Expression of Toll-like receptors in the nervous system

A survey of the literature and some additional experiments

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Degree project in biology, 2007
Examensarbete i biologi, 20p, 2007
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Abbreviations

ALS  Amyotrophic lateral sclerosis
AP   Area postrema
AP-1 Activator-protein 1
BBB  Blood-brain barrier
CARD Caspase recruitment domain
cDNA Complementary DNA
Chp  Choroid plexus
CVO  Circumventricular organ
EAE  Experimental autoimmune encephalomyelitis
HIV  Human immunodeficiency virus
IFN  Interferon
IKB  Inhibitor of NF-KB
IKK-I Inducible IKB kinase
IRAK IL-1R-associated kinase
IRF  IFN regulatory factor
JNK  c-Jun N-terminal kinase
MAPK Mitogen-activated protein kinases
MDA  Melanoma differentiation-associated gene
ME   Median eminence
MDC  Myeloid dendritic cells
MPOA Medial preoptic area
MS   Multiple Sclerosis
MyD88 Myeloid differentiation primary response protein 88
NF-KB Nuclear factor kappa-b
OVLT Organum vasculosum of the lamina terminalis
PAMP Pathogen-associated molecular pattern
PDC  Plasmacytoid dendritic cells
PGN  Peptidoglycan
Poly(I:C) Polyinosonic polycytidylic acid
PRR  Pattern recognition receptor
RIG-1 RNA helicase retinoic acid-inducible gene-1
RIP-1 receptor interacting protein-1
SFO  Subfornical organ
SFV  Semilike Forest Virus
TAK  TGF-β activated kinase
TANK TRAF family member-associated NF-KB activator
TBK  TANK-binding kinase
TIR  Toll IL-1 receptor
TIRAP TIR-domain-containing-adaptor-protein
TLR  Toll-like receptor
TNF  Tumor necrosis factor
TRAM TRIF-related adaptor molecule
TRAF Tumor necrosis factor (TNF) receptor-associated factor
TRIF TIR-domain-containing-adaptor inducing IFNβ
Summary

Toll-like receptors (TLRs) are a part of the innate immune system, which is the first line of defense to pathogens. Thirteen TLRs that are all structurally related and together respond to a broad range of pathogens have been discovered in mammals. The binding to the receptor will start an intracellular signaling pathway resulting in the activation of proinflammatory genes. Binding to certain TLRs induces the activation of type 1 interferons. The Toll receptor was first discovered in *Drosophila* in which it was involved in the development of the dorsoventral polarity. In addition, *Drosophila* with a dysfunctional Toll gene is more susceptible to fungal infections. Later, homologues to the Toll receptor were found in humans. Toll-like receptors seem to play a very important part in immunity since they recognize a great variety of pathogens.

Several pathogens infecting the central nervous system start by entering the peripheral nervous system (PNS) and therefore TLRs could hypothetically be more prevalent as a defense mechanism in the peripheral nervous system than in the central nervous system (CNS). To test the hypothesis I have compared the expression of TLRs in hippocampus (CNS) and trigeminal ganglia (PNS) by purifying RNA, converting it to cDNA, amplifying it and finally visualizing it on a gel. I have also reviewed previous research concerning TLR expression in the nervous system. The literature has shown that most TLRs are expressed in the brain although not in all cell-types. The material on TLR expression in PNS has been limited. From my experiments it seemed like TLRs 7 and 9 were expressed in nerve cells from PNS but not in nerve cells from CNS. The lack of TLRs 7 and 9 in nerve cells in CNS is consistent with previous studies. Although I did not test all TLRs there seemed as at least two of these receptors had a wider expression in PNS.
Introduction

Background

Toll-like receptors (TLRs) are a family of structurally related receptors. Thirteen different receptors have been found in mammals but only ten in humans. They are pattern recognition receptors (PRRs) and all share a leucine-rich repeat recognizing their ligand extracellularly and a Toll IL-1 receptor in their intracellular domain (Figure 1) (Pandey et al. 2006).

Figure 1: General structure of a TLR containing a leucine rich domain extracellularly and a TIR domain intracellularly. Also in the figure is an adaptor molecule containing a TIR domain. Reprinted, with permission, from the Annual Review of Immunology, Volume 24 © 2006 by Annual Reviews www.annualreviews.org

The first Toll-receptor was found in Drosophila melanogaster and was involved in the development of the dorsoventral polarity (Lemaitre et al. 1996). Mutations in the gene coding for the Toll-receptor in adult Drosophila was associated with a higher susceptibility to fungal infections (Hoffmann 2003). The Toll-receptor was first discovered by the German scientists Nüesslein-Volhard and Wieschaus. When they first viewed the strangely developed embryos Nüesslein-Volhard cried out “Toll”, which means “amazing” or “cool” in German. (Opal et al. 2002) Later TLR4, which showed sequence similarity with the Drosophila Toll and therefore got the name Toll-like receptor, was found in humans. Not only the TLR showed homology, but also the molecules in its pathway (Medzhitov et al. 1997). The TLRs can recognize a wide range of infectious organisms. Components of bacteria, viruses etc act as ligands and their binding to the receptors can activate different signaling pathways leading to various cellular responses to the infection.
TLRs are found in most multicellular organisms and are a part of the innate immune system. The immune system can be divided into the innate and adaptive immune system. The innate system has no memory. One of its components, cytokines, are signaling proteins, like hormones, but involved in immune responses. Other examples are the complement system which is a cascade of small proteins helping in clearing an organism from pathogens, and natural killer cells that can kill for example microbial cells (Préhaud et al. 2005). The adaptive immune system has a memory. After an infection, memory B-cells and T-cells will develop recognizing the specific pathogen so that the next time the host is infected by the same pathogen the immune system can protect it much faster. It is only present in vertebrates (Hoffman 2003). The innate and adaptive system can signal to each other and TLRs are also involved in the regulation of the adaptive immune system (Takeda et al. 2003). There has been much research about the adaptive immune system but in recent years there has been more focus on the innate immune system (Germain 2003). The TLRs were discovered in the 1990’s and since then there has been intense research concerning them. They seem to respond to most pathogens and therefore have a very important part in immunity.

