 2nd column). On the other hand TLR7 suppressed TLR2, TLR4 or TLR9 mediated IL-6 and IL-10 release as observed in the TNF-α assay (Fig. 3.2b 4th, 5th 6th column). None of the pair-wise combinations increased levels of the chemokines CXCL1, CCL2 or CCL5 in comparison with single TLR activation by 24 h.

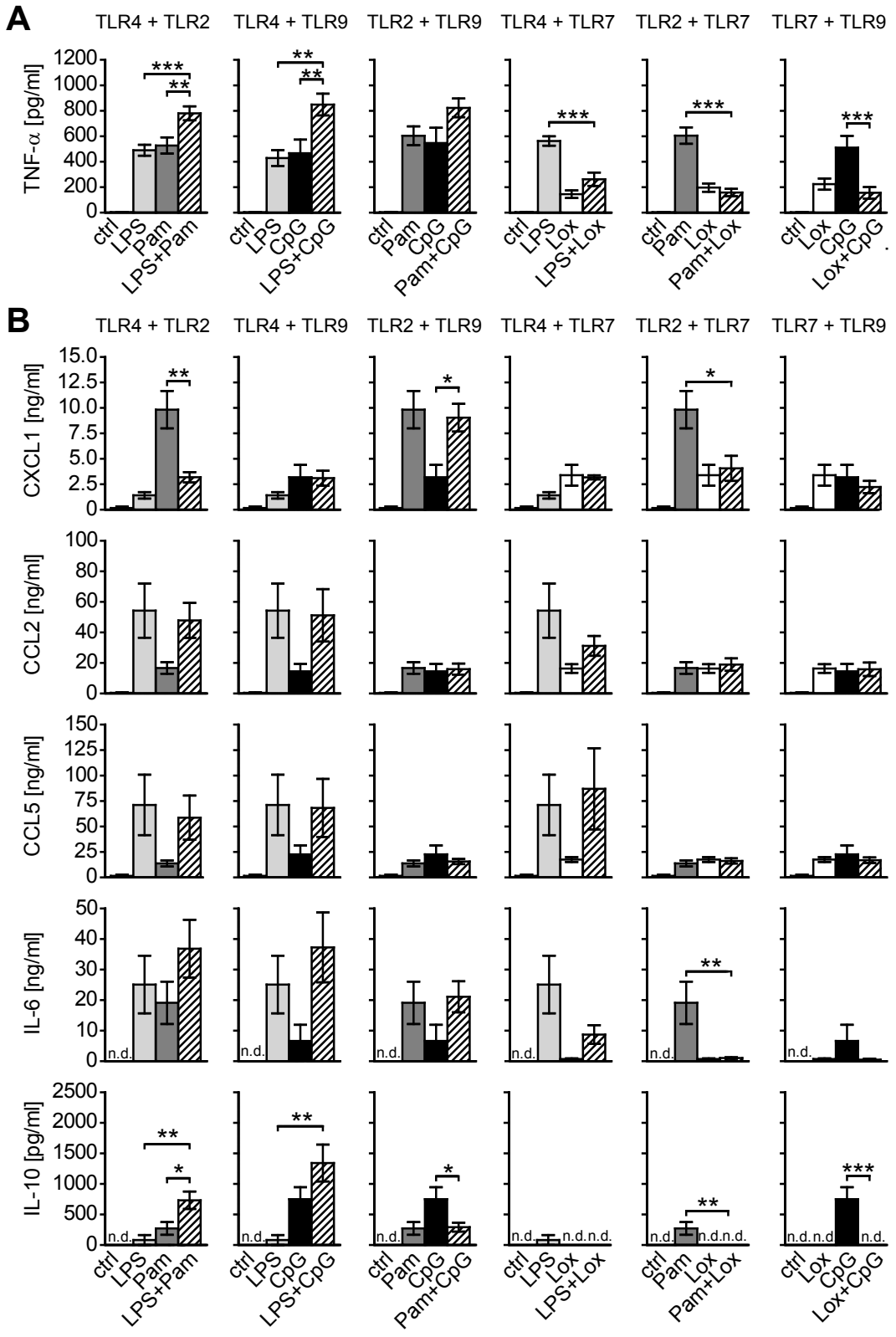

Figure 3.2: Single and combined TLR activation mediates a distinct cytokine and chemokine profile. Microglia were stimulated with TLR specific ligands Pam3CSK4 (Pam, 100 ng/ml), LPS (100 ng/ml), Loxoribine (Lox, 1 mM) and CpG ODN (CpG, 1 μM) alone or with pair-wise stimulation carried out simultaneously. Supernatants were analysed via **(A)** TNF-α ELISA (extract from Fig. 3.1; mean ± SEM of n ≥ 6 independent experiments run with duplicates) **(B)** Flowcytometry-based multiple analyte detection for cytokine and chemokine levels (same samples as used for TNF-α ELISA 24 h after stimulation; mean ± SEM of n = 3 independent experiments) ANOVA with Bonferroni selected pairs between each individual compound and combination; n.d. not detected

3.1.3 Combined TLR activation with exogenous ligands modulates the release of neurotoxic nitric oxide from microglia

In vitro, LPS-stimulated microglia release neurotoxic nitric oxide (NO) (Boje and Arora, 1992; Lehnardt et al., 2006). To further unravel the effect of sole and combined TLR activation on the release of neuroinflammatory and –toxic molecules from microglia the content of NO was measured after several time points (3, 6, 12, 24, 48 and 72 h). Detectable levels were not found until 48 h after single application of the TLR4 ligand LPS and combinations with LPS (Fig. 3.3). While co-stimulation of TLR4 with TLR2 or TLR9 led to a supra additive NO release, while co-stimulation of TLR4 with TLR7 nearly diminished TLR4-induced NO secretion (Fig. 3.3). In detail, simultaneous activation of TLR2 and TLR4 significantly increased NO release by more than 2.5-fold at 48 h (Fig. 3.3) and up to 3-fold by 72 h (control 0.142 µM ±0.198; LPS 12.88 µM ±6.59; Pam3SCK4 0.89 µM ±0.93; LPS+Pam3SCK4 38.4 µM ±14.72, *** vs. LPS and *** vs. Pam3SCK4). The effect of CpG ODN combination with LPS, thereby activating TLR9 and TLR4 respectively, was weaker with merely 1.5-fold more NO at 48 h (Fig. 3.3) and 72 h (control 0.142 µM ±0.198; LPS 12.88 µM ±6.59; CpG ODN 0,15 µM ±0.26; LPS+CpG ODN 19.30 µM ±9.97, *** vs. CpG ODN), and only significant to stimulation with CpG ODN.

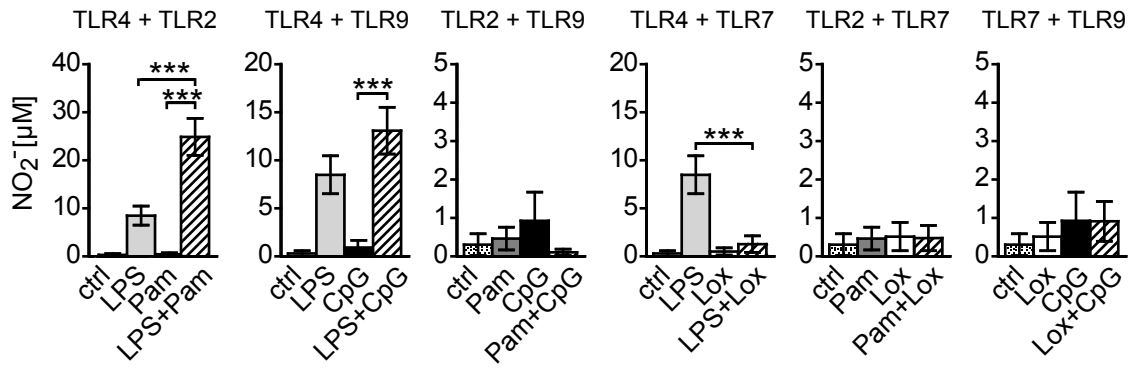

Figure 3.3: Combined activation of distinct TLRs results in an elevated or suppressed release of neurotoxic nitric oxide from microglia. Microglia were stimulated with TLR specific ligands Pam3CSK4 (Pam, 100 ng/ml), LPS (100 ng/ml), Loxoribine (Lox, 1 mM) and CpG ODN (CpG, 1 µM) alone or with pair-wise stimulation carried out simultaneously. The supernatants were collected at several time points (3, 6, 12, 24, 48 and 72 h) and analysed via Griess' reaction for nitrite (NO_2^-) content. Above NO levels after 48 h incubation are displayed (same samples as used for TNF ELISA 48 h after stimulation; mean ± SEM of n = 5 independent experiments run with duplicates). ANOVA with Bonferroni selected pairs of each individual ligand vs. combination; n.d. not detected

3.1.4 Combined activation of TLR2 with Pam3CSK4 and TLR4 with the endogenous ligand HSP60 modulates the inflammatory response of microglia

As several endogenous ligands of TLRs have been identified, the question emerged whether the response of microglia to TLR activation depends on the exogenous or endogenous nature of the TLR agonists confronted with. Heat Shock Protein 60 (HSP60) is a ubiquitously expressed molecular chaperone (Mayer, 2010) essential for maintenance of cellular homeostasis, especially in stress-situations. It is an established endogenous TLR4 agonist (Ohashi et al., 2000). Previously, our group demonstrated in cell cultures assays that HSP60 is released by damaged CNS cells and mediates microglial TLR4-dependent inflammation and neuronal injury (Lehnardt et al., 2008). To further unravel the impact of combined TLR activation on the microglial inflammatory response the endogenous TLR4 ligand HSP60 was applied alone or in combination with the exogenous ligands Pam3CSK4 (TLR2), LPS (TLR4), Loxoribine (TLR7) and CpG ODN (TLR9). Notably, a subject of considerable debate has been whether immunostimulatory effects of endogenous TLR ligands are due to a contamination of the recombinant protein preparations with bacterial products from the microbial expression system (Henderson et al., 2010). Previous control experiments excluded LPS being present in the low endotoxin preparations of HSP60 used in these studies (Lehnardt et al., 2008). Purified microglia were stimulated with each ligand alone or with the TLR4 ligand HSP60 in combination with one of the exogenous TLR ligands. Supernatants were analysed for TNF-α content at several time points (3, 12, 24 and 48 h). Incubation of microglia with HSP60 itself, thereby activating TLR4, did not result in TNF-α secretion at any time point (Fig. 3.4a), in accordance with a report on macrophages (Gao and Tsan, 2003). However, treatment of microglia with HSP60 (TLR4) and Pam3CSK4 (TLR2) resulted in a supra additive release of TNF-α, which was significant to both single applications at 12 h (Fig. 3.4a). TNF-α levels in the supernatants of microglia cultures remained unchanged after combined stimulation with HSP60 (TLR4) and Loxoribine (TLR7) or HSP60 (TLR4) and CpG ODN (TLR9) compared with the single stimulations (Fig. 3.4a). Interestingly, stimulation of TLR4 with both its endogenous and exogenous ligands HSP60 and LPS at the same time did not elicit an elevated TNF-α secretion in comparison with sole stimulation (Fig. 3.4a). Further analysis of the TLR4 and TLR2-induced cytokine/chemokine profile after 12 h stimulation with HSP60 and Pam3CSK4 revealed a significant increase of IL-6 in comparison to stimulation with HSP60 and Pam3CSK4 alone (Fig. 3.4b). In summary, these results indicate, that the reponse intiated by TLR4 might depend on the type of ligand engaged.

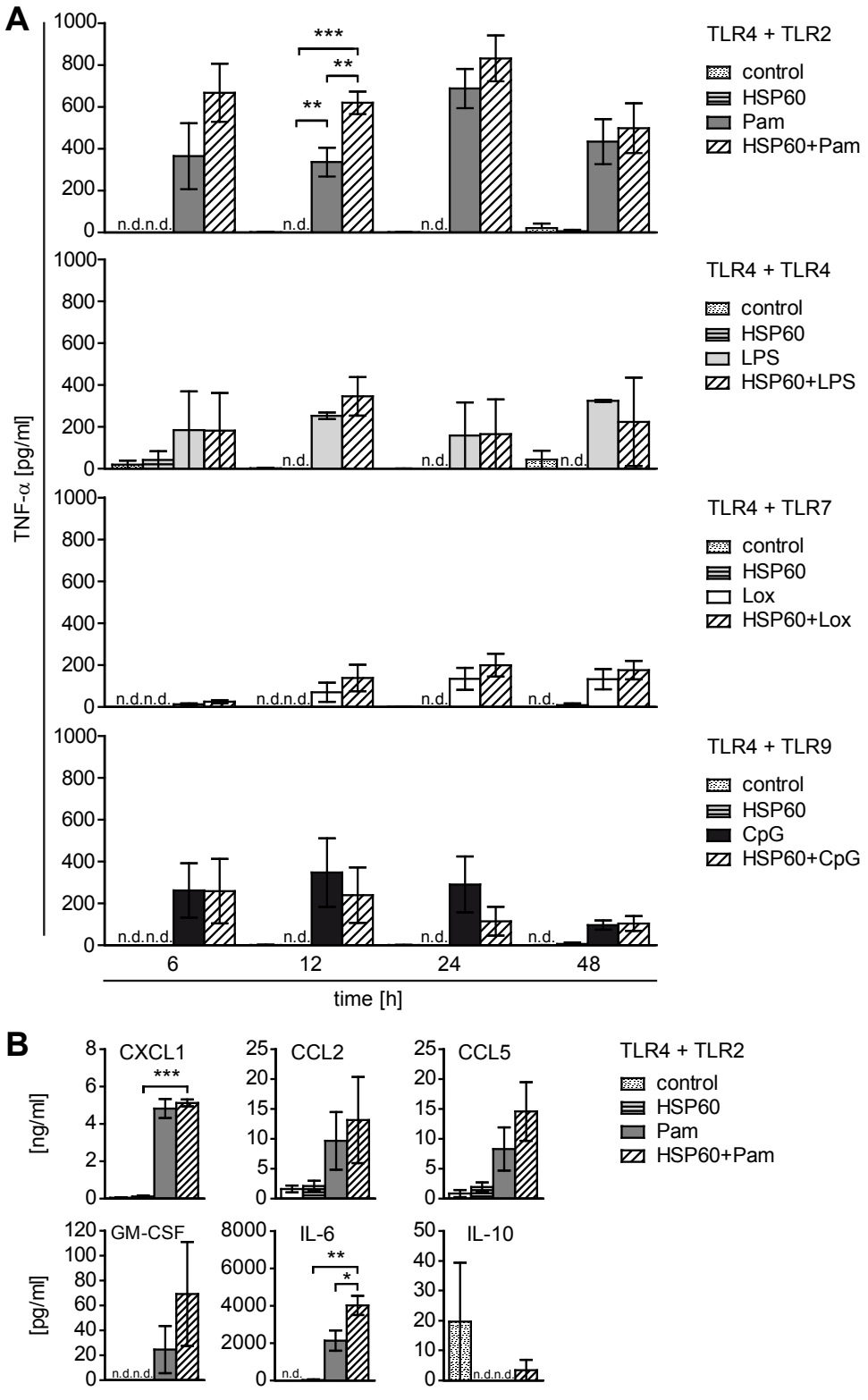

Figure 3.4: The inflammatory response of microglia is enhanced after combined stimulation with the endogenous TLR4 ligand HSP60 and the exogenous TLR2 ligand Pam3CSK4. Microglia were stimulated with TLR specific ligands (10 µg/ml HSP60, 100 ng/ml LPS, 100 ng/ml Pam3CSK4 (Pam), 1 mM Loxoribine (Lox) and 1 µM CpG ODN (CpG)) for the indicated time periods. The pair-wise stimulation was carried out simultaneously. Supernatants were analysed via **(A)** TNF-α ELISA (mean ± SEM of n = 2-4 independent experiments run with duplicates) ANOVA with Bonferroni selected pairs between each individual compound and combination; n.d. not detected **(B)** Flowcytometry-based multiple analyte detection for cytokine/chemokine levels (same probes as used for TNF-α ELISA 12 h after stimulation; mean ± SEM of n = 3 independent experiments run with duplicates); n.d. not detected

3.1.5 Combined activation of microglial TLR2 and TLR4 with exogenous TLR ligands enhances neuronal death *in vitro*

In the following experiments the functional relevance of single and simultaneous pair-wise TLR activation on neuronal survival *in vitro* was investigated. To determine if TLR activation results in inflammation-mediated neuronal death *in vitro* microglia were co-cultured with neurons. Pure neuronal cultures were carried out as a control to determine microglia-dependent neuronal decay. To quantify the number of surviving neurons, cultures were immunostained with an antibody directed against neuronal nuclei (NeuN), a marker for neurons.

Neuronal cultures and co-cultures were incubated with Pam3CSK4 (TLR2), LPS (TLR4), Loxoribine (TLR7) or CpG ODN (TLR9) in single or simultaneous pair-wise combination, as indicated, for 72 h. Applying 1 µM CpG resulted in neurons clumping together thereby impeding quantification, therefore the concentration was reduced by 10-fold.

All TLR agonists affected neuronal survival when neurons were co-cultured with microglia (Fig. 3.5a,b). Yet, incubation with the TLR7 ligand Loxoribine also led to neuronal death in absence of microglia (Fig. 3.5b). As published before neurons exhibit TLR7 and exposure to TLR7 agonists induces cell-autonomous death (Lehmann et al., 2012b; Lehmann et al., 2012a). As compared with pure neuronal cultures activation of TLR2, TLR4 and TLR9 with their specific agonists reduced neuronal survival solely when microglia were present (Fig. 3.5b), as expected (Lehnardt et al., 2008; Iliev et al., 2004). Neuronal survival was further affected by the simultaneous activation of TLR2 and TLR4 and to a lesser extent TLR4 and TLR9 in co-cultures (Fig. 3.5a,b). Subsequent quantification of NeuN-positive cells revealed that compared with single activation, combined activation of TLR2 and TLR4 significantly enhanced microglia-dependent neuronal death. Thereby neuronal viability was reduced by 74% compared with the control co-culture (Fig. 3.5b). Incubation of co-cultures with LPS and CpG ODN, thereby stimulating TLR4 and TLR9, resulted merely in a trend of further reducing neuronal viability as compared with stimulation of each microglial TLR alone (Fig. 3.5b). None of the other TLR ligand combinations increased neuronal death in cultures of neurons alone nor supplemented with microglia in comparison with the respective single application (Fig. 3.5b). These results demonstrate that combined stimulation of microglial TLRs can deteriorate neuronal injury in comparison to activation of a single TLR in microglia.