Ligands

The toll-like receptors can bind a great variety of pathogens like bacteria, protozoa, viruses and fungi (Figure 2). They recognize conserved patterns called pathogen-associated molecular patterns (PAMPs). The TLRs are further divided into subfamilies based on amino acid sequence similarity and genomic structure. As a result, the TLRs within a subfamily recognize the same class of PAMPs (Takeda et al. 2003).

TLR1 can recognize triacyl lipopeptides. It is also reported to associate with TLR2 and these together can respond to soluble factors released from the bacterium *Neisseria meningitides*. TLR2 by itself can be stimulated by many different microbial parts. In particular, peptidoglycan (PGN) from both gram-positive and -negative bacteria, and lipoproteins are recognized by TLR2. TLR6, which is very similar to TLR1, also can associate with TLR2 and together they can recognize microbial lipopeptides. TLRs 1, 2, 6 and 10 all are in the same subfamily and are present in the cytoplasmic membrane. (Takeda et al. 2003) No ligand that binds to TLR10 has been found yet and it is not present in mice (Beutler et al. 2006).

Double-stranded RNA, which is often produced by different viruses, stimulates TLR3. Poly I:C is a synthetic form of dsRNA, which is also recognized by TLR3. TLR3 is present in endosomal compartments. (Takeda et al. 2003) TLR4 is mainly stimulated by lipopolysaccharide (LPS), which is a cell-wall component of gram-negative bacteria. Before binding to TLR4, LPS will bind to LPS-binding protein and the complex is then recognized by CD14, a molecule that is preferentially expressed in monocytes/macrophages and neutrophils. TLR4 can recognize other ligands as well. TLRs 3 and 4 are each subdivided into their own subfamily. (Takeda et al. 2003)

Flagella are motility organelles of bacteria that help them to move. Flagellin is the main protein component of flagella and is also the ligand for TLR5. TLR11 has been found in mice but is only present as a pseudogene in humans. TLR11, which is a relative to TLR5, recognizes components of
uropathogenic bacteria and profilin-like molecule, which is an actin-binding protein, from *Toxoplasma gondii*, which is a parasitic protozoa (Uematsu and Akira 2006).

TLRs 7, 8 and 9, which are all similar, are members of the same subfamily and have an intracellular localization. TLR9 can recognize CpG DNA, both viral and bacterial. In addition it is reported to recognize the malaria pigment hemozoin (Kawai and Akira 2006). The natural ligands of TLRs 7 and 8 are not fully known. TLR7 recognizes synthetic imidazoquinolines-like molecules, which are similar to nucleic acids. Human, but not murine, TLR8 has been reported to respond to imidazoquinolines. In addition human TLRs 7 and 8 can respond to guanosine- or uridine-rich ssRNA from viruses including HIV and influenza (Uematsu and Akira 2006). The ligands for TLR12 and 13 are not known yet (Takeda et al. 2003).

**Intracellular Toll-like receptor signaling**

The stimulation of a TLR results in the activation of various genes involved in the immune system. The TLRs mainly use one of two pathways, the MyD88- dependent or -independent pathway. Nevertheless, there is still much research being done concerning the pathways and the subsequent description is an outline of the pathways.

*The Myeloid differentiation primary response protein 88-Dependent Signaling Pathway*

All TLRs except TLR3 can use the myeloid differentiation primary response protein 88 (MyD88) - dependent signaling pathway (Figure 3 and 4). After a TLR is stimulated by a ligand, MyD88 will
bind to the Toll IL-1 (TIR) domain of the cytoplasmic part of the TLR. TLRs 1, 2, 4 and 6 will also use TIR-domain-containing-adaptor-protein (TIRAP) as an adaptor molecule. Thereafter IL-1R-associated kinase (IRAK) 4 associates with the complex and phosphorylates IRAK1. Tumour necrosis factor receptor-associated factor (TRAF) 6 will then associate with the phosphorylated IRAK. The two together will phosphorylate TGF-β activated kinase (TAK) 1, which will subsequently phosphorylate two molecules from the MAPK family. Furthermore AP-1 will translocate to the nucleus after activation from mitogen-activated protein kinases (MAPKs). In addition TAK-1 will phosphorylate a complex of inhibitor of NF-κB (IκB) kinase and induce ubiquitination-induced degradation of IκB. This will lead to a nuclear translocation of nuclear factor-κappa-b (NF-κB). NF-κB and activator-protein (AP) -1 will together activate genes encoding pro-inflammatory cytokines and chemokines like tumor necrosis factor (TNF) -α, interleukin (IL) -6, IL-12 and IL-1β among others (Naka et al. 2005). Cytokines are proteins signaling between cells involved in immune responses.