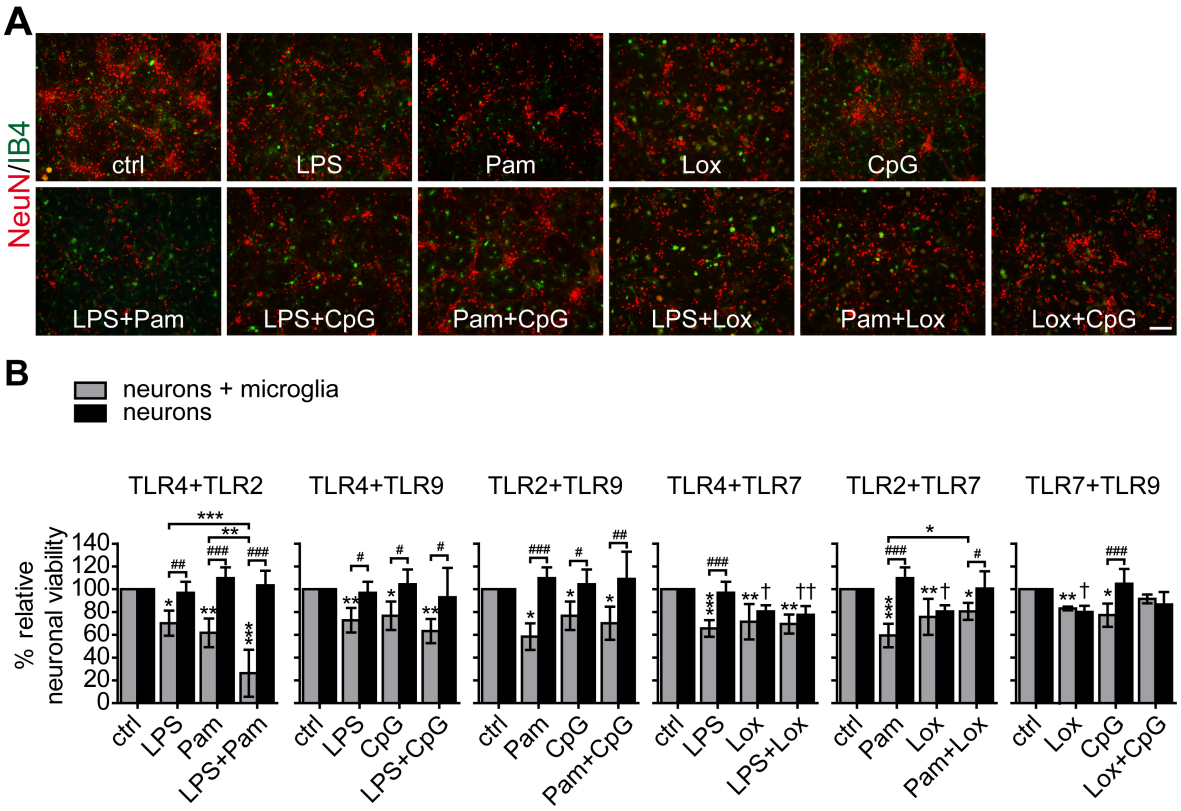

Figure 3.5: Simultaneous challenge of TLR2 and TLR4 with exogenous TLR ligands enhances microglia-dependent neuronal injury *in vitro*. Neurons were cultured alone or supplemented with microglia and stimulated with 100 ng/ml LPS, 100 ng/ml Pam3CSK4 (Pam), 1 mM Loxoribine (Lox) or 0.1 µM CpG ODN (CpG) in single or pair-wise simultaneous application. After 72 h **(A)** cultures were fixed and stained for neuronal nuclei (NeuN, neurons, red), neurofilament (NF, axons, red) and Isolectin IB4 (IB4, microglia, green), representative images (scale bar 100 µm) and **(B)** NeuN-positive cells were quantified, mean ± SD from 4-5 individual experiments with each condition performed in duplicate, ANOVA followed by Bonferroni post-hoc test of control vs. each treatment and of each single vs. pair-wise stimulation within culture testing for treatment effect (neurons: †; co-cultures: *), respectively, 2-way ANOVA with Bonferroni post-hoc test of indicated groups testing if effect is dependent on microglia (#).

3.1.6 The endogenous ligand HSP60 causes neuronal death dependent on microglial TLR4 *in vitro*

Previously, it was demonstrated that HSP60 activates the TLR4 pathway in microglia resulting in neuronal injury *in vitro* (Lehnardt et al., 2008). The CNS cells used for the experiments were isolated from mice of the C3H/HeJ strain, in which TLR4 is expressed but non-functional due to a naturally occurring point mutation (Watson and Riblet, 1974). To verify that HSP60 requires the presence of TLR4 to elicit microglia-dependent neuronal death, I repeated the co-culture experiments using a mouse strain deficient for TLR4 (TLR4$^{-/-}$). Neurons were supplemented with wild-type or TLR4$^{-/-}$ microglia and incubated with HSP60 for 48 h. LPS served as an established TLR4-specific control and markedly reduced neuronal survival in a microglial TLR4-dependent manner (Fig. 3.6a,b). In co-cultures with wild-type microglia a significant loss of 25% NeuN-positive cells compared

with the control occurred after HSP60 stimulation (Fig. 3.6b). Neurons co-cultured with TLR4 $^{-/-}$ microglia were protected from HSP60-mediated neuronal death (Fig. 3.6b). These results verify that HSP60 induces neuronal death via microglial TLR4.

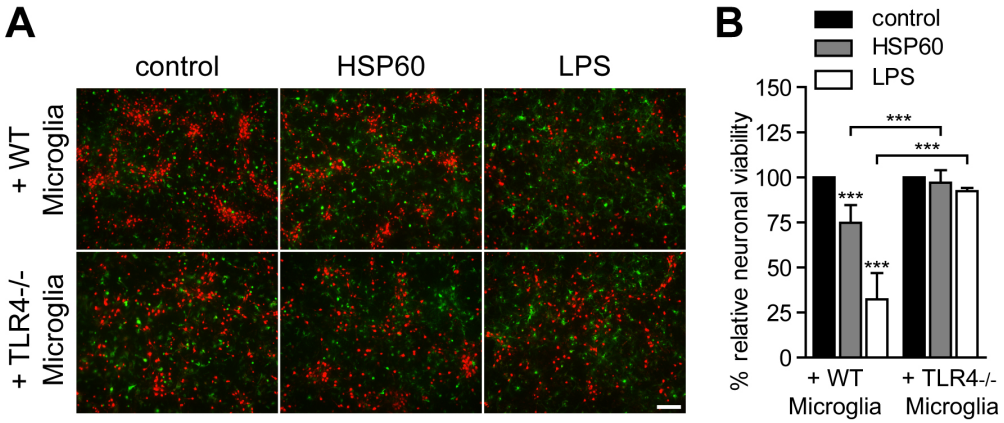

Figure 3.6: HSP60-induced neuronal loss is dependent on microglial TLR4 *in vitro*. Purified neurons and neurons supplemented with microglia derived from wild-type (WT) or TLR4 $^{-/-}$ mice were incubated with 10 µg/ml HSP60 or 10 µg/ml LPS, as indicated. After 48 h cell cultures were fixed and stained with NeuN antibody (neurons; red) and with Isolectin IB4 (IB4, microglia; green). **(A)** Representative images of cultures at 48 h, scale bar 100 µm **(B)** Quantification of NeuN-positive cells, mean ± SD of 3-4 individual experiments with each condition performed in duplicate, ANOVA followed by Bonferroni post-hoc test of each treatment vs. the respective co-culture control, 2-way ANOVA with Bonferroni post-hoc test of indicated groups testing if effect is dependent on microglial TLR4.

3.1.7 Combined activation of TLR4 with HSP60 and TLR7 with Loxoribine enhances neuronal death *in vitro*

Next, the impact of simultaneous stimulation of TLRs on neuronal survival was determined using combinations of the endogenous TLR4 ligand HSP60 together with the four exogenous TLR ligands. Therefore, co-cultures of neurons supplemented with wild-type microglia were stimulated with HSP60 (TLR4), Pam3CSK4 (TLR2), LPS (TLR4), Loxoribine (TLR7) or CpG ODN (TLR9) alone or in simultaneous pair-wise combination, as indicated, and after 72 h surviving neurons were quantified. In co-cultures the combined activation of TLR4 and TLR7 with HSP60 and Loxoribine led to a significant increase in neuronal death by approximately 10% more compared with each single application (Fig. 3.7a). Co-cultures incubated with both TLR4 agonists HSP60 and LPS showed a slight trend of more microglia-dependent neuronal decay in comparison to single stimulus, which did not reach statistical significance (Fig. 3.7a). Compared with single ligand challenge enhanced toxicity was absent when challenging co-cultures with the ligand combinations HSP60 and Pam3CSK4, and HSP60 and CpG ODN for TLR4 and TLR2, and TLR4 and TLR9, respectively (Fig. 3.7a).

Remarkably, in comparison with single ligand application, challenge with HSP60 and Loxoribine amplified neuronal death in co-cultures (Fig. 3.7a), whereas in the previous experiments the combination of LPS and Loxoribine did not (Fig. 3.5), although in both cases

TLR4 and TLR7 were activated. To rule out, that the enhanced toxicity of HSP60 and Loxoribine combination was based on the 10-fold higher HSP60 concentration, that of LPS was adjusted. However, also in this control experiment simultaneous LPS and Loxoribine exposure did not result in an increase of neuronal death compared to single challenge (Fig. 3.7b).

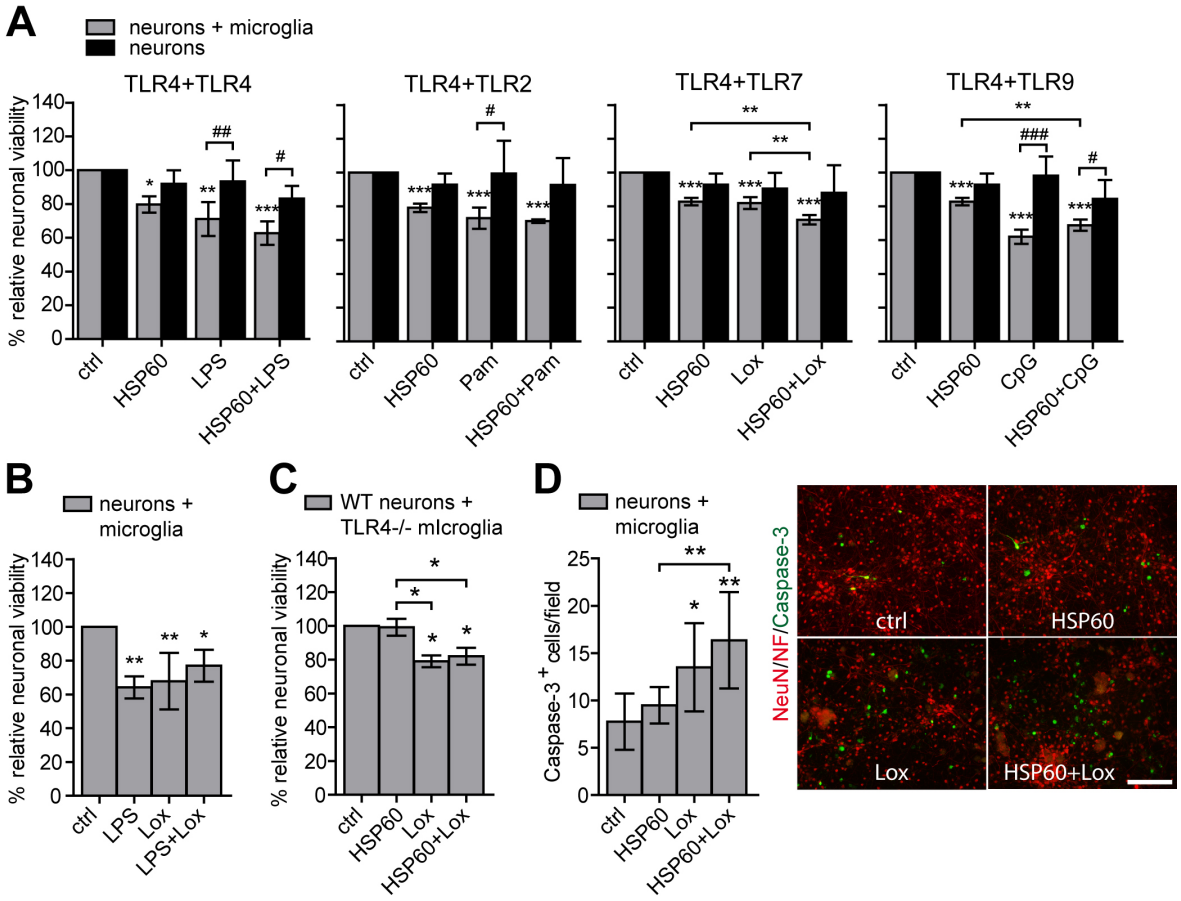

Figure 3.7: Simultaneous challenge of TLR4 and TLR7 with the endogenous ligand HSP60 and exogenous ligand Loxoribine causes increased neuronal loss. Neurons were cultured alone or supplemented with wild-type **(A, B, D)** or TLR4$^{-/-}$ **(C)** microglia and stimulated with **(A)** 1 µg/ml HSP60, 100 ng/ml LPS, 100 ng/ml Pam3CSK4 (Pam), 1 mM Loxoribine (Lox) and 0,1 µM CpG ODN (CpG), **(B)** 1 µg/ml LPS and 1 mM Loxoribine (Lox) or **(C, D)** 1 µg/ml HSP60 and 1 mM Loxoribine (Lox), in single or pair-wise application, as indicated. **(A, B, C)** After 72 h NeuN-positive cells were quantified, mean ± SD from **(A)** 3-4, **(B)** 4 or **(C)** 2 individual experiments with each condition performed in duplicate and averaged, ANOVA followed by Bonferroni post-hoc test of control vs. each treatment and each single vs. pair-wise stimulation within co-culture testing for treatment effect (*) and 2-way ANOVA with Bonferroni post-hoc test of indicated groups testing if effect is dependent on microglia (#) **(D)** After 24 h Caspase-3-positive cells (green with co-staining for NeuN and neurofilament (NF) in red) were quantified with each condition performed in duplicate and averaged, mean ± SEM from 4 individual experiments with ANOVA followed by Bonferroni post-hoc test between control and each treatment and each single to pair-wise stimulation.

To verify that HSP60 contributes to the elevated neuronal death in co-cultures exposed to HSP60 and Loxoribine via activation of microglial TLR4, neurons were co-cultured with microglia from TLR4-deficient mice. The HSP60-induced effect in co-cultures treated with

HSP60 alone as well as with HSP60 and Loxoribine was completely abolished (Fig. 3.7c).

To identify if HSP60 and Loxoribine-mediated increased neuronal death is based on enhanced apoptosis, co-cultures were immunostained 12 h, 24 h and 36 h after stimulation with an antibody against active Caspase-3, a central effector protein in apoptosis. Quantification of active Caspase-3-positive cells revealed that significantly more apoptotic cells were present in Loxoribine-treated co-cultures compared with the control condition (Fig. 3.7d). Exposing co-cultures to HSP60 and Loxoribine together resulted in a sub additive increase in active Caspase-3-positive cells after 24 h in comparison with single ligand application, which did not reach statistical significance (Fig. 3.7d).

In summary, *in vitro*, activation of TLR2, TLR4, TLR7 and TLR9 with their specific exogenous ligands resulted in the microglia-mediated release of inflammatory molecules. Moreover, combined TLR stimulation of TLR4 with TLR2 and TLR4 with TLR9 resulted in an increased release of TNF-α, IL-6, IL-10 and nitric oxide compared to activation of the respective single TLR *in vitro*. In contrast, additional activation of TLR7 suppressed the inflammatory response mediated by TLR2, TLR4, or TLR9, indicating that TLRs individually modulate the immune response. Further, neuronal injury occurred in response to activation of each TLR, whereas only the combination of TLR4 with TLR2 resulted in significantly more neuronal death compared to activation of each TLR alone. Remarkably, when replacing LPS by HSP60, combined activation of TLR4 with TLR7 resulted in an increased loss of neurons compared to single TLR activation. These results point to a complex fine-tuning in TLR-signaling.

3.2 Results (II) – The impact of single and multiple Toll-like receptor activation on the CNS *in vivo*

This part focuses on the relevance of single and combined TLR-stimulation on the CNS *in vivo*. Intrathecal injection is an established model allowing application of TLR ligands to the CNS without exposing and risking damage of the brain (Hoffmann et al., 2007; Lehmann et al., 2012a). Under anesthesia the TLR ligands are injected into the lumbar spinal canal and are delivered to the brain via the cerebrospinal fluid. It has been demonstrated that injection of the TLR2 ligand Pam3CSK4 elicits typical signs of bacterial meningitis in mice, such as infiltration of leukocytes, an increased intracranial pressure and apoptosis of neurons in the dentate gyrus, in a TLR2-dependent manner (Hoffmann et al., 2007). In this present study, the model of intrathecal injection was used to investigate the impact of specific TLR activation in the CNS on neuronal survival. Further, signs of a TLR-induced inflammatory response such as changes in glial cell morphology and in transcription and/or secretion of inflammatory molecules and enzymes in brain tissue were evaluated. Immunhistological

studies are mainly concentrated on the cerebral cortex and corpus callosum as these are areas in the brain, in which commonly features related to CNS pathologies, such as stroke and Alzheimer's disease, are found (personal communication Prof. Dr. Lehnardt).

3.2.1 Relative expression of TLR mRNA in the brain is increased by TLR activation with exogenous ligands

Although TLRs recognize each a different class of pattern-associated molecules, the response they initiate is redundant to a certain extent (Netea et al., 2004). For example, TLR2 is typically classified as a gram-positive bacteria-recognizing receptor as it engages their conserved structural motifs such as the cell wall product peptidoglycan of *S. aureus* (Yoshimura et al., 1999). However, although in response to peptidoglycan alone the expression of inflammatory molecules was impaired in microglia deficient for TLR2, as expected, the response to intact *S. aureus* was qualitatively similar to that of wild-type microglia (Kielian et al., 2005). This indicates that pathogens exhibit several ligands capable of engaging different TLRs, which can substitute for one another. Further, a study on the expression of TLRs in the CNS after challenge with gram-positive bacteria, gram-negative bacteria or a DNA-virus has revealed that not only the TLR thought to recognize the respective pathogen is transcriptionally regulated (Böttcher et al., 2003). Therefore, the influence of a distinct TLR on its own transcription and that of the other TLRs including the central adptor molecule MyD88 in the CNS was investigated.

Mice received an intrathecal injection of the exogenous TLR agonists Pam3CSK4 (TLR2), LPS (TLR4), Imiquimod (TLR7) or CpG-ODN (TLR9) or the carrier (control) and after 12 h whole brain homogenates were analysed for relative mRNA expression levels of TLRs and TLR-associated signaling molecules using commercially available real-time PCR assays.

In the brains of control mice the mRNA expression levels of each TLR differed from one another (Fig. 3.8). In detail, the expression level of TLR3 mRNA was the highest followed by that of TLR5 mRNA. Further, intermediate levels of mRNA for TLR2, TLR6, TLR7, TLR9 and MyD88 and relatively low mRNA levels of TLR1 and TLR8 were present. In the brains of most of the control mice mRNA for TLR4 was nearly undetectable. Interestingly, while intrathecal injection of Pam3CSK4 (TLR2) and CpG ODN (TLR9) resulted in an up-regulation of their respective receptor, injection of LPS (TLR4) or Imiquimod (TLR7) did not influence transcription levels of their engaged TLRs (Fig. 3.8). Further, except for TLR3, all TLR mRNA levels, which were regulated, displayed an up-regulation compared with the control condition (Fig. 3.8). In detail, activation of TLR2 by intrathecal injection of Pam3CSK4 resulted in the up-regulation of TLR1, TLR2 and TLR8 mRNA, while activation of TLR4 by LPS injection led to an increased expression of only TLR1 and TLR2. Injection of the TLR7 ligand Imiquimod influenced mRNA transcription levels of the nucleic acid-

sensing TLRs TLR3, TLR8 and TLR9. While the TLR3 mRNA level was decreased by 2-fold - notably the only down-regulation detected - transcription of TLR8 and TLR9 mRNA was elevated. Activation of TLR9 with CpG ODN induced the up-regulation of TLR1 and TLR2 as well as TLR8 and TLR9 mRNA. As mentioned above, levels of TLR4 mRNA in homogenates of control mice were below the reliable detection limit and they remained unchanged after intrathecal injection of any of the TLR ligands. Regulation of TLR5, TLR6, TLR7 and MyD88 mRNA was unaffected by challenge with any of the TLR ligands compared with the control condition.

In conclusion, TLR activation in the CNS influences the regulation of TLR mRNA expression. Thereby a distinct pattern of regulated TLR mRNAs is induced by different TLRs.