TLRs 7, 8 and 9 signaling (Figure 3) differ slightly from the previous pathway since they can also activate type 1 interferon (IFN) genes. IFN regulatory factor (IRF) 7, which is a transcription factor, will then get activated and translocated to the nucleus where it will induce transcription of IFN-α and IFN-β, which are called type 1 IFN and are cytokines with antiviral activity (Uematsu and Akira 2006).

It has been suggested that, IRF-5 act together with NF-κB as a transcription factor binding to the pro-inflammatory cytokine genes, after activation of TLRs 3, 4, 5 and 7 and possibly other TLRs (Takaoka et al. 2005).

The Myeloid differentiation primary response protein 88-Independent Signaling Pathway

TLRs 3 and 4 use the MyD88-Independent Signaling Pathway (Figure 4). In this pathway TIR-domain-containing-adaptor inducing IFN-β (TRIF) can activate NF-κB either by using TRAF-6 or receptor-interacting protein (RIP) -1. TLR4 also interacts with TRIF-related adaptor molecule (TRAM) when interacting with TRIF. RIP-1 activates the inducible IκB kinase (IKK) complex while TRAF-6 has the same function as in the MyD88-dependent pathway and activates TAK-1 (Kawai et al. 2006). In addition, TRIF is interacting with TANK-binding kinase (TBK) -1 and inducible IκB kinase (IKK-i). They will phosphorylate IRF-3 which will translocate to the nucleus and activate the transcription of type1 IFN genes (Pandey and Agrawal 2006).
Figure 3: The MyD88-dependent pathway and the activation of IRF7 by TLR7, 8 and 9 leading to the transcription of type 1 IFN and inflammatory cytokines. For a more detailed description see text on p. 6-7. Reprinted by permission from Macmillan Publishers Ltd: Cell Death and Differentiation (Kawai and Akira), copyright (2006) www.nature.com/cdd

Figure 4: The MyD88-dependent and -independent pathway leading to the transcription of type 1 IFN and inflammatory cytokines. For a more detailed description see text on p. 6-7. Reprinted by permission from Macmillan Publishers Ltd: Cell Death and Differentiation (Kawai and Akira), copyright (2006) www.nature.com/cdd
Signaling to the adaptive immune system

The TLRs are involved in the signaling to the adaptive immune system through the activation of T-cells. In particular the T helper (T\(_H\))1 response seems to be induced by TLR signaling. T lymphocytes can either express a cluster of differentiation (CD) 4 or CD8 surface molecule. Furthermore, T helper cells (T\(_H\)), which have the CD4 molecule, induce a T\(_H\)1 or T\(_H\)2 response. The T\(_H\)1 response is characterized by its release of proinflammatory cytokines (Berger 2000). The T\(_H\)1 response seems to involve the MyD88-dependent pathway. A mouse deficient in MyD88 immunized with ovalbumin mixed in controlled Freund’s adjuvant failed to elicit a Th1 response (Schnare et al. 2001). Several studies show an involvement of TLRs with the maturation of dendritic cells, which are involved in the activation of naïve T-cells (Takeda et al. 2003). For example the maturation of dendritic cells by TLRs 2 and 4, induced by PGN, is shown (Michelsen et al. 2001). Furthermore, B-cells express most TLRs. They are also activated by ligands to TLRs (Pasare and Medzhitov 2004).

Cells expressing Toll-like receptors

Immune cells

TLRs are expressed in many different cell types in the body. In particular they are expressed in several innate immune cells. Immune cells like monocytes and macrophages express most TLRs except TLR3. In addition NK cells, neutrophils and basophils (Pandey and Agrawal 2006) and granulocytes (Zareember and Godowski 2002) have been reported to express TLRs. Both myeloid dendritic cells (MDCs) and plasmacytoid dendritic cells (PDCs) are found in human blood. MDCs express TLRs 1, 2, 4, 5 and 8 while PDCs express TLR7 and 9. Immature dendritic cells express TLRs 1, 2, 4 and 5 and the expression decreases with maturation caused by exposure to microbes. TLR3 is only expressed in mature dendritic cells (Takeda et al. 2003). TLRs 2, 4, 6 and 8 are expressed in mast cells (Takeda et al. 2003). Adaptive immune cells like B-cells and regulatory T-cells express TLRs as well (Pandey and Agrawal 2006). In T-cells TLRs 1-5 has been detected and TLRs 7 and 9 at lower levels (Caron et al. 2005).

Other types of cells

The intestinal epithelial cells express TLR4 at low levels while TLR5 is only expressed on the basolateral surface (Takeda et al. 2003). TLRs 2 and 4 are expressed in renal epithelial cells (Takeda et al. 2003). Human dermal epithelial cells express TLR4 (Takeda et al. 2003). TLR11 is expressed in kidney and bladder (Uematsu and Akira 2006). Small airway epithelial cells, which are important in protecting the lung from infections, are reported to express TLRs 1-6 constitutively (Ritter et al. 2005). Muscle cells express low constitutive levels of TLRs 1-7 and 9 in humans (Schreiner et al. 2005). According to a study on TLR expression in human tissues (Nishimura and Naito 2005), TLR1 is expressed at high levels in kidney, lung and spleen. TLR2 shows the highest expression in lung and spleen while TLR3 is expressed at the highest level in the placenta. The spleen is where TLR4 is expressed at the highest level. TLR5 shows the highest expression in trachea, placenta, lung, prostate, salivary gland and kidney, although the levels are relatively low. TLR6 is expressed at the highest level in the spleen. TLR7 has the highest
expression levels in the lung, placenta, spinal cord and spleen. Lung and spleen are the organs where TLR8 is expressed at the highest level and TLR9 has the highest expression levels in skeletal muscles and spleen. The spleen and thymus show the highest expression levels of TLR10. In mouse, TLR3 is expressed in lung, brain and kidney (Alexopoulou et al. 2001). Heart, brain and muscle are reported to express TLR2 in humans (Rock et al. 1998).

**RNA helicase retinoic acid-inducible gene-1**

Apart from the TLRs there are several other pattern recognition receptors. One of them is RNA helicase retinoic acid-inducible gene-1 (RIG-1). It was recently discovered and recognizes intracellular viruses. RIG-1 is a receptor residing in the cytoplasm binding dsRNA. It is also suggested to bind ssRNA bearing 5' phosphates (Pichlmair et al. 2006). It contains one helicase domain at its C-terminus and two caspase recruitment domain (CARD) motifs at its N-terminus. The helicase domain is able to unwind dsRNA and the CARD motifs seem to be interacting with signaling molecules leading to the activation of IRF-3 and NF-κB (Figure 5) (Yoneyama et al. 2004). Although the signaling pathway is not fully known it seems like TBK1 and IKK-i are involved in the signaling. They are two molecules also involved in TLR signaling in the MyD88-independent pathway (Perry et al. 2005). Additionally, two receptors showing homology with RIG-1 have been found, the melanoma differentiation-associated gene 5 (Mda5), which also contains two CARD motifs, and LGP2, which does not. Both receptors have a helicase domain (Kawai and Akira 2006). RIG-1 mRNA has been detected with an Affymetrix array in cultured human nerve cells and further an upregulation of the receptor has been observed after Rabies virus infection (Préhaud et al. 2005).

![Figure 5: RIG-1 pathway. For a more detailed description see text. Reprinted by permission from Macmillan Publishers Ltd: Cell Death and Differentiation (Kawai and Akira), copyright (2006) www.nature.com/cdd](image-url)
Reverse Transcriptase

Reverse transcriptase is an enzyme that can transcribe RNA into DNA. Retroviruses, like HIV, have the capability to transcribe their RNA to DNA which they can insert into the host genome (Flint et al. 2003). Therefore it is possible to make complementary DNA (cDNA) from RNA by using reverse transcriptase. The cDNA can then be amplified by polymerase chain reaction (PCR).

Aim

In this project I aim to study the differences in expression of Toll-like receptors in the peripheral nervous system (PNS) and central nervous system (CNS). The presence of more TLRs in PNS than in CNS could be functional since viruses and microbes, infecting the nervous system, usually first infect PNS before entering CNS. Previous unpublished studies on cultured nerve cells have shown that cells from PNS expressed more Toll-like receptors than cells from CNS. I also aimed to make an extensive review of the literature on research on Toll-like receptors in the nervous system.
Results

I Literature review: Toll-like receptors in the nervous system

Toll-like receptors in different cell-types in CNS

The CNS is well-protected from immune cells that can cause harm to the brain. Since the brain, in contrast to the rest of the body, has a limited regenerative capacity after an injury, it is more vulnerable to inflammation. The blood-brain barrier (BBB) prevents many macromolecules from entering into the brain. It consists of endothelial cells that are sealed with tight junctions (Hickey et al. 2001). Furthermore, there is a blood-cerebrospinal fluid barrier that protects the CNS. Additionally, within the CNS anti-inflammatory mediators like transforming growth factor–β (TGF-β) and α-melanocyte stimulating hormone (α-MSH) are produced (Aloisi 2001). The brain has long been thought to have a very limited immune surveillance but today it is known that most immune cells can enter the CNS although the levels of them are much lower than in the rest of the body (Hickey 2001). T-cells are known to be able to cross the BBB (Hickey 2001). There is evidence that B-cells can enter into the brain when the BBB is altered, but it is not known whether they can enter under normal circumstances (Hickey 2001). Both dendritic cells, which stimulate T-cells, and macrophages are detected in the meninges and choroid plexus (Aloisi 2001). Monocytes are circulating white blood cells that carry out phagocytosis and develop into macrophages when entering tissues. Microglia, which is one of the most important types of immune cells of the CNS, probably originate from the monocyte/macrophage lineage and enters the CNS before birth (Hickey 2001). Microglia can secrete cytokines and neurotrophic factors. They are also able to carry out phagocytosis and are important in reparation. Nevertheless, all their functions are not known yet (Aloisi 2001).

Microglia

Microglia is the cell-type within the CNS with the broadest TLR expression. TLRs 1-9 have been detected in both mouse and human. (Table 1 and 2)

Expression levels in mouse

The cultured microglia showed different expression levels in the studies performed. TLRs 2 and 6-8 showed the highest levels, TLRs 4-5 middle levels whereas TLRs 1, 3 and 9 showed the lowest levels (Olson and Miller 2004). Another study on microglia in vitro detected the highest transcription level of TLR2, medium levels of TLRs 4 and 7 and the lowest levels of TLRs 1-4, 6, 7 and 9 while TLR5 were not detected at all.