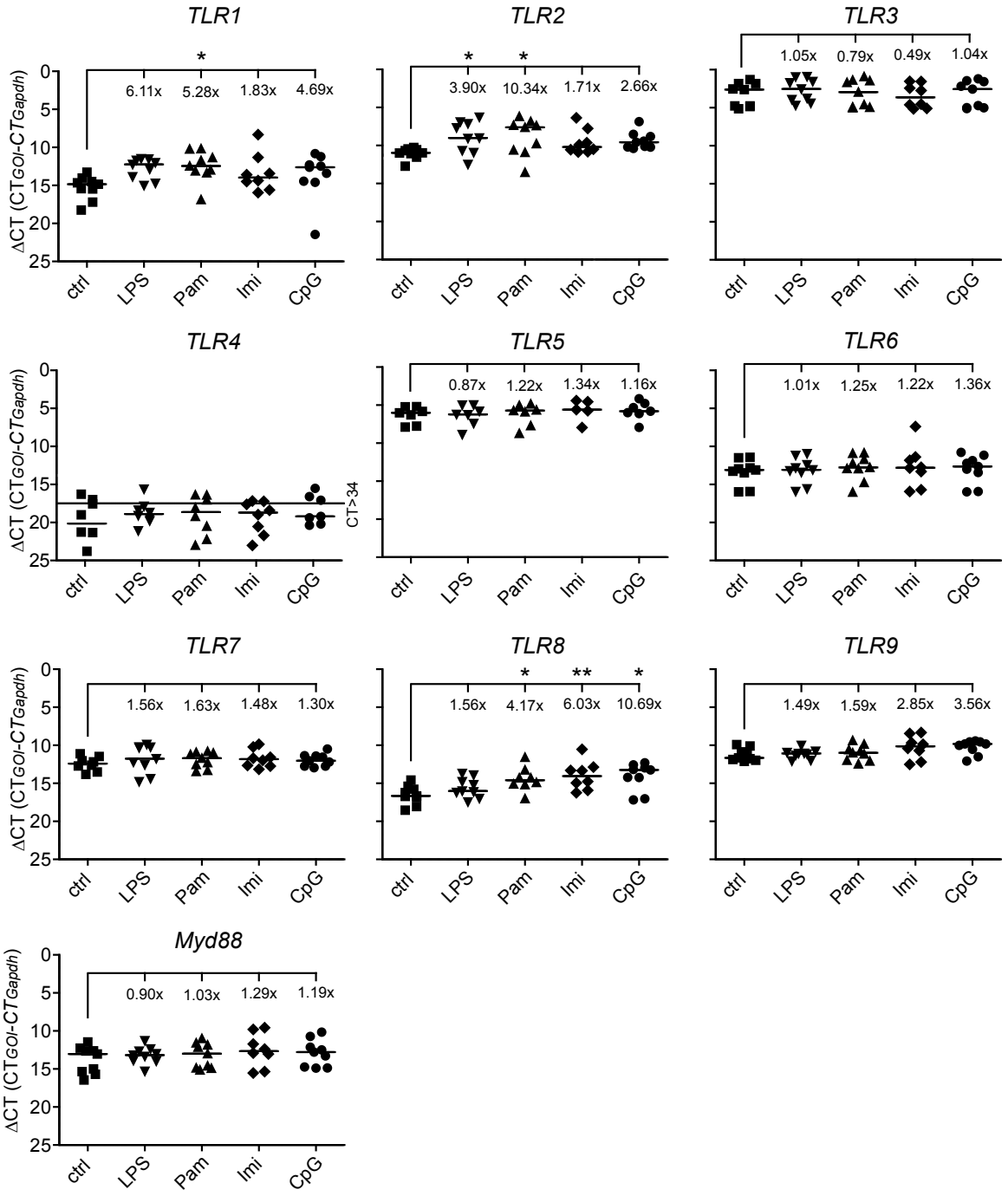

Figure 3.8: Regulation of relative mRNA levels of TLR1-9 and the central adaptor molecule MyD88 in the murine brain after intrathecal injection of specific exogenous TLR ligands. Mice received intrathecal injection of either 10 µg Pam3CSK4 (Pam, TLR2, n = 9), 10 µg LPS (TLR4, n = 9), 10 µg Imiquimod (Imi, TLR7, n = 8) or 10 µg CpG ODN (CpG, TLR9, n = 9) or carrier as a control (ctrl, n = 9) and were sacrificed after 12 h. Brain tissue homogenates were analysed for relative mRNA expression levels. Data are presented as delta cycle threshold (ΔCT=CT$_{geneofinterest(GOI)}$-CT$_{Gapdh}$) of each mouse with median per group on reverse scale to visualize increase/decrease of relative mRNA levels. Fold change (under brackets with values $\geq$2 x and $\leq$0.5 x expressing biological significance) was calculated using the 2^{-ddCT} method with the median of each group, setting the control to 1, ANOVA of log2 transformed ΔCT values followed by Bonferroni post-hoc test of control vs. treatment (stars above brackets indicating statistical significance). Fold increase for TLR4 mRNA is not displayed due to cycle threshold (CT) values greater 34 (data not reliable). It should be noted that statistical significance often does not reflect biological significance of a group due to the large variation of ΔCT values among the mice.

3.2.2 Activation of TLR2, TLR7 and TLR9 in the CNS with exogenous ligands causes neuronal and axonal damage *in vivo*

To assess the role of TLR stimulation on neuronal survival in the brain in vivo the four exogenous TLR-ligands Pam3CSK4 (TLR2), LPS (TLR4), loxoribine (TLR7) or CpG-ODN (TLR9) or the carrier (control) were injected intrathecally and after 3 days, the brains were sectioned and stained with antibodies against neuronal nuclei (NeuN, neurons) and neurofilament (NF, axons). Evaluation of NeuN-positive cells (verified by subsequent quantification) and NF-positive fibers in the cerebral cortex revealed a robust loss of neurons and axons after activation of TLR2, TLR7 and TLR9 (Fig. 3.9a,b). In detail, compared with control mice, exposure of the CNS to the TLR2 ligand Pam3CSK4 resulted in a loss of 12.1% NeuN-positive cells (Fig. 3.9b). In addition, neurons demonstrating pyknosis (cell shrinkage), an indicator of apoptosis were observed (Fig. 3.9a upper panel). Further, the amount of NF-positive fibers was greatly diminished in the cerebral cortex of 9 out of 11 mice (Fig. 3.9a lower panel). Upon Loxoribine-induced activation of TLR7 numbers of cortical neurons were reduced by 11.9% compared with control mice (Fig. 3.9b) and 7 out of 9 mice displayed signs (fragmentation and loss) of severed axons (Fig. 3.9a lower panel). A considerable loss of 17% NeuN-positive cells was found upon challenge with the TLR9 agonist CpG ODN compared with control mice (Fig. 3.9b). Further, signs of axonal injury were apparent in the cerebral cortex of 7 out of 10 CpG ODN treated mice (Fig. 3.9a lower panel). After activation of TLR4 with LPS neuronal numbers were decreased slightly by 5.2% (not significant) compared to control mice (Fig. 3.9b) and minor signs of axonal injury were present in 8 out of 10 mice compared with the control mice (Fig. 3.9a lower panel).

As mentioned above upon challenge with Pam3CSK4 (TLR2) NeuN-positive cells displayed pyknosis (Fig. 3.9a upper panel). To identify if apoptosis contributes to cell death in the cerebral cortex after TLR activation, the brain sections of all groups were stained by TUNEL (detection of a late-stage of apoptosis). Mice having received Pam3CSK4 (TLR2) injection had 4-fold more TUNEL-positive cells in the cerebral cortex compared with control mice (Fig. 3.9c). Interestingly, injection of LPS (TLR4) caused an increase of TUNEL-positive cells by 3-fold (Fig. 3.9c). The amount of TUNEL-positive cells in the cerebral cortex of mice challenge with Loxoribine or CpG ODN did not differ from control (Fig. 3.9c).

In summary, these data imply that activation of TLR2, TLR7 and TLR9 in the brain has detrimental consequences in the CNS affecting neuronal cell survival and integrity of axons.

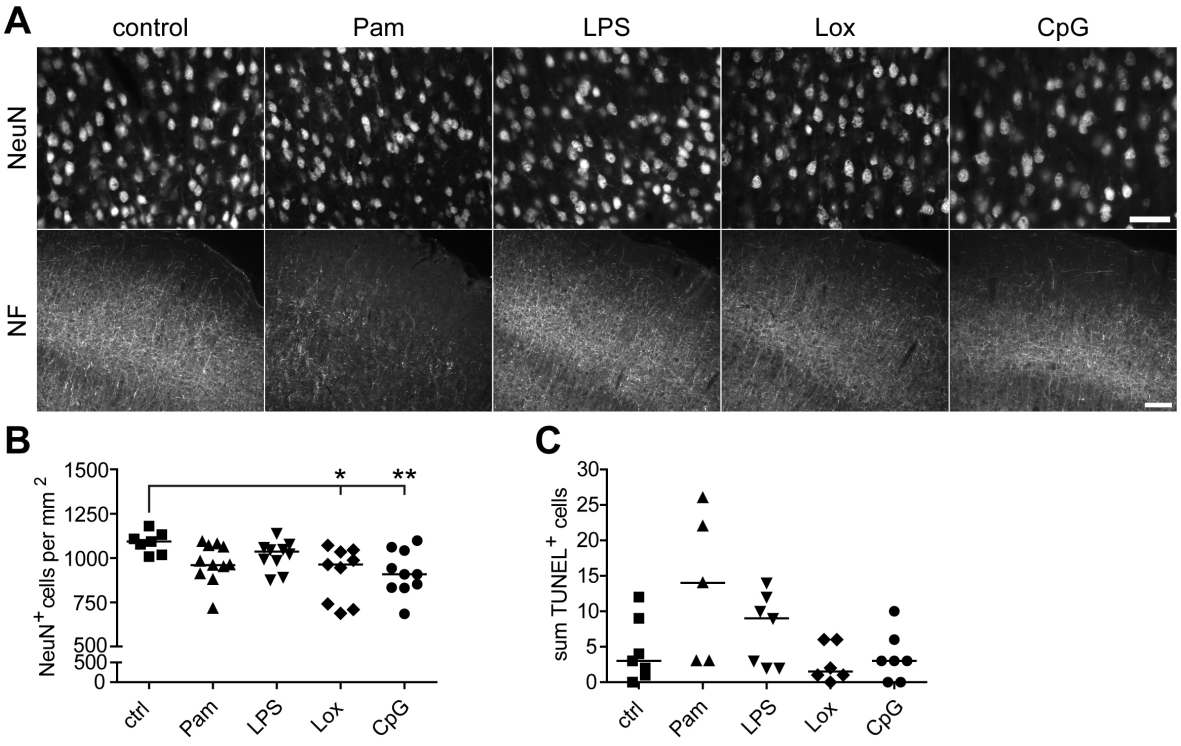

Figure 3.9: Activation of TLRs by intrathecal injection of specific exogenous TLR ligands provokes neuronal injury in the cerebral cortex. Activation of TLR2, TLR7 or TLR9 causes profound loss of neurons and axons. Mice received intrathecal injection of either 40 µg Pam3CSK4 (Pam, TLR2, n = 11), 10 µg LPS (TLR4, n = 10), 136 µg Loxoribine (Lox, TLR7, n = 9) or 10 µg CpG ODN (CpG, TLR9, n = 10) or carrier as a control (ctrl, n = 7) and were sacrificed after 3 d. **(A)** Representative images of the cerebral cortex stained for neuronal nuclei (NeuN, neurons) and neurofilament (NF, axons), scale bars 100 µm, inset 50 µm. Quantification of **(B)** NeuN-positive cells and **(C)** TUNEL-positive cells in the cerebral cortex; median of group with Kruskal-Wallis followed by Bonferroni post-hoc test of control vs. treatment.

3.2.3 Challenge with exogenous TLR ligands *in vivo* elicits a pro-inflammatory response in the CNS

To determine the inflammatory properties of TLR activation in the CNS in vivo the morphology of microglia in the cerebral cortex, the regulation of mRNA levels of inflammatory molecules and the influx of leukocytes into the CSF was evaluated. To this end, brain sections (3 d after injection; see 3.2.2) were probed with an antibody against the microglial marker ionized calcium adaptor binding molecule 1 (Iba1) and relative mRNA levels in brain tissue homogenates (12 h after injection; see 3.2.1) were determined by real-time PCR. Also, the number of leukocytes in the CSF (12 h after injection; see 3.2.1) was quantified in a Rosenthal cell chamber.

Resting microglia in the parenchyma of the healthy brain exhibit a small cell body and their processes are fine with complex branching, which is visible in the cerebral cortex of the control mice (Fig. 3.10a). In the cerebral cortex of mice challenged by intrathecal injection of Pam3CSK4, thereby activating TLR2, Iba1-positive microglia displayed a pronounced activated (amoeboid) morphology as apparent by retracted short, thick processes

(Fig. 3.10a). Like-wise, although to a lower degree, the processes of Iba1-positive microglia exposed to intrathecal LPS (TLR4), Loxoribine (TLR7) or CpG ODN (TLR9) appeared thicker and less complex and therefore more prominent compared with those in the control brains (Fig. 3.10a).

Upon TLR-induced activation of microglia *in vitro* several inflammatory molecules are up-regulated (Olson and Miller, 2004; Bsibsi et al., 2002). Therefore, the expression levels of mRNA for the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 and the nitric oxide-producing enzyme iNOS as well as the anti-inflammatory cytokines IL-4 and IL-10 were determined by real-time PCR. Intrathecal injection of Pam3CSK4, LPS, and CpG ODN, thereby activating TLR2, TLR4 and TLR9, respectively, led to an up-regulation of TNF-α and IL-1β mRNA relative to control (Fig. 3.10b). Stimulation of TLR7 by Imiquimod did not influence the expression of these pro-inflammatory cytokines (Fig. 3.10b).

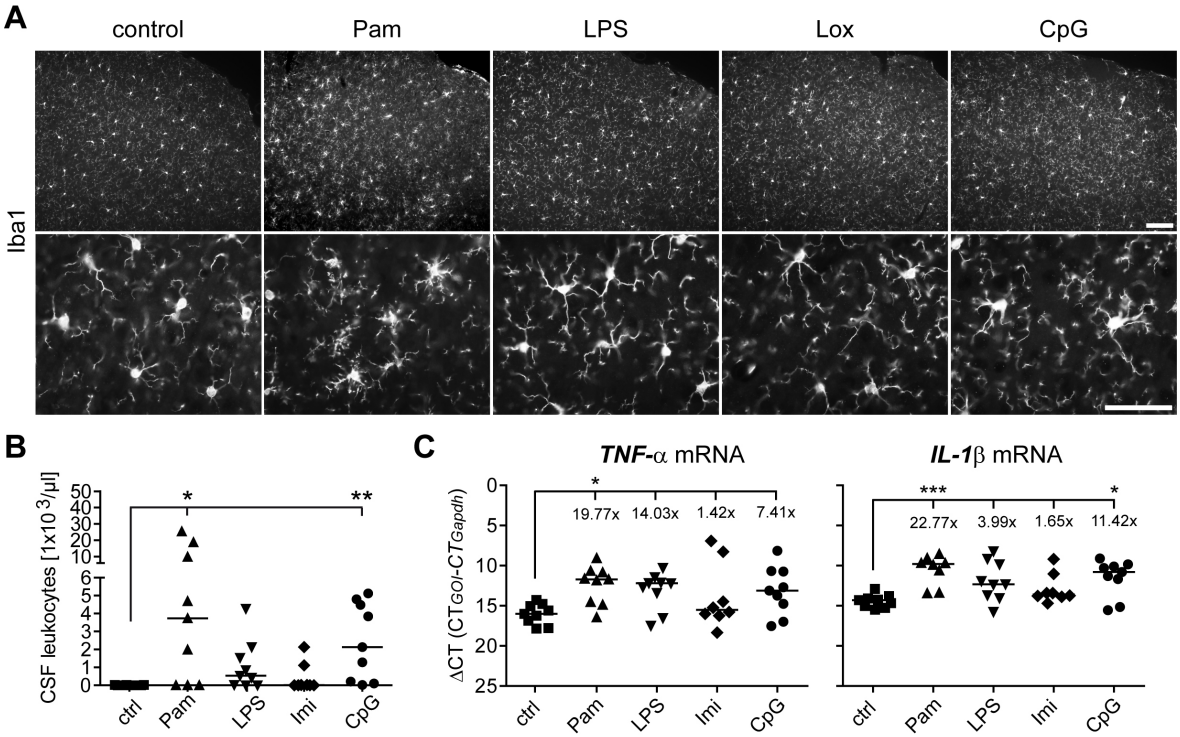

Figure 3.10: Challenge with exogenous TLR ligands *in vivo* elicits an inflammatory response in the CNS. (A) Representative images of the cerebral cortex stained for ionized calcium adaptor binding molecule 1 (Iba1, microglia) from mice 3 d after receiving intrathecal injection of 40 µg Pam3CSK4 (Pam; n = 11), 10 µg LPS (n = 10), 136 µg Loxoribine (Lox; n = 9) or 10 µg CpG ODN (CpG; n = 10) or carrier (control [ctrl], n = 7); scale bars 100 µm, higher magnification 50 µm. **(B)** Relative mRNA expression levels of pro-inflammatory cytokines in brain tissue and **(C)** quantification of infiltrating leukocytes in the cerebrospinal fluid (CSF) of mice 12 h after intrathecal injection of one of the four exogenous TLR ligands (10 µg Pam3CSK4 [Pam], n = 9; 10 µg LPS, n = 9; 10 µg Imiquimod [Imi], n = 8 or 10 µg CpG ODN [CpG], n = 9) or carrier (control [ctrl], n = 9). **(B)** Levels of mRNA were determined by real-time PCR, Data are presented as ΔCT per animal with median of group; ANOVA of log2 transformed ΔCT values followed by Bonferroni post-hoc test of control vs. treatment **(C)** CSF was obtained from the cisterna magna and samples containing blood were excluded from analysis. Median with Kruskal-Wallis followed by Dunn's multiple comparison test of control vs. treatment.

Infiltration of peripheral macrophages and leukocytes is restricted in the brain until they are attracted by signals generated in response to infection or injury (Ransohoff et al., 2003). Accordingly, leukocytes were not present in the CSF of control mice (Fig. 3.10c). Activation of TLR2, TLR4 and TLR9 by intrathecal injection of Pam3CSK4, LPS, and CpG ODN, respectively, led to an infiltration of peripheral immune cells into the CSF (Fig. 3.10c). The largest amount of leukocytes was found in the CSF of mice challenged with the TLR2 Ligand Pam3CSK4 (Fig. 3.10c). Activation of TLR7 in the CNS *in vivo* did not provoke recruitment of leukocytes (Fig. 3.10c).

These results point to a distinct inflammatory response induced by TLR2, TLR4, TLR7 and TLR9 in the CNS, in which cells of the intrinsic innate immune system and cells of the peripheral adaptive immune system participate.

3.2.4 The host-derived TLR4 agonist HSP60 induces neuronal injury in the CNS dependent on a functional TLR4 pathway *in vivo*

To study if the host-derived TLR4 agonist HSP60 is capable of inducing CNS damage *in vivo*, it was administered to mice by intrathecal injection. The protein serum albumin exhibits a similar molecular weight of 60 kDa as HSP60 does and served as a negative control. After 3 days, brains were sectioned and immunostained for markers of neurons (NeuN) and axons (NF) to study signs of neurodegeneration. Neuronal integrity was unaffected in brains of mice receiving intrathecal injection of serum albumin as compared to non-operated mice (naïve; Fig. 3.11b). Importantly, the cerebral cortex of HSP60-injected mice revealed a significant reduction of NeuN-positive cells (Fig. 3.11a). Subsequent quantification confirmed a significant loss of 10.1% NeuN-positive cells in comparison to the serum albumin-treated control group (Fig. 3.11b). Seven out of ten of the HSP60-treated wild-type mice showed a clearly distinguishable loss of axons in the corpus callosum (Fig. 3.11c) and cerebral cortex (data not shown) compared to control injected mice.