Expression levels in humans

The level of expression differed between the studies. According to the study on cultured cells (Bsibsi et al. 2002) TLRs 2 and 3 were expressed at the highest levels, TLRs 1, 4-8 at medium levels while 9 at the lowest level. In another study on cultured cells TLRs 1, 2, 4 and 7 were expressed at higher levels than TLRs 3, 5, 6, 8 and 9. Expression of TLR10 was not detected at all. In addition, differences in expression level between individuals were found (Jack et al. 2005).
Astrocytes

In mouse astrocytes the TLR expression is as extensive as in microglia and TLRs 1-9 have been detected, whereas in human only TLRs 1-5 and 9 have been found. (Table 3 and 4)

Expression levels in mouse
In cultured astrocytes, levels of TLR3 mRNA were the highest of all TLRs detected followed by TLRs 1 and 6 mRNA while TLRs 7 and 8 mRNA were at the lowest levels of them all (Carpentier et al. 2005). Another study also in vitro detected the highest level of TLR7 mRNA whit TLRs 2, 3, 8 and 9 mRNA at intermediate levels and TLRs 1, 4, 5 and 6 mRNA at the lowest levels (McKimmie and Fazakerley 2005).

Expression levels in human
One study (Farina et al. 2004) only detected mRNA from TLR3 in vitro. In addition, another study detectd TLR3 mRNA at the highest level and TLRs 1, 4, 5 and 9 mRNA at lower levels (Jack et al. 2005). Bsibsi et al. (2002) found TLRs 2 and 3 mRNA, the latter at the highest level. On the other hand, Bsibsi et al. (2006) found TLR4 mRNA at the highest level in vitro, thereafter TLR2, followed by TLR3 while TLR1 at the lowest level. They did not analyze the presence of the other TLRs.

Neurons

In mouse neurons TLRs 1-4 and 6 have been found. In human only TLRs 1-4 have been detected but then the others have not been tested. (Table 5 and 6)

Oligodendrocytes

Oligodendrocytes express the fewest TLRs. TLRs 1-4 have been detected in mouse. (Table 7)
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<th>TLR</th>
<th>Ability to respond to different compounds</th>
<th>mRNA In vitro</th>
<th>Protein In vitro</th>
<th>mRNA In vivo</th>
<th>Protein In vivo</th>
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For details concerning in vitro and in vivo or mRNA and protein see text in table.
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<td>Yes, with immunostaining (Bibsi et al. 2002) and with flow cytometry (Jack et al. 2005)</td>
<td>No, not with semi-quantitative PCR (Jack et al. 2005) -</td>
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<td>TLR5</td>
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<td>TLR10</td>
<td>No, not with semi-quantitative PCR (Jack et al. 2005)</td>
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No data concerning in vivo studies or studies of stimulated cells were found in the literature.
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<th>TLR</th>
<th>Ability to respond to different compounds</th>
<th>mRNA in vitro</th>
<th>Protein in vivo</th>
<th>mRNA in vitro</th>
<th>Protein in vivo</th>
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<td>-</td>
<td>Yes</td>
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No data concerning in vivo or in vitro studies of expression of TLRs 5-9 were found in the literature.

1 For details concerning in vivo and in vitro or mRNA and protein see text in table.

2 For details concerning in vivo and in vitro or mRNA and protein see text in table.

TLR4 mRNA with Affymetrix U133 Array

TLR4 Protein

TLR3 mRNA with Affymetrix U133 Array

TLR3 Protein

TLR2 mRNA with Affymetrix U133 Array

TLR2 Protein

TLR1 mRNA with Affymetrix U133 Array

TLR1 Protein
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1 No data concerning in vivo studies or studies of stimulated cells were found in the literature
**Toll-like receptors in different areas of CNS**

Using quantitative RT-PCR on a whole mouse brain, mRNA from TLRs 1-9 and 13 were found. Compared to the spleen the CNS showed lower levels of all TLR mRNA except for TLR3 for which the mRNA levels were equal in both organs. Levels of TLR transcripts were also measured after infection with *Semilike Forest Virus* (SFV) and an upregulation of TLRs 1-3, 7-9 and 13 was observed. Also different mice strains showed a great variety in mRNA levels of different TLRs. (McKimmie et al. 2005)

A study (Mishra et al. 2006) on mouse brain showed the presence of transcripts of TLRs 1-9, with GEArray analysis, and an upregulation of all TLRs except TLR5 after infection with *M. corti*. However, not all TLRs showed expression on a protein-level with in situ IF microscopy. TLRs 1, 3, 4 and 9 were not found as proteins. TLR2 showed the highest protein expression of all TLRs in normal mouse brain. After infection with *M. corti* all TLRs except TLR5 were found as proteins in the CNS. Astrocytes expressing TLR2 were found at the highest levels in periventricular and leptomeningeal areas while neurons and microglia expressing TLR2 were mostly found in the parenchyma. TLR6 expression showed a similar pattern.

The circumventricular organ (CVOs), which includes the organum vasculosum of the lamina terminalis (OVLT), the subfornical organ (SFO), the median eminence (ME) and the area postrema (AP), do not have a blood brain barrier. Instead large molecules can enter. Additionally, the choroids plexus (chp) and the leptomeninges are highly vascularized (Nguyen et al. 2002). Since these areas are in contact with the blood stream they are more exposed to infectious organisms, toxins etc and therefore should have a larger need for immune surveillance and consequently these areas have been more extensively studied.