To test whether TLR4 is required for HSP60-induced neuronal cell death *in vivo*, TLR4 $^{-/-}$ mice were subjected to the same protocol. Subsequent immunohistochemical analysis revealed that neuronal numbers of the cerebral cortex did not differ between HSP60- and serum albumin-treated animals (Fig. 3.11b). Similarly, six out of seven TLR4 $^{-/-}$ mice did not display any major loss or injury of axonal structures after HSP60 injection compared to SA treated mice (Fig. 3.11c).

The adaptor molecule MyD88 is central in TLR signalling upon ligand engagement (Watters et al., 2007). A requirement for MyD88 in HSP60 induced neuronal cell loss has been shown *in vitro* (Lehnardt et al., 2008). To elucidate the role of MyD88 in HSP60-mediated CNS damage *in vivo*, experiments with intrathecal injection of HSP60 were repeated using MyD88-deficient (MyD88 $^{-/-}$) mice. Remarkably, naïve and serum albumin-

treated MyD88 $^{-/-}$ mice displayed more NeuN-positive cells in the cerebral cortex than in that of wild-type mice (Fig. 3.11a,b), suggesting a role in neurogenesis, as has been demonstrated for TLR2 and TLR4 (Rolls et al., 2007). HSP60-treated MyD88 $^{-/-}$ mice displayed a loss of 9.7% NeuN-positive cells compared with MyD88 $^{-/-}$ mice receiving the control protein serum albumin; however, the reduction did not reach statistical significance (Fig. 3.11b). In addition, axonal structures in the corpus callosum of MyD88 $^{-/-}$ mice injected with HSP60 remained intact as compared with the control groups of MyD88 $^{-/-}$ mice (Fig. 3.11c).

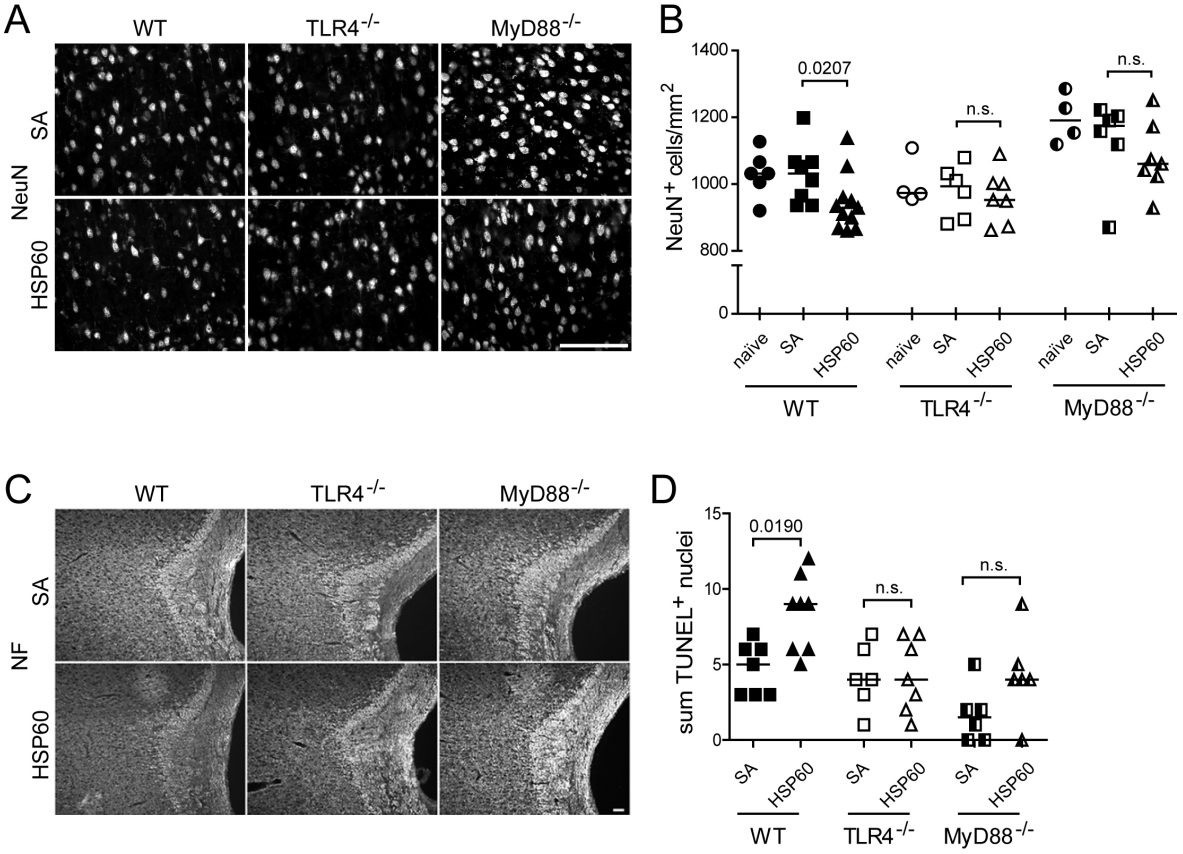

Figure 3.11: HSP60 causes TLR4-dependent and MyD88-involving CNS damage *in vivo*. Intrathecal injection of HSP60 results in a significant loss of neurons in the cerebral cortex and of axonal structures in the corpus callosum associated with apoptosis in wild-type, but not TLR4 $^{-/-}$ or MyD88 $^{-/-}$ mice. Wild-type (WT), TLR4 $^{-/-}$ and MyD88 $^{-/-}$ mice received intrathecal injection of 40 µg HSP60 or 40 µg serum albumin (SA) and were sacrificed after three days. Naïve mice were not subjected to surgery. The cerebral cortex and corpus callosum were evaluated for signs of neuronal and axonal loss. (WT/SA n = 8, WT/HSP60 n = 11, TLR4 $^{-/-}$/SA n = 6, TLR4 $^{-/-}$/HSP60 n = 7, MyD88 $^{-/-}$/SA n = 6, MyD88 $^{-/-}$/HSP60 n = 7) **(A)** Representative images and **(B)** quantification of neuronal nuclei (NeuN)-positive neurons in the cerebral cortex. **(B)** Median, Mann-Whitney-U-Test. **(C)** Representative images of the corpus callosum, visualized by immunostaining with neurofilament (NF) **(D)** Quantification of TUNEL-positive cells in the cerebral cortex. Median, Mann-Whitney-U-Test. Scale bars 100 µm

To analyse whether the observed injurious effects induced by HSP60 are associated with apoptosis in the CNS, brain sections were stained by TUNEL. Quantification of TUNEL-positive cells revealed a small, but significant increase of 1.8 times more apoptotic cells in the cerebral cortex of wild-type mice after HSP60 challenge compared to treatment with serum

albumin (Fig. 3.11d). In line with the results from quantification of neurons and evaluation of axonal integrity, numbers of apoptotic cells were not increased in the cerebral cortex of TLR4 $^{-/-}$ mice, but slightly, although not statistically significant, increased in MyD88 $^{-/-}$ mice compared to the respective serum albumin control (Fig. 3.11d). Taken together, the above results demonstrate that HSP60 mediates neuronal injury *in vivo* and imply an obligatory role of TLR4, and a partial involvement of the adaptor molecule MyD88.

3.2.5 Subsequent challenge with HSP60 does not amplify TLR-induced inflammation and neuronal damage

As demonstrated above activation of different TLRs in the CNS resulted in CNS tissue injury. It has been demonstrated that endogenous TLR ligands are liberated from injured cells (Yu et al., 2010). During CNS infection the host immune response considerably contributes to neuronal damage (Weber and Tuomanen, 2007) and after CNS insults injury-induced inflammation is involved in secondary tissue damage (Giulian and Vaca, 1993). Therefore, it was tested whether exposure to the endogenous TLR ligand HSP60 contributes to a previous TLR-induced insult. I hypothesized that additional activation of TLRs by their endogenous ligands fuels the TLR-response to exogenous ligands, thereby amplifying CNS damage.

Intrathecal injection of the exogenous TLR ligands Pam3CSK4 or Loxoribine, thereby activating TLR2 and TLR7, respectively, was followed by an intrathecal injection of HSP60 the next day. Water in which Pam3CSK4 and Loxoribine were dissolved and cell culture grade PBS in which HSP60 was dissolved, were injected as carrier controls. Mice were sacrificed after another 3 days and the brains were subjected to immunohistological analysis.

Quantification of NeuN-positive cells in the cerebral cortex of mice after sole activation of TLR2 with Pam3CSK4, TLR7 with Loxoribine or TLR4 with HSP60 revealed a loss of 4.4%, 5.7% and 9.3%, respectively, in comparison with control mice (Fig. 3.12a). Further, as compared with control mice combined injection of Pam3CSK4 and HSP60 or Loxoribine and HSP60 resulted in a reduction of NeuN-positive cells by 6.5% and 8.2%, respectively (Fig. 3.12b). However, subsequent injection of HSP60 to Pam3CSK4 or Loxoribine did not result in increased neuronal death compared with challenge to each TLR ligand alone (Fig. 3.12a,b). As axonal damage can precede to neuronal death (Raff et al., 2002), the cerebral cortex was examined for the integrity of NF-positive fibers. However, Pam3CSK4- and Loxoribine-induced axonal injury was not increased by the additional injection of HSP60 (Fig. 3.12c,d upper panel). Further, the amount and morphology of Iba1-positive microglia in the cerebral cortex of HSP60-treated mice resembled those in the control (Fig. 3.12c,d lower panel). Notably, in contrast to the previous *in vivo* experiment (Fig. 3.10a),

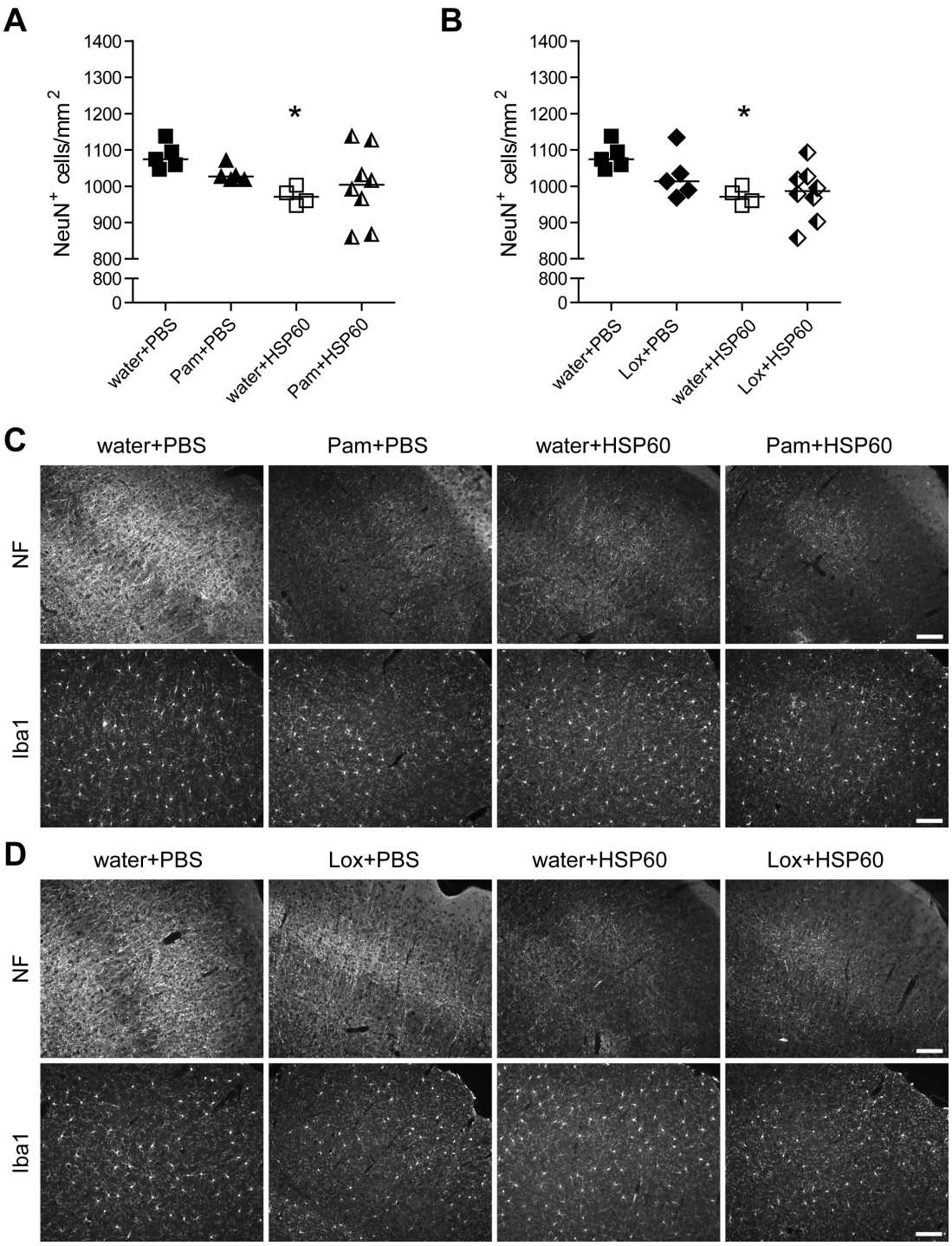

Figure 3.12: Subsequent challenge with HSP60 does not exacerbate TLR-induced CNS damage or inflammation in the cerebral cortex. Neuronal and axonal loss mediated by activation of TLR2 or TLR7 with exogenous agonists is not amplified by subsequent exposure to the endogenous TLR4 ligand HSP60. Mice received 2 subsequent intrathecal injections. On day one 40 µg Pam3CSK4 (Pam) or 136 µg Loxoribine (Lox) or carrier (H2O), were injected. On day two 40 µg HSP60 or carrier (PBS) were injected and all mice were sacrificed after another 3 days (H2O/PBS n = 7; H2O/HSP60 n = 4; Pam/PBS n = 5; Lox/PBS n = 5; Pam/HSP60 n = 8; Lox/HSP60 n = 8. **(A, B)** Neuronal nuclei (NeuN)-positive neurons were quantified in the cerebral cortex, median with Kruskal-Wallis followed by Bonferroni post-hoc test of control vs. treatment. **(C, D)** Representative images of the cerebral cortex stained for neurofilament (NF, axons/dendrites) and ionized calcium adaptor binding molecule 1 (Iba1, microglia) scalebars 100 µm, (inset 50 µm).

in the cerebral cortex of mice challenged with the TLR2 agonist Pam3CSK4 most of the microglia displayed a morphology resembling more a resting state (Fig. 3.12c,d lower panel). In addition, microglia appeared to have decreased in number. A similar situation was observed in the cerebral cortex of Pam3CSK4-HSP60-treated mice as well as Loxoribine-treated and Loxoribine-HSP60-treated mice (Fig. 3.12c,d lower panel).

These results demonstrate that CNS inflammation and damage initiated by activation of TLR2 and TLR7 is not exacerbated by the subsequent activation of TLR4 with the endogenous ligand HSP60 in this experimental set-up.

3.2.6 CNS injury caused by challenge with HSP60 includes demyelination dependent on TLR4 and MyD88

A former study revealed that activation of TLR4 by LPS causes oligodendrocyte injury *in vitro* and *in vivo* (Lehnardt et al., 2002). To evaluate if the endogenous ligand HSP60 has the capacity to induce injury of oligodendrocytes and/or loss of myelin *in vivo*, brain sections were probed with antibodies directed against adenomatous polyposis coli (APC) and myelin basic protein (MBP) as markers for oligodendrocytes and myelin, respectively. Wild-type mice challenged with HSP60 showed a major loss of oligodendrocytes in the cerebral cortex compared to mice treated with serum albumin (Fig. 3.13a). Quantification confirmed this observation with a loss of 65.3% APC-positive cells (Fig. 3.13b). Whereas the cerebral cortex of serum albumin-injected mice displayed a widespread distribution of MBP, in those of HSP60-injected mice a reduction in MBP immunoreactivity, a sign of demyelination, was observed (Fig. 3.13c). To determine the role of TLR4 and MyD88 in oligodendrocyte injury and in demyelination induced by HSP60 *in vivo*, brain sections were stained with the antibodies mentioned above. None of the TLR4$^{-/-}$ nor MyD88$^{-/-}$ mice that received HSP60 exhibited loss of oligodendrocytes or fragmentation of MBP-positive structures compared to the respective serum albumin control (Fig. 3.13a,c). Quantitative analysis confirmed that survival of APC-positive cells in the cerebral cortex of TLR4$^{-/-}$ and MyD88$^{-/-}$ mice was not compromised by injection of HSP60 (Fig. 3.13b). These data suggest that intrathecal HSP60 is toxic towards oligodendrocytes dependent on a functional TLR4 and MyD88 pathway *in vivo*.

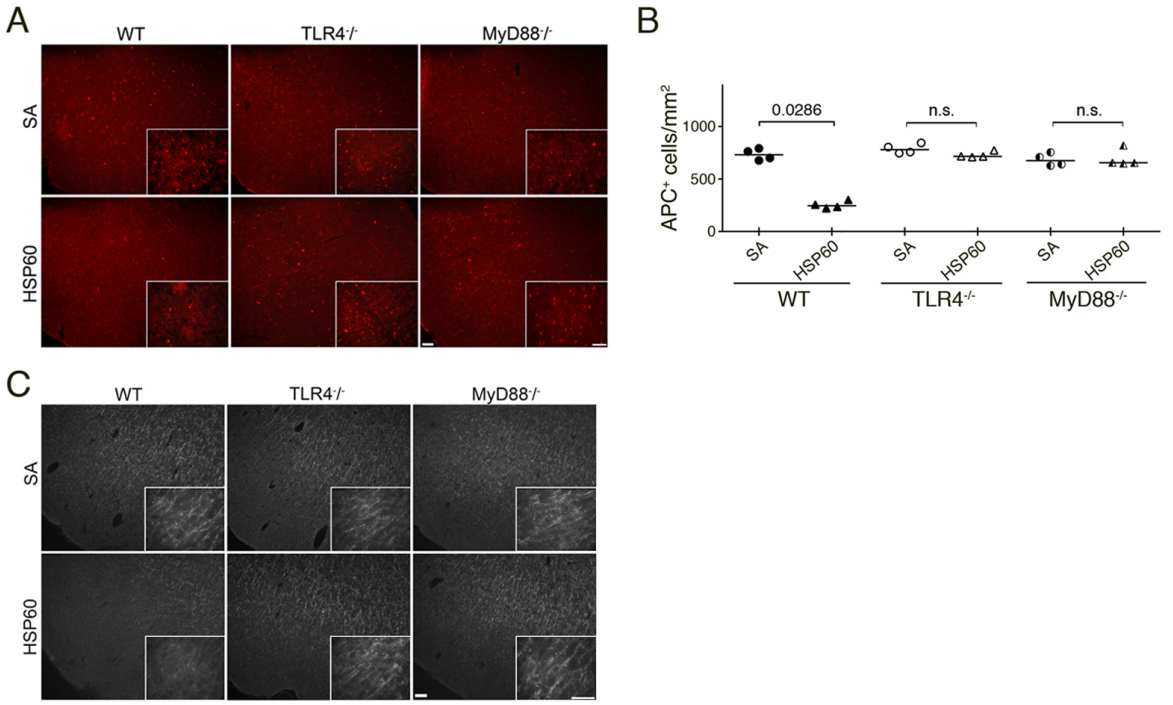

Figure 3.13: Challenge with HSP60 results in demyelination dependent on TLR4 and MyD88 *in vivo*. Three days after intrathecal injection of 40 µg HSP60 or 40 µg serum albumin (SA) coronal sections of wild-type (WT, HSP60 n = 4, SA n = 4), TLR4 $^{-/-}$ (HSP60 n = 4, SA n = 4) and MyD88 $^{-/-}$ (HSP60 n = 4, SA n = 4) mice were evaluated for the effect of HSP60 on myelination. **(A)** Representative images of the cerebral cortex of WT, TLR4 $^{-/-}$, and MyD88 $^{-/-}$ mice probed with an antibody directed against adenomatous polyposis coli (APC, oligodendrocytes). Scale bar 50 µm. **(B)** Quantification of APC-positive cells in the cerebral cortex of WT, TLR4 $^{-/-}$, and MyD88 $^{-/-}$ mice. Median, Mann-Whitney U test of indicated groups. n.s. not significant **(C)** Representative images of the cerebral cortex of WT, TLR4 $^{-/-}$, and MyD88 $^{-/-}$ mice stained for myelin basic protein (MBP, myelin). Scale bar 50 µm.

3.2.7 Glial cells of mice treated with HSP60 do not display major signs of activation

The inflammatory response generated by activated microglia was identified as a major cause of TLR4-induced neuronal injury *in vitro* (Lehnardt et al., 2003; Lehnardt et al., 2008). To examine if exposure to HSP60 results in activation of the innate immune cells of the CNS *in vivo*, brain sections from the HSP60- and serum albumin-treated wild-type mice were immunostained for microglia with an antibody directed against Iba1. As compared with serum albumin-treated mice signs of microglial activation such as shortening and thickening of processes and cell proliferation were absent in the cerebral cortex of HSP60-treated mice (Fig. 3.14a). Quantification of Iba1-positive cells in the cerebral cortex confirmed that the number of microglia was not explicitly higher after injection of HSP60 in comparison with serum albumin (Fig. 3.14a). Likewise, cells positive for CD11b, another marker for macrophages/microglia, displayed a similar morphology and number in the cerebral cortex of both groups (data not shown), additionally indicating that peripheral macrophages did not enter the CNS parenchyma. Astrocytes, another glial cell type of the CNS, also contribute

to various forms of brain injury (Raivich et al., 1996). To evaluate the role of astrocytes in HSP60-associated CNS damage *in vivo*, brain sections were probed with anti-glial fibrillary acidic protein (GFAP). However, activation of astrocytes, which can be characterized by enhanced intensity of the GFAP signal or proliferation and accumulation of GFAP-positive cells, was not observed in the cerebral cortex of mice challenged with HSP60 (Fig. 3.14b). Accordingly, numbers of GFAP-positive cells quantified, did not differ among HSP60- and serum albumin-treated groups (Fig. 3.14b).

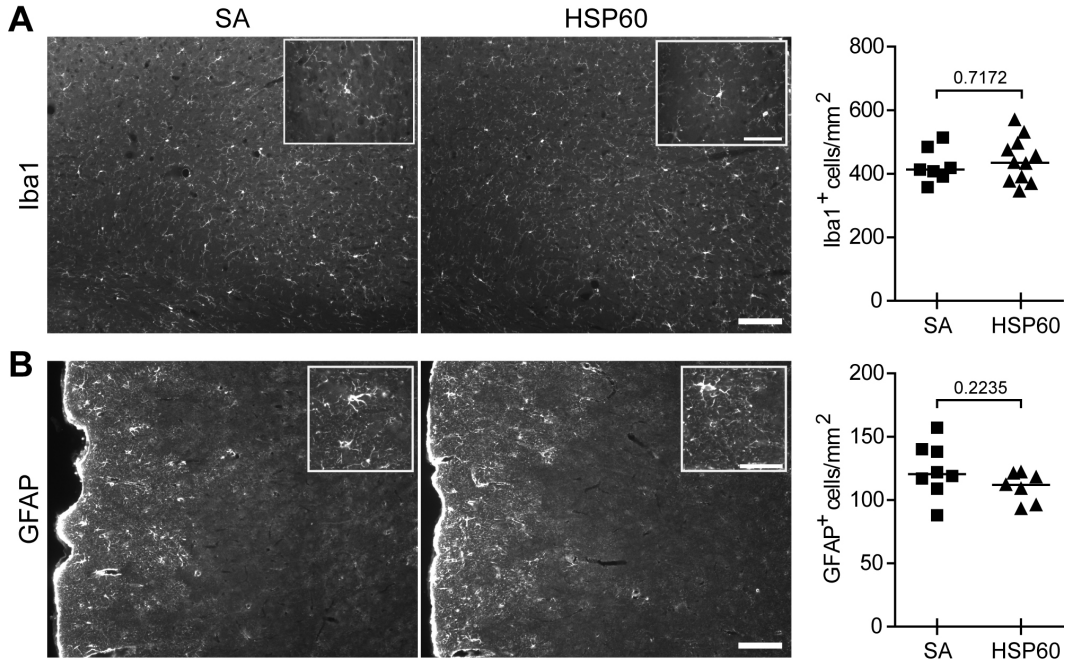

Figure 3.14: Brains challenged with HSP60 do not display major signs of glial activation *in vivo*. The cerebral cortex was evaluated for signs of microglial and astrocyte activation 3 d after injection of 40 µg HSP60 (n = 11) or 40 µg serum albumin (SA, n = 8). **(A)** Representative images and quantification of Iba1-positive microglia in the cerebral cortex of wild-type mice, median, Mann-Whitney-U-Test. **(B)** Representative images and quantification of GFAP-positive astrocytes in the cerebral cortex of wild-type mice, median, Mann-Whitney-U-Test. Scale bars 100 µm, insets 50 µm

3.2.8 Intrathecal injection of HSP60 does not result in release of cytokines or chemokines in the brain *in vivo*

Upon activation cells of the innate immune system respond by rapidly secreting immunostimulatory molecules. The question was therefore whether HSP60 triggers the release of inflammatory mediators in the brain. Therefore, wild-type mice received an intrathecal injection of HSP60 or serum albumin. Further, mice subjected to intrathecal injection of the exogenous TLR4 ligand LPS or sham surgery (insertion of the syringe but no injection) served as a positive and negative control, respectively. After 12 h lysates of whole brains were analysed with the flowcytometry-based multiplex assay and ELISA for the presence of pro-inflammatory cytokines and chemokines. Inflammatory molecules such as TNF-α,

GM-CSF, IFN-γ and IL-10 were not detected in brain homogenates of any of the investigated groups (data not shown). As expected, injection of the positive-control LPS induced a marked inflammatory response (Szczepanik and Ringheim, 2003), with elevated protein levels of typical inflammatory molecules such as IL-α, IL-1β, IL-6, CXCL1, CCL2, CCL3 and CCL5 compared with sham mice (Fig. 3.15). In contrast, activation of TLR4 with HSP60 did not induce an increased production of any of the inflammatory mediators as compared with the control groups (Fig. 3.15).

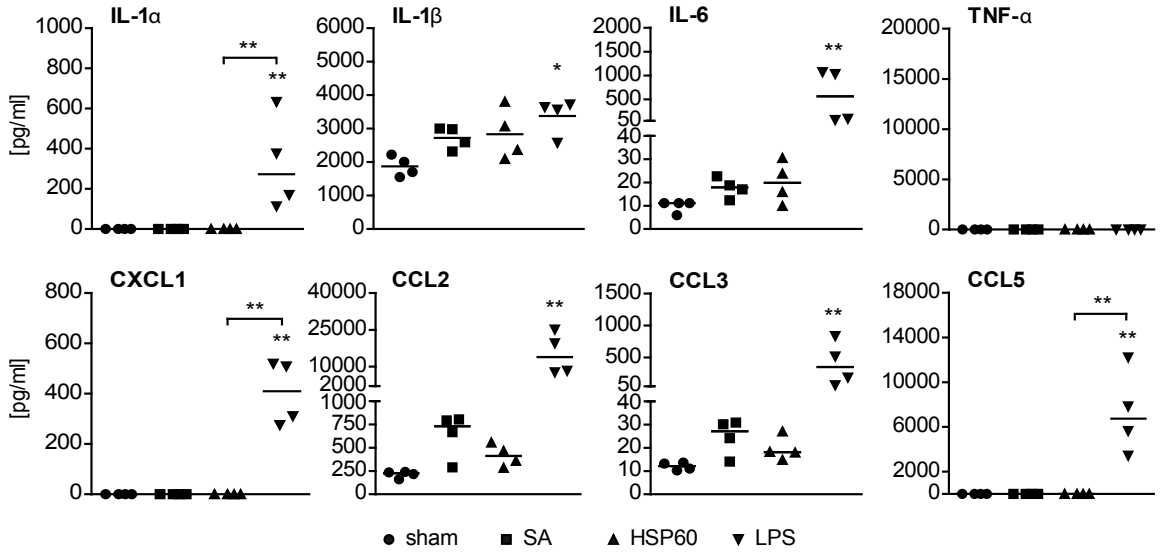

Figure 3.15: Stimulation of the CNS with HSP60 *in vivo* does not trigger a considerable inflammatory response. Wild-type mice were subjected to intrathecal injection of 40 µg HSP60, 40 µg serum albumin (SA) or 10 µg LPS or sham surgery. After 12 h brain lysates were analysed for protein levels of cytokines and chemokines by flowcytometry-based multiple analyte detection and IL-1β ELISA, median; Kruskal-Wallis followed by Dunn's selected pairs; SA- and LPS-treatment vs. sham and HSP60- vs. SA-treatment.

3.2.9 Nitric oxide and transcription levels of neurotoxic metabolite-producing enzymes are unchanged in the CNS in response to HSP60 challenge

Reactive oxygen species released during an inflammatory burst contribute to neuronal injury (Chao et al., 1992; Brosnan et al., 1994). In particular, upon activation of microglial TLR4 with HSP60, nitric oxide (NO) is released and acts neurotoxic *in vitro* (Lehnardt et al., 2008). Further, exposure of microglia to necrotic neurons induces the expression of the enzymes inducible nitric oxide synthase (iNOS), indoleamine 2,3-dioxygenase (Ido), cyclooxygenase-2 (Cox-2) and glutaminase (Gls), the latter participating in enhanced microglia-mediated neurotoxicity *in vitro* (Pais et al., 2008). These pro-inflammatory enzymes are involved in the generation of the potentially neurotoxic NO (iNOS), quinolic acid (Ido), and glutamate (Gls) and the inflammation-related and vasodilation-mediating prostaglandin (Cox-2). To further elucidate the inflammatory response to HSP60 *in vivo*,

brain lysates were analysed for mRNA expression of enzymes mentioned above and for content of NO. Injection of the positive-control LPS led to a 2.3-fold increase of Cox-2 mRNA and a 6.9-fold increase of iNOS mRNA (Fig. 3.16a), whereas NO itself was undetectable at this time point (Fig. 3.16b). Injection of the endogenous TLR4 ligand HSP60 did not cause any changes in expression of the investigated mRNAs or production of NO (Fig. 3.16a,b).

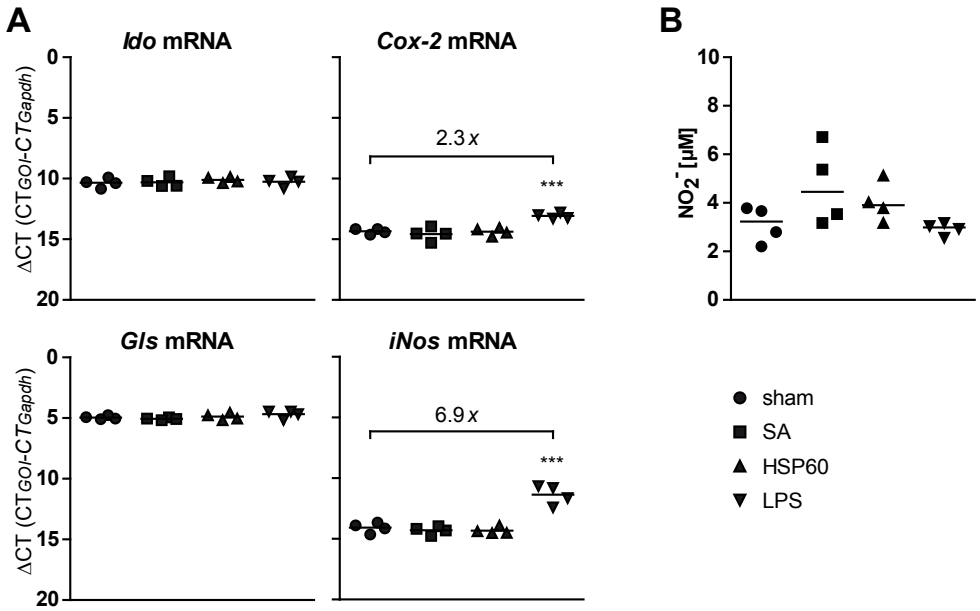

Figure 3.16: Expression of neurotoxic metabolite-producing enzymes and production of nitric oxide are not enhanced in brains challenged with HSP60 *in vivo*. Wild-type mice received intrathecal injection of 40 µg HSP60 (n = 4), 40 µg serum albumin (SA, n = 4) or 10 µg LPS (n = 4) or sham surgery (n = 4). After 12 h brain lysates were analysed for **(A)** relative mRNA expression levels of idolamine 2,3-dioxygenase (Ido), cyclooxygenase-2 (Cox-2), glutaminase (Gls) and inducible nitric oxide synthase (iNOS) and **(B)** content of nitrite (NO_2^-). **(A)** Levels of mRNA were determined by real-time PCR, ΔCT per animal with median of group; ANOVA of log2 transformation followed by Bonferroni selected pairs with SA- and LPS-treatment vs. sham and HSP60- vs. SA-treatment. Bars above groups indicate fold increase relative to sham calculated with medians. **(B)** Determination of nitrite levels by Griess' reaction; median; Kruskal-Wallis followed by Dunn's selected pairs with SA- and LPS-treatment vs. sham and HSP60- vs. SA-treatment.

3.2.10 Intrathecal injection of HSP60 does not change mRNA expression levels of cytokines and chemokines in the CNS

An obvious activation of glia was not observed 3 d after intrathecal injection of HSP60. Further, secretion of cytokines, chemokines and NO, and expression of mRNA from neurotoxic metabolite-producing enzymes was not detected by 12 h post HSP60 challenge. Therefore, the experiment was repeated with additional time points, and the fold change of various genes encoding molecules that are typically involved in inflammation-related CNS pathologies was evaluated (Ransohoff and Brown, 2012; Jaerve and Müller, 2012). To this end, wild-type mice were injected intrathecally with HSP60 or with serum albumin or LPS that each served as a negative control or positive control, respectively. After 6 h, 12 h and 3 d

whole brain lysates were analysed by real-time PCR. Intrathecal injection of the exogenous TLR4 ligand LPS resulted in a robust up-regulation of the cytokines TNF-α and IL-1β and the chemokines CCL2 and CCL5 at every time point tested (Fig. 3.17). In contrast, none of the tested pro-inflammatory genes were up-regulated after intrathecal injection of HSP60 compared to serum albumin at any time point observed (Fig. 3.17). However strikingly, 6 h after challenge with HSP60 the mRNA level of TGF-β was drastically down-regulated, which was not the case for LPS (Fig. 3.17). TGF-β is a growth factor and has neuroprotective properties as it can suppress microglial cytotoxicity (Merrill and Zimmerman, 1991) and induce synthesis of neurotrophins (Buchman et al., 1994).

In summary, *in vivo*, the impact of TLR activation in the murine brain was evaluated after intrathecal injection of the respective exogenous ligands for TLR2, TLR4, TLR7 and TLR9. Real-time PCR analysis revealed that activation of each TLR leads to a distinct change in the mRNA expression pattern of TLR1-9 in the murine brain. Furthermore, immunohistological analysis of brain sections revealed that activation of TLR2, TLR7 and TLR9 causes neuronal and axonal loss in the cerebral cortex. This was in part accompanied by a neuroinflammatory response as indicated by microglia displaying an activated morphology, by the influx of leukocytes into the cerebrospinal fluid and by elevated levels of TNF-α and IL-1β mRNA in brain homogenates. Furthermore, intrathecal injection of HSP60 resulted in injury and loss of neurons and oligodendrocytes in a TLR4-dependent and MyD88-involving manner. In contrast to the robust generation of inflammatory molecules induced by injection of the exogenous TLR4 ligand LPS, signs of HSP60-mediated activation of the immune system in the CNS were not measurable at the time points evaluated. However, early after exposure of the CNS to HSP60 a marked down-regulation of the neuroprotective growth factor TGF-β was detected. In conclusion, TLRs mediate neuroinflammation and -degeneration in the CNS.

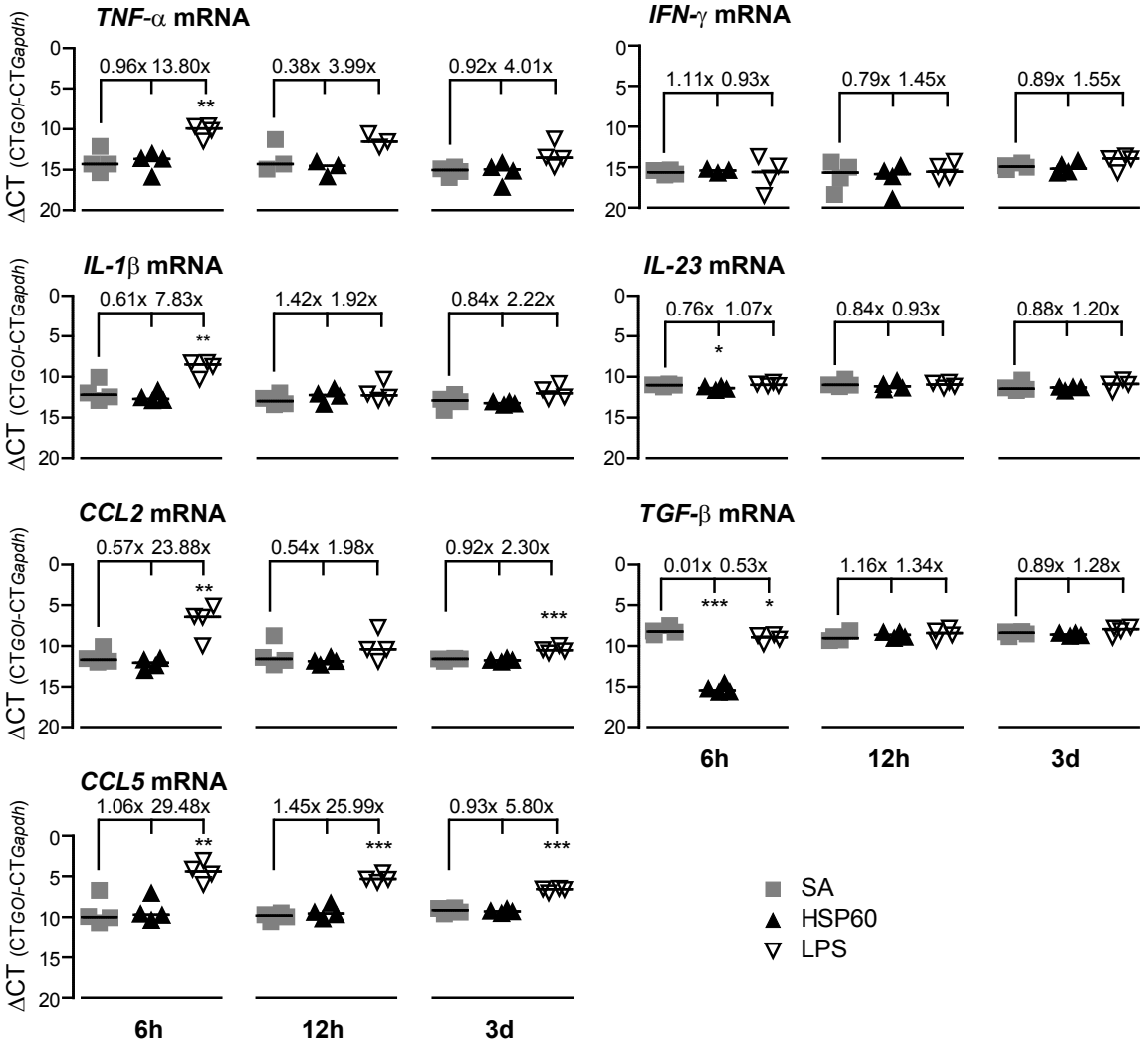

Figure 3.17: In contrast to LPS, HSP60 does not induce a considerable inflammatory response in the brain *in vivo*. Wild-type mice received intrathecal injection of 40 µg HSP60 (n = 4), 40 µg serum albumin (SA, n = 4), or 10 µg LPS (n = 4). After 6 h, 12 h, and 72 h brain lysates were analysed for the expression of various genes encoding pro-inflammatory molecules, as indicated, by real-time PCR. ΔCT per animal with median of group; ANOVA of log2 transformation followed by Bonferroni-selected pairs; versus SA for each time point. Bars above groups indicate fold increase calculated with the median.