**TLR2**

In mice, TLR2 mRNA has been detected in various areas using in situ hybridization. A hybridization signal was found in the chp, around the lateral ventricle and the SFO. Moreover, it was found at the the fimbria, stria terminalis, the optic tract and at the inferior cerebellar peduncle (Laflamme et al. 2001 and Zekki et al. 2002). In addition TLR2 transcripts have been detected in the anterior paraventricular nucleus of the thalamus, the supraoptic nucleus, the ependymal tanycytic lining cells of the ventral third ventricle, the paraventricular nucleus of the hypothalamus and in some cells in the lateral hypothalamic area. Injection of LPS increased the level of TLR2 in structures accessible by the bloodstream and additionally in parenchymal structures (Laflamme et al. 2001).

**TLR4**

In rats, TLR4 mRNA was found, using in situ hybridization, in areas accessible by the bloodstream including the leptomeninges and the CVO like OVLT, SFO, ME and AP. In addition it was found in some parts of the parenchyma like the supramammillary nucleus, cochlear nucleus, the posterior hypothalamic nucleus and the ventrolateral medulla. Furthermore, parenchymal structures like the MPOA, arcuate nucleus, the dorsomedial hypothalamic nucleus, the paraventricular nucleus of the
hypothalamus, the ventromedial hypothalamus surrounding the aqueduct and central canal and the dorsovagral complex also showed a TLR4 mRNA hybridization signal (Laflamme and Rivest 2001).

According to another study (Chakravarty and Herkenham 2005) in mouse, using in situ hybridization, TLR4 mRNA was found at the highest level in the cph and meninges. Furthermore TLR4 transcripts were found spread around the parenchyma. The TLR4 mRNA appeared in isolated cells and around fenestrated capillaries in CVOs like SFO, ME and AP. Additionally TLR4 expression was detected in the ventricular ependyma.

The Allen Brain atlas (http://www.brain-map.org) is a database showing all genes expressed in the mouse brain. They have used in situ hybridization to detect the transcripts. In addition to TLRs 1-9 it shows the expression of TLRs 11-13. In particular TLR12 seems to be widely expressed in the brain.

PNS

The research concerning TLR expression in the peripheral nervous system is not as extensive as the research about the central nervous system. However, some experiments have been performed. TLR4 and the receptor CD14 have been detected at a protein-level with immunohistochemistry in human and rat trigeminal sensory neurons (Wasachi and Hargreaves 2006). Both TLR4 mRNA and protein have been found in the rat nodose ganglion with RT-PCR and Western blot (Hosoi et al. 2005). Constitutive expression of TLR2 protein is detected on human cultured Schwann cells with flow cytometry and in vivo using immunofluorescence staining. The same investigators detected expression of TLR2 in nerve cells using immunofluorescence (Oliviera et al. 2003).

TLRs and neurodegeneration

Many of the studies concerning TLR expression concerned the role of TLRs in neurodegeneration. Some of their results are summarized as follows:

Mixed cell cultures from rat forebrain treated with LPS show loss of microglia, oligodendrocytes and axons. In addition, for neuronal loss to occur microglia is required. Furthermore, mice with a defect TLR4 gene do not show any cell loss, implying that the cell damage is mediated by activation of TLR4 signaling pathways (Lehnardt et al. 2003). TLR2 were found to be involved in neurodegeneration induced by group B Streptococcus. Also in this study microglia seemed to be required. Moreover, the neural loss was indicated to be due to apoptosis as examined by tunel assay (Lehnardt et al. 2006). Cell death in hippocampal areas and the dentate gyrus were observed after treatment with the TLR9 agonist CpG DNA. In addition, microglia seem to be needed for the neural loss in vitro. The neurodegeneration starts in neurites before spreading. TNF-α and iNOS have been found to be involved in neural damage (Iliev et al. 2003). The presence of TLR3 protein was detected with immunohistochemical staining in Purkinje cells in patients with ALS and Alzheimer’s disease (Jackson et al. 2006). White matter from healthy humans and Multiple Sclerosis (MS) patients compared in vivo using immunohistochemistry showed higher TLRs 3 and 4 protein expression levels in MS patients (Bsibsi et al. 2002).
Experimental autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is a mouse model of the human disease MS, which is characterized by demyelination. EAE was shown to be induced by several ligands to TLRs like pertussis toxin, mediated by TLR4 (Kerfoot et al. 2004) and mycobacteria and LPS (Hansen et al. 2005). The hybridization signal of TLR2 was stronger in certain areas like the chp, the leptomeninges under the optic tract, the periamygdaloid cortex and areas lining the cochlear nucleus in mice with EAE as compared with healthy mice. The presence of the LPS receptor CD14 was also observed (Zekki et al. 2002). Higher levels of TLRs 1, 2, 4 and 6-9 were found CNS in mouse with EAE. Mice deficient in TLR9 developed a weaker and more delayed disease than wt mice while mice deficient in MyD88 did not develop the disease at all. Deficiency in TLR2 on the other hand had no effect on EAE development (Prinz et al. 2006).

Neuroprotection

TLRs are also reported to be involved in neuroprotection. Cultured human astrocytes treated with poly I:C, activating TLR3, induces many neuroprotective molecules (Bsibsi et al. 2006). In addition, treatment with poly I:C suppresses EAE in mice possibly mediated through IFN-β (Touil et al. 2006). Furthermore, mice with Alzheimer’s disease showed a greater level of amyloid β-protein (Aβ) when also having a TLR4 defective gene after stimulation with LPS (Tahara et al. 2006).