3.3 Results (III) – The physiological relevance of the endogenous TLR4 ligand HSP60 in the CNS *in vivo*

When endogenous intracellular molecules such as HSP60 are present in the extracellular environment they are regarded as foreign and activate the innate immune system (Kono and Rock, 2008), which in turn likely is involved in causing secondary damage. The results above demonstrate that intrathecal injection of HSP60 is capable of compromising the survival of neurons and oligodendrocytes. To pinpoint HSP60 as a potential endogenous danger signal in the CNS, it was necessary to determine its presence in the CNS. Moreover,

the physiological event of cell damage was approached to determine whether HSP60 is exposed to the extracellular space and is therefore available to TLR4. To this end, mice were subjected to a middle cerebral artery occlusion (MCAo), which is a mouse model of cerebral ischemia and generates a defined brain injury. Sham-operated mice, which underwent all surgical procedures except induction of ischemia itself, served as a negative control.

3.3.1 HSP60 protein is located to neurons and increased after cerebral ischemia

To study the localisation of HSP60 in the healthy and injured CNS mice were subjected to MCAo, or sham-operated (performed by Odilo Engel at the Center for Stroke Research, Charité-Universitaetsmedizin Berlin) and after 1 d, 2 d, 4 d and 14 d the brains were removed for immunohistological analysis. Coronal sections were probed with an antibody directed against HSP60 and co-stained with markers for neurons (NeuN), microglia (Iba1), astrocytes (GFAP) and oligodendrocytes (APC). Although HSP60 is considered being expressed ubiquitously (Mayer, 2010), in the brains of sham-operated mice and in the contralateral intact hemisphere of mice subjected to MCAo, HSP60 was detected predominantly in neurons and located to the cytoplasma (Fig. 3.18a). However, some oligodendrocytes displayed a weak immunoreactivity of HSP60 (Fig. 3.18a). A signal for HSP60 was absent in microglia and astrocytes (Fig. 3.18a). Immunoreactivity for HSP60 was clearly enhanced 1 d after MCAo in the infarct area and surrounding lesion-associated region of stroked mice as compared with the contralateral hemisphere and with brains of sham-operated mice (Fig. 3.18a). Western blot analysis of pure neuronal cultures confirmed a robust expression of HSP60 in neurons (Fig. 3.18b). Moreover, after exposing neurons to the TLR7 ligand Imiquimod, thereby inducing cell-autonomous apoptosis (Lehmann et al., 2012b), the amount of HSP60 was clearly increased in comparison with untreated neurons (Fig. 3.18b). Consistent with the *in vivo* results described above, HSP60 was absent in cultured microglia. However a faint expression was observed after incubation with Imiquimod (Fig. 3.18b).

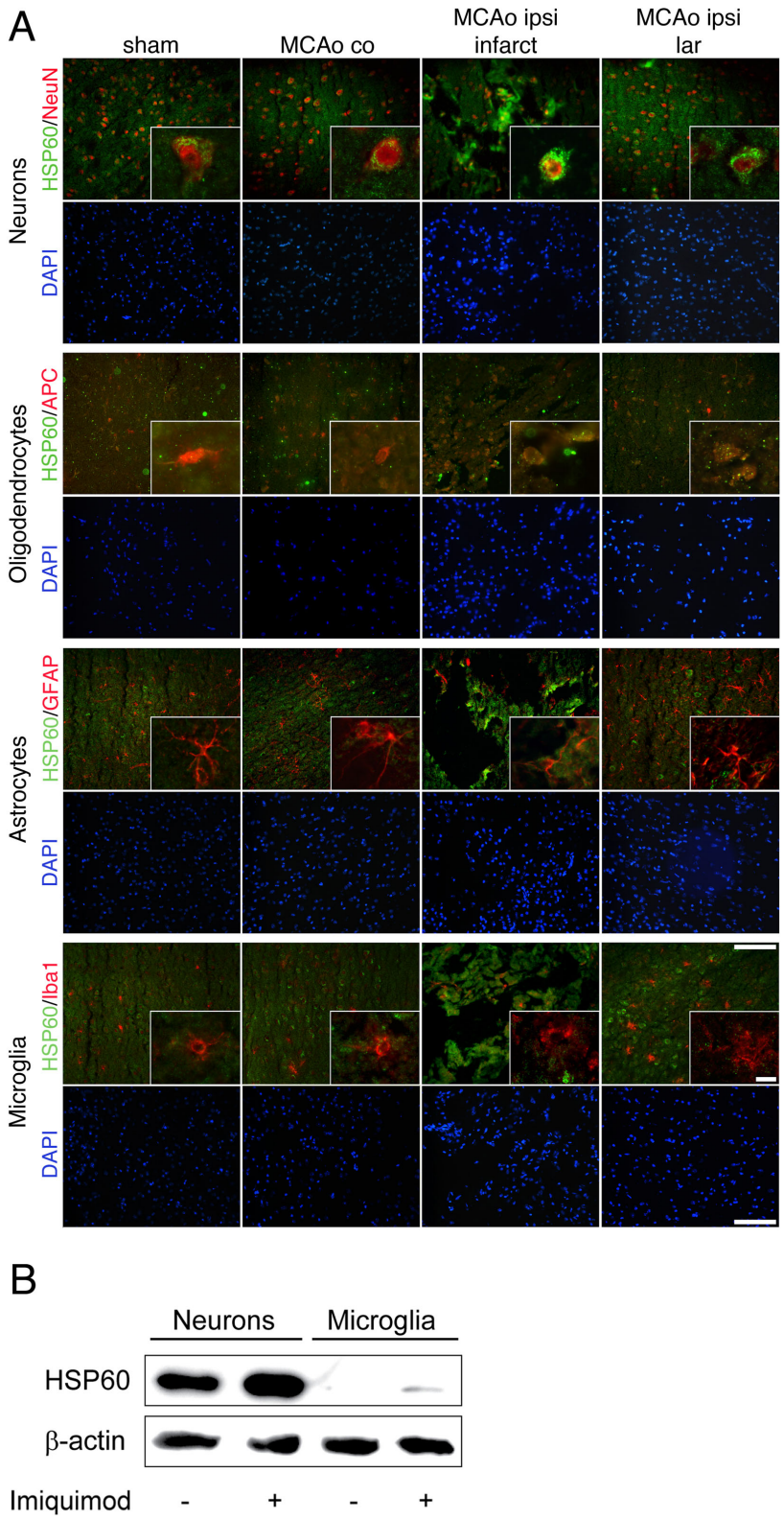

Figure 3.18: HSP60 is predominantly present in neurons of the CNS. (A) Immunofluorescence images of the contralateral (co) brain hemisphere, and of the ipsilateral (ipsi) brain hemisphere with the stroked area (infarct) and lesion-associated region (lar) of mice 1d after MCAo. Sham-operated animals served as a negative control. Tissues were double-stained with anti-HSP60 and with anti-NeuN, anti-APC, anti-Iba1 and anti-GFAP antibodies to mark neurons, oligodendrocytes, microglia and astrocytes, respectively. Nuclei were stained with DAPI. Scale bars, 50 µm. **(B)** Immunoblots of lysates from cultured neurons and microglia, both incubated with 10 µg/ml imiquimod or PBS (control) for 12 h or 3 h, respectively, probed with an antibody directed against HSP60. β-actin served as loading control.

3.3.2 Endogenous HSP60 is released in CNS injury

To determine whether endogenous HSP60 is released during injurious processes in the CNS *in vivo*, cerebrospinal fluid (CSF) obtained from the cisterna magna of the mice subjected to MCAo and sham surgery was analysed by ELISA. At 1 d after the ischemic insult, elevated levels of HSP60 were detected in the CSF of stroked mice compared with the CSF of control mice (Fig. 3.19); however, these data did not reach statistical significance.

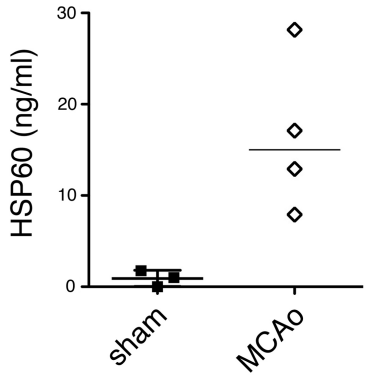

Figure 3.19: The amount of HSP60 is increased in the cerebrospinal fluid of stroked mice. Cerebrospinal fluid (CSF) from mice subjected to focal cerebral ischemia (1 d after MCAo, n = 4) and from control mice (1 d after sham surgery, n = 3) was assayed for HSP60 concentrations by ELISA. Median, Mann-Whitney u test, p = 0.0651.

In summary, endogenous HSP60 was detected in the CNS and was predominantly located in neurons. Upon CNS injury, HSP60 was markedly increased in neurons of the affected area. Moreover, elevated levels of HSP60 were detected in the CSF, suggesting its release by damaged neurons. These results underline that in principle endogenous HSP60 can serve as a danger signal to the innate immune cells of the brain, thereby triggering neurodegenerative processes.

Discussion

4.1 Distinct regulation of Toll-like receptor expression in the CNS upon specific Toll-like receptor activation

Toll-like receptors are constitutively expressed in the CNS. In addition, several studies have demonstrated that TLRs are dynamically and differentially regulated during CNS development (Kaul et al., 2012) and pathologies such as stroke (Lehnardt et al., 2007a; Caso et al., 2007), traumatic brain injury (Babcock et al., 2006; Zhang et al., 2012b), Morbus Alzheimer (Walter et al., 2007; Frank et al., 2009) and Multiple Sclerosis (Bsibsi et al., 2002; Prinz et al., 2006). Whereas the exact inducers of TLR expression during the events mentioned above are not known, it is likely that transcription of TLRs during CNS infections might be initiated in part by pathogen-mediated activation of TLRs themselves (Böttcher et al., 2003; Koedel et al., 2003). Further, TLRs, although they bind molecules of diverse structures, respond in a redundant manner, as they share the same signaling pathways (Netea et al., 2004). For example, TLR2 is established as the major receptor for gram-positive bacteria since it detects their characteristic cell wall components. Yet, studies suggest that TLR2 might be dispensable in the recognition of whole gram-positive bacteria in the brain (Koedel et al., 2003). Therefore the impact of distinct TLR activation on the regulation of TLR expression in the CNS *in vivo* was investigated. A substantial constitutive expression of mRNA for TLR1-9, except TLR4, and the central adaptor molecule was detected in the healthy brain of control animals, whereas at different levels (Fig. 3.8). The highest mRNA level of all TLRs tested was found for TLR3, the receptor for double stranded RNA, and could be largely attributed to the abundant population of astrocytes, which exhibit a marked expression of TLR3 *in vitro* (Jack et al., 2005; Farina et al., 2005; Bsibsi et al., 2006). In addition, microglia (Olson and Miller, 2004) and neurons (Cameron et al., 2007) express TLR3. Brian tissue has a limited regenerative capacity and since pathogens are capable of eliciting devastating consequences, the concept of redundancy among TLRs is of particular concern in the brain. A substantial repertoire of available pattern recognition receptors ensures a rapid ignition of an effective immune response upon infection of the CNS parenchyma.

Interestingly, after 12 h the activation of a given TLR did not necessarily influence its own transcription and did not regulate TLR expression per se (Fig. 3.8). None of the signaling molecules included in the expression analysis were regulated, not even the central adaptor molecule MyD88. Nevertheless, all TLR mRNAs, which displayed a fold change to control, were up-regulated and each will be discussed later on in the appropriate context.

An advantage of elevated TLR availability would be an increased sensitivity of the innate immune cells towards pathogens and tissue damage. For example, it was demonstrated that elevated TLR4 protein on the cell surface of microglia results in increased binding of LPS. Furthermore, activation of NF-κB and secretion of IL-1β, IL-6, TNF-α and NO were enhanced (Parajuli et al., 2012). However, increased sensitivity could result in over-activation of the immune response and increased severity of the insult. For example, an increase of TLR8 was observed in purified neurons suffering oxygen and glucose deprivation and ischemic mice additionally subjected to a TLR8 agonist suffered increased mortality, neurological deficit, infarct size and T cell infiltration (Tang et al., 2013). Alternatively, as demonstrated with TLR2, TLRs might be post-translationally modified to a soluble form and released into the extracellular space, serving as a decoy receptor to capture TLR ligands before they can engage functional membrane-bound TLRs (LeBouder et al., 2003). Such a regulatory mechanism could contribute to dampening the immune response, thereby minimizing inflammation-mediated tissue damage. It has to be taken into account that leukocytes had already infiltrated the CSF (Fig. 3.10b) and possibly migrated into the parenchyma at this time point. The change in levels of the TLR mRNAs detected in the brain homogenates could be influenced by their expression on the leukocytes. Which points to the draw-back of this experimental design – the cells in which TLR up-regulation has taken place were not determined. However, the distinct and dynamic expression of TLRs upon TLR activation described in this study and during CNS pathologies as reported by other studies (Lehnardt et al., 2007a; Babcock et al., 2006; Frank et al., 2009; Böttcher et al., 2003) point to the relevance of TLRs in the CNS.

4.2 The impact of TLR2 and TLR9 activation on the CNS

Analysis of TLR transcription revealed a strong up-regulation of TLR2 in the murine brain by activation of TLR2, TLR4 and TLR9 (Fig. 3.8). Constitutive expression of TLR2 is found on microglia (Jack et al., 2005; Lehnardt et al., 2006), astrocytes (Lehnardt et al., 2006; Bsibsi et al., 2002) and oligodendrocytes (Lehnardt et al., 2006; Bsibsi et al., 2002). An up-regulation of TLR2 has been described in several experimentally induced CNS pathologies such as cerebral ischemia (Lehnardt et al., 2007a), traumatic brain injury (Babcock et al., 2006; Zhang et al., 2012b) and Alzheimer's disease (Frank et al., 2009), and has been predominantly detected in microglia/macrophages of the affected brain areas (Lehnardt et al., 2007a; Ziegler et al., 2007; Zhang et al., 2012b; Frank et al., 2009). However, an up-regulation of TLR2 has also been observed in cortical neurons soon after experimental ischemia (Tang et al., 2007; Ziegler et al., 2007). It has been proposed that up-regulation of TLR2 is a sign of microglial activation (Lalancette-Hébert et al., 2009) and is part of a non-specific neuroinflammatory effector phase in neurodegenerative diseases (Letiembre et

al., 2009). TLR2 is under transcriptional control of NF-κB (Johnson and Tapping, 2007), therefore any type of stimulus causing activation of NF-κB may be responsible for the non-specific up-regulation of TLR2.

Transcription levels of the viral and bacterial DNA-sensing TLR9 were only affected by activation of the viral ssRNA-sensing TLR7 and TLR9 themselves (Fig. 3.8). Expression of TLR9 has been detected in microglia and at low levels in astrocytes in vitro (Jack et al., 2005; Bsibsi et al., 2002). *In vivo* studies have described an up-regulation of TLR9 in mouse models of parasitic or viral infection (Mishra et al., 2006; Böttcher et al., 2003) as well as in the mouse model of Alzheimer's disease (Frank et al., 2009) or multiple sclerosis (Prinz et al., 2006). The location of increased TLR9 protein was investigated only in the case of the parasite infection and found in infiltrating immune cells present in the ventricles and meninges (Mishra et al., 2006). Just like TLR2, transcription of TLR9 is regulated by NF-κB activation (Takeshita et al., 2004). However, as mRNA levels of TLR2 and TLR9 were increased by activation of different TLRs (Fig. 3.8), additional transcriptional control mechanisms must exist.

Toll-like receptors are a key element in pathogen-recognition by innate immune cells. Together, TLRs bind a large array of pathogen-associated molecules and initiate an inflammatory response, which is redundant to a certain degree (Netea et al., 2004). Activation of microglial TLR2, TLR4 or TLR9 with the specific exogenous ligands resulted in the secretion of the same cytokines and chemokines *in vitro* (Fig. 3.2). In particular the cytokine TNF-α and the chemokines CCL2 and CCL5 were detected (Fig. 3.2a,b). Similarly, *in vivo* microglia displayed an activated morphology and the stimulation of TLR2, TLR4 or TLR9 led to induction of the pro-inflammatory cytokines TNF-α and IL-1β (Fig. 3.10a,c). This is in line with the observed leukocyte influx upon activation of the respective TLRs (Fig. 3.10b). TNF-α production by microglia was demonstrated to be critical for leukocyte recruitment to the CNS upon LPS exposure (Zhou et al., 2006). Moreover, TNF-α and IL-1β likely alter blood-brain-barrier permeability and induce expression of critical adhesion molecules on CNS vascular endothelium, which are required for entry of leukocytes into the CNS (Esen et al., 2004), while the chemokines CCL2 and CCL5 propagate adhesion of leukocytes to the brain microvasculature (dos Santos et al., 2005). However, in addition to triggering protective immunity, the release of inflammatory molecules from microglia can cause neuronal death. *In vitro*, TLR2-, TLR4- and TLR9-induced loss of neurons required the presence of microglia (Fig. 3.5). Microglia-dependent TLR-induced neuronal death has been reported before and was in part ascribed to the release of the neurotoxic molecules TNF-α and NO. For example, in the presence of microglia CpG ODN was highly toxic to neurons, resulting in dramatically reduced neurite density. Treatment of the co-cultures with an inhibitor of iNOS or blockage of the TNF receptor prior to CpG ODN challenge was

neuroprotective to a certain degree (Iliev et al., 2004). Similarly, whole heat-inactivated Group B *Streptococci* induced neuronal apoptosis by a TLR2-dependent release of NO from microglia (Lehnardt et al., 2006). In this study specific activation of TLR2 *in vivo* by injection of Pam3CKS4 resulted in a dramatic loss of axonal structures with a less pronounced reduction in neuronal numbers (Fig. 3.9a,b). Loss of severed axons can precede to disintegration of the cell body itself (Raff et al., 2002). Accordingly, some of the surviving neurons displayed nuclear shrinkage, a feature of apoptosis, indicating onset of cell death. The massive loss of axons (Fig. 3.9a) and the amoeboid morphology of the microglia (Fig. 3.10a) observed in the cerebral cortex at day 3 after intrathecal injection of Pam3CSK indicate a rapid removal of the debris by a marked phagocytic activity of the microglia. Several reports have illuminated the impact of TLR2 in CNS pathologies. For example, TLR2 $^{-/-}$ mice subjected to MCAo exhibited a smaller infarct size and since the lesion-associated microglia of the wild-type mice exhibited increased TLR2, it was postulated that the stroke-induced CNS damage is propagated by TLR2 signaling in microglia (Lehnardt et al., 2007a; Ziegler et al., 2007). Similarly, TLR2 $^{-/-}$ mice with traumatic brain injury exhibit a decreased expression of inflammatory cytokines, which is associated with less neurological deficits (Yu and Zha, 2012). These studies point to a deleterious function of TLR2 in injury-induced inflammatory processes. On the other hand, in experimental Alzheimer's disease TLR2 $^{-/-}$ mice have increased Aβ levels in the brain that correlate with accelerated memory deficits (Richard et al., 2008). Furthermore, *in vitro* microglial uptake of Aβ was mediated by activation of TLR2, demonstrating that modulation of the innate immunity contributes to Morbus Alzheimer (Chen et al., 2006).

Activation of TLR9 in the murine brain led to a marked neuronal and axonal loss (Fig. 3.9a,b). Recently the impact of TLR9 activation in the murine brain was investigated. In contrast to this present study, CpG ODN was chronically administered by intraventricular infusion to investigate the impact of chronic inflammation. Notably, infusion of CpG ODN led to the loss of ependymal integrity, and increased microglial activation and axonal damage in the vicinity of the ventricle and a deficit in spatial memory. The magnitude of the observations was more severe after long-term (4 weeks) than short-term (1 week) exposure and was ascribed to ongoing microglia-mediated neuroinflammation (Tauber et al., 2009). However, in this study at hand a single injection of CpG ODN was sufficient enough to cause neuroinflammation and –degeneration (Fig. 3.9, Fig. 3.10). In contrast, TLR9 activation can be beneficial as vaccination of Alzheimer mice with CpG ODN ameliorated the disease-related pathology by markedly reducing amyloid burden in the cortex (Scholtzova et al., 2009). In summary, signaling via TLR2 as well as TLR9 can mediate neuroinflammation and trigger neuronal and axonal injury. However, it appears that depending on the pathological context in which TLRs are activated, their influence can likewise be beneficial.

4.3 Activation of TLR7 in the CNS causes neuronal injury in a cell-autonomous manner

Three days after intrathecal injection of Loxoribine, thereby activating TLR7, a decline in neuronal numbers and damage of axonal structures in the cerebral cortex of mice was observed (Fig. 3.9a,b). Surrounding microglia displayed a minor activated morphology (Fig. 3.10a). In addition, activation of TLR7 did not enhance the expression of the pro-inflammatory molecules TNF-α and IL-1β or provoke an influx of peripheral immune cells to the CNS by 12 h post injection (Fig. 3.10b,c). Similarly, *in vitro* the secretion of cytokines and chemokines from Loxoribine-challenged microglia was relatively low (Fig. 3.2). Moreover, microglia were dispensable in TLR7-induced neuronal death *in vitro* (Fig. 3.5) in accordance with recently published data (Lehmann et al., 2012a; Lehmann et al., 2012b). Previously, our group has demonstrated that TLR7 is expressed on neurons of the cerebral cortex (Lehmann et al., 2012a; Lehmann et al., 2012b). Moreover, *in vivo* activation of TLR7 with the agonists single-stranded RNA 40 (ssRNA40, human deficiency virus-derived) or lethal-7b (let7b, notably, an endogenous microRNA and a newly identified agonist) triggered death of cortical and striatal neurons with loss of axons in the cortex, corpus callosum and hippocampus (Lehmann et al., 2012a; Lehmann et al., 2012b). Importantly, several experiments revealed that TLR7-induced neuronal cell death occurred in a cell-autonomous fashion. In a crucial *in vivo* experiment specifically cortical neurons were replenished with TLR7 by *in utero* electroporation of TLR7$^{-/-}$ mice to verify that neurons are directly harmed by let-7b via TLR7 and not through TLR7 activation of any other CNS cell (Lehmann et al., 2012a). In another experiment prior elimination of microglia from the brain did not protect cortical neurons from let7b-mediated toxicity (Lehmann et al., 2012a). However, TLR7-induced cell-autonomous neuronal cell death is amplified by the presence of microglia *in vitro* (Lehmann et al., 2012b). *In vitro* very low levels of cytokines and chemokines were detected and *in vivo* mRNA levels of TNF-α and Il-1β remained unchanged after activation of TLR7 (Fig. 3.1, 3.2, 3.10c). Activation of TLR7 in immune cells can result in cytokine production via NF-κB; however it primarily leds to the generation of the type I interferons IFN-α and IFN-β via activation of the IRF transcription factors (Yamamoto et al., 2002b). As type I IFNs mediate antiviral properties, possibly, since TLR7 senses viral ssRNA, the CNS immune cells encounter viral infection by primarily secreting IFN-α and/or IFN-β to block virus spread (van den Pol et al., 2014). In accordance, it has been shown that intracerebroventricular inoculation of the TLR7 agonist Imiquimod results in a weak cytokine, yet strong IFN-β response in the murine brain (Butchi et al., 2011).

Notably, the other 2 viral nucleic acid-sensing TLRs TLR3 and TLR8 have also been detected on murine neurons (Cameron et al., 2007; Ma et al., 2006). TLR3 is concentrated

in the growth cones with stimulation resulting in rapid collapse and irreversible inhibition of neurite extension. TLR8 is localized to the cell body and axons and its activation inhibits neurite outgrowth and causes apoptosis (Ma et al., 2006). Interestingly, in this present study expression of TLR8, which is phylogenetically similar to TLR7 and likewise a ssRNA-detecting TLR, was markedly up-regulated upon TLR2, TLR7 and TLR9 activation, whereas TLR7 mRNA levels remained unchanged, even after activation of TLR7 itself (Fig. 3.8). Other studies have revealed that while during an experimental parasite infection of the CNS elevated TLR8 is present in infiltrating immune cells found in the ventricles and meninges and in axons originating from granular neurons (Mishra et al., 2006), after an ischemic event TLR8 is enhanced in cortical neurons (Tang et al., 2013). Moreover, ischemic mice additionally subjected to a TLR8 agonist suffered increased mortality, neurological deficit, infarct size and T cell infiltration (Tang et al., 2013), pointing to detrimental consequences of TLR up-regulation. Death-mediated signaling of TLR3, TLR7 and TLR8 in neurons has been found to be independent of NF-κB, the classical terminal effector in microglial signaling and inflammation (Cameron et al., 2007; Ma et al., 2006; Lehmann et al., 2012a). However, the pathway mediating TLR7-induced neuronal death includes activation of the canonical TLR signaling molecule IRAK-4 (Lehmann et al., 2012a). Currently, our group is investigating the role of the TLR adaptor molecule MyD88-5, which is preferentially expressed in neurons at high levels and has been linked to neuronal death upon deprivation of oxygen and glucose (Kim et al., 2007) but not to TLR activation itself. Possibly, viral-detecting TLRs on neurons enable them to initialize apoptosis upon viral infection to arrest viral replication and protect adjacent cells supporting the innate immunity in controlling virus spread within the CNS.

4.4 Activation of TLR4 in the CNS: a double-edged sword

It is striking that depending on the ligand used, activation of the same receptor, in this case TLR4, resulted in a different outcome regarding inflammation and injury. Intrathecal injection of the exogenous ligand LPS induced a robust inflammatory response but no discernible CNS injury, while intrathecal injection of the endogenous ligand HSP60 caused neuronal death as well as demyelination but failed to mediate neuroinflammation. A detrimental as well as a beneficial influence of TLR4 on CNS pathologies has been demonstrated before. For example, TLR4$^{-/-}$ or mutant mice have reduced infarct sizes compared with wild-type mice after an ischemic insult (Cao et al., 2007; Caso et al., 2007; Tang et al., 2007; Kilic et al., 2008; Hyakkoku et al., 2010). Patients with a TLR4 polymorphism, which exhibit a blunted TLR4 mediated inflammatory response, have a reduced risk of late-onset AD (Minoretti et al., 2006). On the other hand, TLR4 mutant mice exhibit a higher Aβ load, a major hallmark of Morbus Alzheimer (Tahara et al., 2006;

Jin et al., 2008). Further, TLR4$^{-/-}$ mice present more severe EAE symptoms than wild-type mice, indicating a regulatory role of TLR4 on priming autoimmune IL-17-producing T cells (Marta et al., 2008). Moreover, upon nerve injury TLR4 proved to be critical for clearing of inhibitory myelin debris thereby allowing rapid nerve regeneration (Boivin et al., 2007).

As outlined above TLR4 plays a critical role in determining the pathological outcome of several neurological disorders. Interestingly, TLR4 mRNA was rare to undetectable in the brain homogenates of control mice as well as of those challenged with each of the four TLR ligands (Fig. 3.8). Similarily, a former *in situ* hybridization study revealed that TLR4 mRNA is present in the rat brain at low to moderate levels (Laflamme and Rivest, 2001). Similarly, in human white matter TLR4 was nearly undetectable (Bsibsi et al., 2002). Possibly, low levels of TLR4 are sufficient to induce signaling, since functional TLR4 signaling was observed in peripheral innate immune cells despite low detectable surface expression (Visintin et al., 2001). Importantly, application of the TLR4 specific ligand LPS did cause a profound inflammatory response in the mouse brain (Fig. 3.15-3.17) and the destructive effect of HSP60 on neurons and oligodendrocytes was dependent on TLR4 (Fig. 3.11, Fig. 3.13). An up-regulation of TLR4 was not detected upon activation of any of the four TLRs (Fig. 3.8), although an increased expression has been observed during infectious diseases of the murine brain with gram-positive bacteria, gram-negative bacteria, viruses or a parasites (Böttcher et al., 2003; Mishra et al., 2006). Further, in mouse models of stroke, traumatic brain injury, Alzheimer's and Parkinson's disease and brain tissue of MS patients, levels of TLR4 mRNA and protein were augmented in the regions affected by the respective diseases, predominantly on microglia (Hyakkoku et al., 2010; Tang et al., 2007; Chen et al., 2008; Zhang et al., 2012b; Walter et al., 2007; Panaro et al., 2008; Bsibsi et al., 2002). Possibly, transcription of TLR4 is regulated by mechanisms other than TLR activation. Whereas it is established that neurons express TLR3, TLR7 and TLR8 (Cameron et al., 2007; Lehmann et al., 2012b; Ma et al., 2006), it is controversial if neurons likewise exhibit TLR4. It was reported that expression of TLR4 in cultured cortical neurons renders these cells vulnerable to ischemia (Tang et al., 2007). However, *in vitro* TLR4-mediated neuronal loss was not cell-autonomous but required the presence of microglia (Fig. 3.5, Fig. 3.6) as neurons do not express TLR4 (Lehnardt et al., 2002; Lehnardt et al., 2003; Lehnardt et al., 2008). Since TLR expression in neurons might depend on the disease context, further studies are necessary to solve this controversy. However, although the results reveal that TLR4 is essential for HSP60-induced neurodegeneration and demyelination *in vitro* and *in vivo* (Fig. 311, Fig. 3.13), it must be kept in mind that, besides TLR4, other receptors are capable of recognizing HSP60. HSP60 is bound to the cell surface of macrophages even in the absence of TLR4 (Habich et al., 2002). The scavenger receptor LOX-1 on dendritic cells was identified to bind HSP60 (Delneste et al., 2002). Recently,

microglial LOX-1 was linked to propagating HSP60-mediated neurotoxicity (Zhang et al., 2012a). Also, the protein TREM2 was identified as an HSP60 receptor with engagement resulting in an increased phagocytic activity of a microglial cell line (Stefano et al., 2009) which, notably, has been described as non-inflammatory phagocytosis (Takahashi et al., 2005). Finally, TLR2, expressed in a fibroblast cell line, responded to HSP60 as determined in an NF-κB reporter assay (Vabulas et al., 2001). As demonstrated using the exogenous ligand Pam3CSK4, activation of TLR2 triggered neuronal cell death *in vitro* and *in vivo* (Fig. 3.5, Fig. 3.9) and could therefore participate in HSP60-mediated TLR4-dependent neurodegeneration.

I demonstrated here that HSP60 triggers injury of neurons and oligodendrocytes in a TLR4-dependent fashion *in vivo* (Fig. 3.11, Fig. 3.13) and loss of neurons dependent on microglial TLR4 *in vitro* (Fig. 3.6). A subject of considerable debate has been whether immunostimulatory effects of heat shock proteins are due to contamination of the recombinant protein preparations with bacterial products from the microbial expression system (Henderson et al., 2010). In particular it was discussed that more likely LPS and not the recombinant HSP60 acted on macrophages as these do not secrete TNF-α upon HSP60 incubation (Gao and Tsan, 2003). Likewise, upon challenge with HSP60 levels of TNF-α and other cytokines and chemokines remained unaltered in supernatants of microglia(Fig. 3.4) and brain lysates (Fig. 3.15) in comparison with LPS (Fig. 3.2, Fig. 3.15). However, previous extensive control experiments excluded that LPS triggers the neurotoxic effects induced by our HSP60, notably low-endotoxin, preparations (Lehnardt et al., 2008). The neurotoxic effect of the HSP60 preparation was undiminished although it was treated with an antagonist of LPS (Lehnardt et al., 2008). Vice versa, the neurotoxic effect of HSP60 could be abrogated by using denaturing approaches of subjecting it to heat or to the proteolytic enzymes protein K or trypsin (Lehnardt et al., 2008). Further, a LPS concentration sufficient enough to cause neuronal death to the same extent as the HSP60 preparation did, clearly exceeded the theoretical maximal content of LPS – as tested in an independent laboratory - in the HSP60 preparation (Lehnardt et al., 2008). Additionally, HSP60 derived from injured CNS cells demonstrated a neurotoxic effect considerably similar to that induced by recombinant HSP60 (Lehnardt et al., 2008). In this context it is noteworthy, that in contrast to intrathecal injection of recombinant HSP60, which resulted in significant neuronal loss and demyelination in the cerebral cortex (Fig. 3.11, Fig. 3.13), LPS itself did not affect the survival of neurons *in vivo* (Fig. 3.9).

Nearly all diseases and insults of the CNS are accompanied or followed by inflammation. Microglia are the sentinels of pathological settings in the CNS and rapidly initiate an inflammatory cascade (Kreutzberg, 1996). As demonstrated *in vitro* HSP60-induced neuronal injury required the presence of microglia (Fig. 3.6), which release neurotoxic nitric oxide (Lehnardt et al., 2008). However, CNS damage induced by intrathecal HSP60 was not ac-

companied by major activation or proliferation of glial cells (Fig. 3.14), release of nitric oxide (Fig. 3.16)or a considerable inflammatory response (Fig. 3.15, Fig. 3.17) in the brain *in vivo*. Nevertheless, a subtle response, that was not detected in this experimental set-up, cannot be excluded as microglia and astrocytes constantly monitor their proximity and respond to even minor changes in their microenvironment (Nimmerjahn et al., 2005). Possibly, the neuroinflammatory or –toxic molecules analysed accumulated at other time points than those observed or the local concentrations were sufficient enough to affect neurons but were diluted by analysing whole brain. Further, HSP60 might induce the Type I interferons IFN-α and IFN-β. However, challenge with HPS60 triggered injurious processes in the cerebral cortex in a TLR4-dependent manner (Fig. 3.11). Several studies have suggested that TLR4-induced neuroinflammation has a pivotal role in the CNS, as the neuroprotective effects in TLR4 $^{-/-}$ mice is likely due to the lack of TLR4-mediated activation of microglia. For example, in experimental Parkinson's disease TLR4 $^{-/-}$ mice were less vulnerable to dopaminergic cell death and displayed a decreased number of activated microglial cells (Noelker et al., 2013). In studies on experimental stroke mice defective or deficient of TLR4 repeatedly display reduced infarct volumes in comparison with wild-type mice (Cao et al., 2007; Caso et al., 2007; Tang et al., 2007; Kilic et al., 2008; Hyakkoku et al., 2010; Hua et al., 2009). These mice with a disrupted TLR4 pathway exhibit less macrophages/microglia in the stroked area and a decreased NF-κB translocation (Hyakkoku et al., 2010; Hua et al., 2009) or less amounts of TNF-α and IL-6 (Cao et al., 2007). Further, the enzymes iNOS (Kilic et al., 2008; Caso et al., 2007) and Cox-2 (Caso et al., 2007) were lower, and inhibition of both enzymes in wild-type resulted in a better outcome and reduced infarct volumes (Caso et al., 2007). In this model of specific TLR4 activation the indicators of neuroinflammation mentioned above were evaluated. Whereas the exogenous TLR4 ligand LPS induced expression of Cox-2, iNOS, TNF-α, IL-6 amongst others (Fig. 3.15, Fig. 3.16) and activation of microglia (Fig. 3.10a), the endogenous TLR4 ligand HSP60 did not (Fig. 3.14, Fig. 3.15, Fig. 3.165), although it did cause severe CNS damage, which was not the case for LPS.

However of all the enzymes, cytokines, chemokines and neurotoxic metabolites tested, one candidate did stand out. Soon after injection of HSP60, but not of LPS, the expression of TGF-β drastically dropped (Fig. 3.17). The growth factor TGF-β has multifaceted properties in the CNS (Flanders et al., 1998). It has a survival-promoting effect, which may occur directly on neurons as well as indirectly by inducing synthesis of neurotrophins such as NGF or NT-3 (Buchman et al., 1994). Further, TGF-β can suppress microglial cytotoxicity (Merrill and Zimmerman, 1991) and deactivate macrophages (Tsunawaki et al., 1988). TGF-β is implicated in acute and chronic degenerative CNS diseases (Flanders et al., 1998) and therefore down-regulation of TGF-β by HSP60 could at least in part account for the neurodegenerative effects observed.

Activation of TLR4 by its exogenous ligand LPS or endogenous ligand HSP60 led to

different outcomes in the CNS and might be due to the initiation of opposing subsequent pathways. TLR4 is the only TLR, which uses both the MYD88- and TRIF-pathway (Yamamoto et al., 2003). The recruitment of the adaptor molecule MyD88 results in NF-κB activation and the production of a wide array of inflammatory cytokines and chemokines, and the transcription of enzymes, such as iNOS. These inflammatory mediators and transcripts were not increased in the brain upon challenged with HSP60. The recruitment of TRIF leds to activation of the IRF transcription factors and production of IFN-α and IFN-β (Sasai and Yamamoto, 2013), which can act neurotoxic in high levels (Reder and Feng, 2014). Intrathecal injection of HSP60 into TRIF-deficient mice could reveal whether the HSP60 activates the TRIF-pathway upon TLR4-binding. However, it is possible that the dose is critical whether TLR4 mediates a beneficial of deleterious response and remains to be elucidated *in vivo*.

4.5 Combined activation of Toll-like receptors results in a fine-tuned response

As discussed above specific activation of TLRs is capable of causing CNS injury *in vitro* and *in vivo*. Single TLRs were exposed to their respective specific ligands to analyze what impact each TLR has. However, host cells and pathogens comprise several molecular patterns recognized by TLRs and therefore they are likely to be present in a mixture. Hence, it can be assumed that they engage more than one TLR during pathological events in the CNS. Consequently, the impact of combined TLR activation on inflammation and neurodegeneration in the CNS was analysed in this study. Cultures of pure microglia were used to evaluate the impact of combined TLR activation on the neuroinflammatory response. Further, cultures of neurons with and without microglia allowed determining what impact the modulated neuroinflammatory response has on neuronal survival.