TLR8 and development

Ma et al. (2006) showed that TLR8 could be involved in brain development. As analyzed with Western blot in mouse, TLR8 first appeared at embryonic day 12 and increased until postnatal day 21, when it suddenly decreased. This time period coincides with that of the completion of neurogenesis and axonogenesis. In addition immunohistochemical studies show that the regional localization of TLR8 changes through development. Furthermore, stimulation with the synthetic ligand R-848 to TLR8 inhibits the outgrowth of neurites from cultured neurons.
II Experimental investigation

In addition to the literature overview, which contained relatively little observations in vivo and from PNS, I also performed some experiments in vivo from both CNS and PNS.

Expression of TLRs and signaling molecules in trigeminal ganglia and hippocampus

I tested the expression of the genes TLRs 3, 4, 7, 9, IRF3, 5, 7, MyD88, TRAF-6 and RIG-1 with RT-PCR on tissues from trigeminal ganglia (PNS) and hippocampus (CNS). β-actin, which is expressed in all cells, was also tested to be able to compare RNA levels in the different samples (Figure 6). The tissues I examined contained various cell-types. The tissue from hippocampus contained nerve cells and microglia, astrocytes and oligodendrocytes. Whereas the tissue from trigeminal ganglia contained nerve cells and Schwann cells. The glial cells are more abundant than the nerve cells in both PNS and CNS. I detected all the genes in both hippocampus and trigeminal ganglia (Figure 7 and 8).

β-actin

Figure 6: Expression of β-actin (263 bp) in trigeminal ganglia and hippocampus. PCR was run for 30 cycles. Lane L contains ladder (100 bp), lane 1 contains trigeminus ganglia sample 1, lane 2 contains trigeminus ganglia sample 2, lane 3 contains hippocampus sample 1, lane 4 contains hippocampus sample 2 and lane C contains control that contained water instead of template.

TLR3 TLR4 TLR7 TLR9

Figure 7: Expression of TLRs 3 (205 bp), 4 (325 bp), 7 (338 bp) and 9 (339 bp) in trigeminal ganglia and hippocampus. PCR was run for 40 cycles in a and 35 cycles in b. Lane L contains ladder (100 bp), lane 1 contains trigeminus ganglia sample 1, lane 2 contains trigeminus ganglia sample 2, lane 3 contains hippocampus sample 1, lane 4 contains hippocampus sample 2 and lane C contains control that contained water instead of template.
Expression of TLRs in nerve cells and glia cells in trigeminal ganglia and hippocampus

To analyze more specifically the cell-type expressing the TLRs and RIG-1, I dissected areas from hippocampus and trigeminal ganglia with laser containing nerve cells or glial cells respectively (Figure 11 and 12). Thereafter I performed a RT-PCR. I made the dissection twice. In the experiment after the first dissection I detected RIG-1 in all samples, TLR4 in glial cells from hippocampus and in both nerve cells and glial cells from trigeminus ganglia (Figure 9). I could not find TLRs 7 and 9 in any of the samples (Figure 10). In the experiment after the second dissection I detected TLR4 solely in nerve cells and glial cells from PNS and TLRs 7 and 9 exclusively in nerve cells from PNS (Figure 11).

Expression of TLRs in nerve cells and glia cells in trigeminal ganglia and hippocampus

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Figure 8: Expression of MyD88 (315bp), RIG-1 (212bp), TRAF-6 (241bp), IRF3 (293bp), IRF5 (222bp) and IRF7 (336bp) in trigeminal ganglia and hippocampus. PCR was run for 40 cycles in a and 35 cycles in b. Lane L contains ladder (100 bp), lane 1 contains trigeminus ganglia sample 1, lane 2 contains trigeminus ganglia sample 2, lane 3 contains hippocampus sample 1, lane 4 contains hippocampus sample 2 and lane C contains control that contained water instead of template.

Figure 9: Expression of β-actin (263 bp), TLR4 (325 bp) and RIG-1(212 bp) in hippocampus and trigeminal ganglia after first dissection of areas containing nerve cells or glial cells. PCR was run for 33 cycles in a, 38 cycles b-c. Lane L contains ladder (100 bp), lane 1 contains nerve cells from hippocampus, lane 2 contains glial cells from hippocampus, lane 3 contains nerve cells from trigeminus ganglia, lane 4 contains glial cells from trigeminus ganglia and lane C contains control that contained water instead of template.
Figure 10: Expression of TLRs 7 (338 bp) and 9 (339 bp) in hippocampus and trigeminal ganglia after first dissection of areas containing nerve cells or glial cells. PCR was run for 43 cycles. Lane L contains ladder (100 bp), lane 1 contains nerve cells from hippocampus, lane 2 contains glial cells from hippocampus, lane 3 contains nerve cells from trigeminus ganglia, lane 4 contains glial cells from trigeminus ganglia and lane C contains control that contained water instead of template.