Production of TNF-α, NO and other inflammatory molecules was determined in pure microglial cultures after stimulation of each TLR alone and in combination. Combined stimulation of TLR4 plus TLR2 and TLR4 plus TLR9 caused additive secretion of TNF-α, IL-6, IL-10 and NO from microglia compared to activation of the respective single TLR (Fig. 3.2). On the other hand, combined stimulation with ligands specific for TLR2 and TLR9 did not trigger such an enhanced response. A synergistic effect of TLR4 and TLR2 activation on TNF-α secretion has been observed before in macrophages (Sato et al., 2000; Beutler et al., 2001). In line with this, it was postulated that confirmation of one microbial product through another might serve as a safety-mechanism for the organism (Beutler et al., 2001). Further, *in vivo* it was observed that in comparison with single injection, intracisternal injection of LPS or peptidoglycan (TLR2 agonist) together with CpG ODN has a synergistic effect on the incidence of meningitis and influx of inflammatory cells

(Deng et al., 2001). Whether TLR activation in microglia results in a synergistic response might depend on which TLR-signaling pathways are initiated simultaneously. It has been described that simultaneous activation of the MyD88- and TRIF-signaling pathway results in a synergistic production of TNF-α and IL-6 by mouse bone-marrow-derived macrophages (Bagchi et al., 2007). On the other hand, actvation of the MyD88-pathway with different TLR ligands did not (Bagchi et al., 2007). This would explain why combined stimulation of TLR4 (MyD88/TRIF) with TLR2 (MyD88), but not TLR2 (MyD88) with TLR9 (MyD88), yielded an enhanced secretion of TNF-α and NO from microglia. Further, the prolongation of TNF mRNA half-life might account in part for the synergistic TNF-α production (Gao et al., 2001), whereas an up-regulation of mRNA for the enzyme iNOS could be responsible for the increase in NO (Gao et al., 1999).

The relationship between TLR-dependent microglial activation and neurodegeneration *in vitro* has been reported (Lehnardt et al., 2006; Iliev et al., 2004; Lehnardt et al., 2003) and was in part ascribed to the release of the neurotoxic molecules TNF-α (Iliev et al., 2004) and NO (Boje and Arora, 1992; Lehnardt et al., 2008; Lehnardt et al., 2007a; Chao et al., 1992). Combined activation of TLR4 with TLR2 and TLR4 with TLR9 by their respective specific exogenous agonists enhanced neuronal death in neuron-microglia-cultures compared with exposure to the single agonists (Fig. 3.5). The decrease in neuronal survival was clearly dependent on the presence of microglia. Further, the impact of combined TLR activation on the microglial inflammatory response is reflected in the results of the co-culture experiments. For example, levels of secreted inflammatory molecules hardly changed after simultaneous activation of TLR2 and TLR9 in microglia (Fig. 3.2) and in comparison to single TLR stimulation neuronal survival was not further compromised (Fig. 3.5). However, activation of both TLR4 and TLR2 had a synergistic effect, namely production of pro-inflammatory molecules (Fig. 3.2) and decreasing neuronal viability (Fig. 3.5). These results indicate that in principle simultaneous activation of multiple TLRs in the CNS can exert a detrimental effect on neuronal survival through an exaggerated inflammatory response.

On the other hand, TLR2, TLR4 and TLR9 signaling were remarkably suppressed by combined TLR7 activation. Parallel stimulation of TLR7 did not only suppress secretion of inflammatory mediators from microglia (Fig. 3.2), but also appeared to abolish TLR2-, TLR4- and TLR9-induced neuronal cell death in co-cultures (Fig. 3.5). Interactions among TLR7 and TLR9 have been studied before with conflicting results. Natural and synthetic ligands of TLR7 inhibited TLR9 induced IFN-α secretion from plasmacytoid dendritic cells and B cells, which was found unlikely due to kinetic up-take advantages (Berghöfer et al., 2007) and was accounted to a reduced expression of the transcriptional factor IRF 7 (Marshall et al., 2007). In turn, another group suggested that Imiquimod-mediated TLR7 signaling did not affect CpG ODN-mediated TLR9 signaling in microglia. Yet, Imiquimod might hinder binding of CpG ODN in addition to inhibition of TLR9 by physical interaction

with TLR7 (Butchi et al., 2010). As the reports are contradicting, it remains elusive by which mechanism TLR7 suppresses the TLR9 response as well as that of TLR2 and TLR4. Studies on interaction of TLR7 with TLR2 or TLR4 are not at hand; however, a comparison between inactivated *Mycobacterium butyricum* and its purified components lipoteichoic acid and Pam3CSK4 (agonists of TLR2) has been performed (Gambhir et al., 2012). It was observed that whole bacteria was less efficient in provoking the release of TNF-α, IL-6 and NO from dendritic cells and macrophages. In addition, in contrast to the TLR2 agonists, which initiated a rapid and prolonged DNA-binding of NF-κB, whole bacteria failed to increase NF-κB activity to such an extent (Gambhir et al., 2012), emphasizing that signaling of a single TLR can be compromised by other TLRs or factors. Possibly, since TLR2 and TLR4 recruit Mal in addition to MyD88 (Yamamoto et al., 2002a), TLR7 signaling is initiated faster and therefore impedes consecutive TLR2 and TLR4 signaling. Moreover, TLRs compete for the same signaling molecules and as the signaling complex assembled for TLR2 and TLR4 requires additional proteins, the TLR7 pathway might be more rapid and "favorable". In addition, it cannot be ruled out, that other inflammatory molecules than those tested accumulated or that the outcome depends on the dose of each ligand used in the combination.

As demonstrated *in vitro*, combined TLR activation is capable of enhancing microglial activation, which in turn amplifies neuronal injury. It is widely accepted that inflammatory processes belong to the main contributors of brain tissue damage. During CNS infection the host immune response considerably contributes to neuronal damage (Weber and Tuomanen, 2007) and after CNS insults injury-induced inflammation is involved in secondary tissue damage (Giulian and Vaca, 1993). Commonly, immune responses are self-limiting, but a chronic inflammatory response could further exacerbate CNS injury (Glass et al., 2010). In line with this, continuous intracerebroventricular infusion of the TLR9 ligand CpG ODN leads to pronounced microglial activation and axonal injury (Tauber et al., 2009). Further, mice receiving an intracerebral injection of high-mobility group box-1 (HMGB1), another endogenous TLR4 ligand, shortly after experimental stroke exhibited increased edema, infarct area and neurologic deficits, whereas TLR4 $^{-/-}$ mice were protected (Yang et al., 2011). Similarly, young mice receiving a systemic LPS injection prior to a normally harmless hypoxic-ischemic insult experience severe loss of axons and neurons in the corpus callosum and underlying structures (Lehnardt et al., 2003). Systemic infections are related with sustained aggravation in many diseases of the CNS. Moreover, patients with CNS infections have an approximately 3 times higher risk of stroke (Chien et al., 2013) and there is a growing body of evidence that bacterial and viral infections trigger neurodegenerative and autoimmune CNS diseases (De Chiara et al., 2012). The initial pathogen-mediated inflammatory response may be perpetuated by immunstimulatory molecules released by the host itself. To test whether TLR-induced CNS injury is

exacerbated by subsequent TLR exposure to an endogenous ligand, an initial intrathecal injection of the exogenous ligands for TLR2 (Pam3CSK4) or TLR7 (Loxoribine) was followed by HSP60. Injection of each of the three TLR agonists alone provoked a considerable loss of neurons and injury of axons in the cerebral cortex as expected (Fig. 3.12). However, additional injection of HSP60 failed to deteriorate Pam3CSK4- or Loxoribine-induced CNS injury (Fig. 3.12). In addition, in mice challenged with HSP60 subsequent to Pam3CSK4 or Loxoribine, it could be observed, that the morphology of the microglia resembled more a resting state (Fig. 3.12). This *in vivo* study possibly reflects the tolerance paradigm found in *in vitro* studies, where it was observed that immune cells stimulated with a TLR ligand respond hyporesponsively to subsequent TLR stimulation (Sato et al., 2000; Dalpke et al., 2005). A variety of inhibitory strategies causing tolerance to subsequent stimulation, such as decreased degradation or dissociation of signaling molecules and enhanced expression of inhibitory proteins, have been elucidated (Fan and Cook, 2004). In the setting of CNS ischemia, the brain is resistant to a serious ischemic episode if a previous sub-threshold ischemic event has occurred; a phenomenon termed ischemic preconditioning (Stetler et al., 2014). Moreover, another phenomenon termed cross-tolerance can be elicited by other forms of sub lethal stress such as hypothermia (Nishio et al., 2000) or even challenge with TLR ligands. As such, systemic application of LPS, Pam3CSK4, or CpG ODN, activating TLR4, TLR2, and TLR9, respectively, prior to an experimental ischemic episode results in neuroprotection (Rosenzweig et al., 2007; Stevens et al., 2008; Hua et al., 2008). Thereby-although the pre-conditioning stimuli did elevate TNF-α levels-subsequent down-regulation of TNF-α levels emerged to be crucial in mediating the neuroprotective effect in comparison to the control mice (Rosenzweig et al., 2007; Stevens et al., 2008). Likewise, TLR4$^{-/-}$ mice subjected to ischemic pre-conditioning are not protected from subsequent cerebral ischemia as they display reduced NF-κB activity as well as decreased TNF-α expression compared to wild-type mice (Pradillo et al., 2009). Mice can also be protected by pre-conditioning with the TLR7 ligand Gardiquimod, which, interestingly, is not dependent on TNF-α, but on IFN signaling (Leung et al., 2012). Overall, these studies point to the complex inflammatory processes in the brain of which a delicate balance of pro-inflammatory signals and regulatory mechanisms is essential for tissue integrity. Whereas an insufficient TLR response could increase susceptibility to infection or fail to eliminate the pathogens, disproportionate signaling might result in excessive tissue damage or autoimmune diseases. Therefore a tightly controlled TLR signaling allows the host to respond properly to pathogens and tissue damage. However, TLR signaling is only a fraction of the complex molecular and cellular events occurring in CNS pathologies and its contribution might depend on the nature of the CNS insult.

4.6 The TLR4 ligand HSP60 serves as a danger signal in the CNS

Initially, TLRs were ascribed to detect the presence of pathogens based on their conserved structures. Soon, it became clear that TLRs can sense danger signals derived from the host itself. Under physiological conditions, TLRs might not be confronted with their endogenous ligands or only to a minimum extent. During pathological conditions the liberation or secretion of these molecules from injured or dying cells signals danger to the organism. Repeatedly, TLR2 or TLR4 deficient mice subjected to an ischemic insult, notably a non-infectious situation, displayed less destruction of the CNS tissue compared with wild-type mice (Lehnardt et al., 2007a; Ziegler et al., 2007; Caso et al., 2007; Hua et al., 2009). It was postulated that the engagement of TLRs by endogenous ligands must be responsible for the deteriorated outcome. HSP60, an endogenous ligand of TLR4, is capable of triggering neurodegeneration and demyelination in the CNS as demonstrated here (Fig. 3.11, Fig. 3.13). Previous work has revealed that HSP60 is released by injured CNS cells *in vitro* (Lehnardt et al., 2008). To evaluate whether HSP60 is present in the brain and is released into the extracellular space of the CNS by damaged cells *in vivo*, thereby being available to TLR4, mice were subjected to an ischemic insult. Evaluation of brain sections from sham-operated mice revealed that HSP60 was particularly detectable in neurons. Neurons have an extraordinarily high metabolic rate, which is dependent on mitochondrial function. To cover the energy needs hundreds of mitochondria are present in neurons (Knott et al., 2008). Since HSP60 is associated with mitochondria (Mayer, 2010), it is plausible that a marked presence of HSP60 was found in neurons (Fig. 3.18). It has been demonstrated that high amounts of HSPs are released from necrotic but not apoptotic cells (Basu et al., 2000; Lehnardt et al., 2008). Apoptosis is a programmed cell death producing apoptotic bodies, which phagocytic cells quickly engulf before the contents spill out and cause damage to the surrounding cells (Bruce Alberts, 2008). Accordingly, supernatants derived from apoptotic neuronal cultures were to a lesser extent toxic than supernatant from necrotic cells (Lehnardt et al., 2008). Thus, injury-induced disintegration of neurons thereby liberating high amounts of HSP60 serves as a potent danger signal in the microenvironment. In this study, a model of stroke which sets a defined injury to the CNS verified that HSP60 is released into the extracellular space as the CSF of stroked mice contained explicitly more HSP60 than that of sham-operated mice (Fig. 3.19). Strikingly, elevated concentrations of soluble HSP60 have also been measured in the CSF of children suffering severe traumatic brain injury (Lai et al., 2006). However, it is possible that in addition to HSP60, other endogenous ligands, such as HSP70 and β-defensin, are released from injured CNS cells and activate TLRs and other receptors (Asea et al., 2002; Biragyn et al., 2002), thereby contributing to CNS injury. In line with this, HMGB1, an-

other endogenous ligand of TLR4, was found in the CSF of rats subjected to MCAO and immunohistostaining and -blotting revealed that HMGB1 originated from neurons (Qiu et al., 2008). Likewise, let-7b, a microRNA abundant in the brain, predominantly expressed in neurons and identified as an endogenous ligand of TLR7, is elevated in the CSF of Alzheimer patients (Lehmann et al., 2012a). Whereas HSP60 and HMGB1 might contribute in the initial stages of the inflammatory response (Qiu et al., 2008), let-7b acts directly on neurons causing cell-autonomous death (Lehmann et al., 2012a).

In addition to the ischemia-induced release of HSP60, it was further found to be increased in intact cortical neurons of the infarct-region (Fig. 3.18). The finding that HSP60 is overexpressed in injured neurons matches the results of other studies. In hippocampal neurons HSP60 mRNA is increased after transient cerebral ischemia (Abe et al., 1993). Also, elevated levels of HSP60 mRNA and protein are detectable in brain tissue homogenates of the ipsilateral hemisphere compared to the contralateral hemisphere after permanent focal cerebral ischemia, and it was suggested to be a mechanism to protect proteins from aggregation and degradation during mitochondrial stress (Wagstaff et al., 1996). However, it has been demonstrated that HSP60 can be translocated to the surface of damaged and dying cells (Chen et al., 1999), with elevated amounts of HSP60 serving as a danger signal to dendritic cells, T cells (Osterloh et al., 2004) and microglia (Lehnardt et al., 2008). Thus, in addition to liberation of HSP60 from necrotic neurons during the ischemic insult, possibly the increased amount of HSP60 found in neurons after the insult is released at a later time point by active secretion or delayed death of the cells. Therefore, the acute and an additional delayed release of HSP60 with binding to TLR4, in addition to other neurotoxic factors, could account for exacerbated damage following ischemia. Thus, HSP60 seems to be an adequate candidate for signaling danger to TLR4 in the brain. Moreover, in other neurodegenerative and demyelinating CNS diseases such as Alzheimer's disease, Parkinson's disease, and Multiple Sclerosis extracellular danger signals could contribute to disease progression. However, it cannot be ruled out that endogenous ligands may also induce repair mechanisms *in vivo*.

4.7 Toll-like receptors as therapeutic targets in CNS pathologies

Currently, TLRs are used or are intended to be used as therapeutic targets, as they have a strong potential for prevention or intervention of infections, especially sepsis, or cancer (Savva and Roger, 2013; Vacchelli et al., 2013). Imiquimod, an agonist of TLR7, is prescribed as a topical anti-viral agent for e.g. external warts (Savva and Roger, 2013). Further, modified ligands or antibodies as well as antagonists directed against TLRs are under development or even being tested in clinical trials these days (Savva and Roger, 2013; Vacchelli et al., 2013). As acute and chronic inflammation is a key element in CNS patholo-

gies, targeting TLRs in the CNS is promising in generating protective immune responses to cancer or modulating neuroinflammation and/or suppressing neurodegeneration (Marsh and Stenzel-Poore, 2008). However, as demonstrate here single activation of TLRs in the CNS can have a major fatal impact on CNS integrity. TLRs are expressed on immune cells as well as neurons and can elicit inflammation as well as mediate cell-autonomous death. Moreover, TLRs cannot only substitute for one another, they can influence the outcome mediated by one another, what should be considered when developing antagonists for single TLRs. Thus, the complexity of TLRs in CNS inflammation and injury brings up the question how to target this family of pattern-recognition receptors to achieve an adequate therapeutic strategy. To date, the exact role of the innate immunity as well as the whole range of mediators involved in the very early events of CNS pathologies have not been identified, and additional research is essential.

4.8 Conclusions and Future Directions

It is evident that activation of TLRs in the CNS can cause neuroninflammation and neurodegeneration. However, the results indicate that the degree of neuroinflammation and neurodegeneration in the CNS depends on which TLR is engaged. Further, when more than one TLR is engaged, the outcome likely depends on the combination and timing of TLR activation. Moreover, the type of agonist might have an additional influence. These are important factors as several pathogen- and host-derived TLR ligands exist. During a neuropathological setting, these ligands could engage different TLRs and modulate the outcome. As demonstrated on purified microglia, although the tested TLRs share common signaling cascades, only some synergized, whereas others antagonized one another when activated simultaneously. This fine-tuning of the inflammatory response also had an impact on neuronal survival in co-cultures. Further, *in vivo* subsequent challenge with different TLR agonists did not exacerbate neuronal injury. It can be assumed that microbial structures liberated from disintegrated pathogens and/or endogenous molecules discharged from injured host cells persist after the termination of infection or insult, serving as agonists for TLRs. As excessive activation of the immune response in the brain is destructive, control mechanisms must exist to restrict continuous neurotoxic insults. Thus, it appears that the extent of TLR-induced CNS injury is dependent on the physiological and pathological context in which TLRs are activated. Possibly, whereas activation of the innate immunity by PAMPS during an infection cannot be further stimulated by closely timed subsequent challenge of TLRs, release of DAMPS from CNS cells suffering an ischemic insult or traumatic injury leads to TLR-mediated secondary damage. Here HSP60 has been verified as such an endogenous agonist, which is increased in neurons and present in the extracellular space after CNS injury. Moreover, intrathecal HSP60 is capable of causing neurodegeneration and demyelination in a TLR4-dependent manner. It is therefore proposed that neuronal damage provides a sufficient amount of endogenous agonists for TLR-mediated neurotoxicity, driving a vicious cycle of neurodegeneration. A malfunction in the mechanisms regulating inflammation could account for chronic inflammation in which TLRs may be involved.

A detailed understanding of how the intrinsic innate immunity is involved in both CNS disorders and health is crucial, when reducing excessive neuroinflammation and its effects is intended. Using co-cultures allowed discriminating whether the presence of microglia is obligatory for TLR-induced neuronal injury *in vitro*. *In vivo*, microglial activation and the presence of inflammatory mediators in the CNS were evaluated, whether TLR-induced brain damage is dependent on microglia could not be elucidated. This is of particular interest as activation of TLR4 with its endogenous ligand HSP60 caused prominent CNS injury, whereas an activated morphology of microglia and mediators of an inflammatory response were absent. The mouse strain CD11b-HSVTK (Heppner et al., 2005) enables

a nearly complete ablation of microglia in the adult CNS (Grathwohl et al., 2009). Thus, whether microglia are essential for TLR-induced CNS injury *in vivo* could be approached by intrathecal injection of the TLR agonists into these microglia-depleted mice with subsequent immunohistological analysis.

Further, although TLRs are receptors of the innate immunity, it is appreciated that neurons constitutively express TLRs, which have been linked to inhibition of axonal growth and cell-autonomous death (Ma et al., 2006; Cameron et al., 2007; Lehmann et al., 2012b). It is intriguing to study how come TLRs in neurons all mediate death and if the adaptor molecule MyD88-5, which is highly expressed in neurons and has been linked to neuronal death induced by oxygen and glucose deprivation (Kim et al., 2007), is responsible for initiation of this detrimental pathway upon activation of neuronal TLRs. This could be approached by performing viability assays with cultures of purified neurons from MyD88-5$^{-/-}$ mice and by subsequent immunoprecipitation assays and western blots to decipher the signaling cascade.

Taken together, the findings of this study demonstrate that single and combined activation of TLRs influence the pattern and extent of TLR expression, of neuroinflammation and neurodegeneration in the CNS, a mechanism by which the intrinsic innate immunity might co-determine the diversity of CNS diseases. Further, as demonstrated with the endogenous TLR4 ligand HSP60, the release of TLR ligands during CNS cell damage, a common event in many forms of brain disease, bi-directionally links CNS injury to neurodegeneration and demyelination *in vivo*.

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Berlin,

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