Figure 11: Expression in hippocampus and trigeminal ganglia of β-actin (263 bp), TLRs 4 (325 bp), 7 (338 bp) and 9 (339 bp) after second dissection of areas containing nerve cells or glial cells. PCR was run for 43 cycles. Lane L contain ladder (100 bp), lane 1 contain nerve cells from hippocampus, lane 2 contain glial cells from hippocampus, lane 3 contain nerve cells from trigeminus ganglia, lane 4 contain glial cells from trigeminus ganglia and C contain control that contained water instead of template.
Table 9: Overview of my experiments concerning expression of TLRs 4, 7, 9 and RIG-1 in nerve cells and glial cells in CNS and PNS respectively.\(^1\)

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\(^1\) Levels of expression are roughly estimated from fragment sizes and normalized to β-actin. Levels are shown with (+), +, ++.
Discussion

TLR expression in the nervous system

Thirteen TLRs are found in mammals of which TLR10 is not present in mice and TLR11 is not present in humans. Many recent studies concerning TLRs in the CNS show that most of them are expressed in the brain. TLRs 1-9 are all expressed in human brain (Table 2, 4, 6 and 7) and in addition to TLRs 1-9, TLR13 is detected in mouse brain (McKimmie et al. 2005). TLR10 has not been found so far in the human brain. The presence of TLRs 11 and 12 have not been studied in the nervous system since they in addition to TLR13 are the most recently discovered TLRs. Nevertheless, the Allen Brain Atlas (http://www.brain-map.org), which is a database with all genes expressed in the mouse brain, show the expression of TLRs 11 and 12.

Microglia is the cell-type with the broadest TLR expression (Table 1 and 2), which is not surprising since microglia have many immune functions (Aloisi et al. 2001). The TLR expression in astrocytes is as broad as the expression in microglia in mice (Table 3) whereas in humans the expression is narrower (Table 4). The TLR expression in human neurons is not fully studied. Only TLRs 1-4 are detected in human neurons (Table 6), but the presence of the others has not been analyzed. Mouse neurons have been studied more extensively and in addition to TLRs 1-4 also TLR6 is found. Also TLR7 protein could be detected after infection with the parasite *Mesocestoides corti* (Table 5). Oligodendrocytes contain the fewest expressed TLRs of all CNS cell types (Table 7).

Most experiments have been performed in vitro, so the actual TLR expression in vivo remains to be clarified. There are some contradictory studies on the TLR expression in particular cell-types (Table 1-5). There could be many reasons for this. Different methods differ in sensitivity. Also the same method can vary in detection depending on the antibody used or the primer in a PCR. Furthermore, cultured cells could show different expression even though they are of the same cell-type, depending on where in the brain they have their origin and their age as well as the culture conditions employing various factors in the media and surface coatings. There is also a study (Ma et al. 2006) showing that TLR8 expression changes during development. In addition, the level of expression differs between the studies. This could also be a result of different strains of mice being tested, since differences in TLR expression between mice strains has been previously observed (McKimmie et al. 2005). The level of expression also seems to differ between individuals at least in humans (Jack et al. 2005).

TLR expression in the brain has been particularly abundant in different areas in the CVO, the chp and the leptomeninges. These areas are more exposed to factors in the circulating blood than the rest of the brain, since they lack a BBB.

Experiments

In nervous tissue containing mixed cell-types I could find transcripts encoding all examined mRNAs. These were TLRs 3, 4, 7 and 9, MyD88, TRAF-6, IRFs 3, 5 and 7 and RIG-1. This is in accordance with published studies in the CNS, since both microglia and astrocytes express TLRs 1-9 in mice in vitro (Table 1 and 3). My studies show that these in vitro observations are valid in
MyD88 is an adaptor molecule used by all TLRs except TLR3, while IRF3 is involved in TLR3 signaling and IRF7 in TLR7 and TLR9 signaling. TRAF-6 is involved in all TLR signaling pathways. IRF5 seems to be involved in TLR signaling by at least TLRs 3, 4, 5 and 7 (Takaoka et al. 2005). RIG-1 is another pattern recognition receptor residing in the cytoplasm (Yoneyama et al. 2004).

To further analyze which cell-types express the various TLRs, I also tested transcripts of TLRs in tissue containing either neurons or glial cells. By comparing fragment sizes from the PCR and normalize them to β-actin it seems like the TLR4 expression level is higher in glial cells than nerve cells in both CNS and PNS (Table 9). Although I did not detect TLR4 in nerve cells from hippocampus, it could still have been expressed but not detected due to low expression levels in nerve cells. Likewise the expression level in nerve cells from PNS was low (Table 9). Expression of TLR4 in both nerve cells and glial cells was found in previous studies (Table 1-7). After the second dissection the neurons in PNS expressed more TLRs than the neurons in CNS. The expression of TLRs 7 and 9 could not be found in the neurons in CNS (Figure 10) but in the neurons in PNS (Figure 11). The lack of TLRs 7 and 9 in CNS nerve cells is consistent with previous studies in vitro. TLR7 protein has been detected in mouse neurons in the CNS after exposure to M. corti (Table 5). TLR7 recognizes ssRNA from viruses like influenza (Uematsu and Akira 2006).

The two experiments I performed on tissue containing either neurons or glial cells did not give the same results. In the second experiment I did not purify enough RNA from the samples from CNS and therefore I did not detect any of the TLRs there. Furthermore in the second experiment I detected TLRs in PNS that I did not detect in the first experiment. It is difficult to judge the amount of RNA from PNS from the two different experiments (Figure 9 and 11). The photographs have different levels of exposure and the compounds used for staining are different. If there was more RNA from PNS in the second experiment it could be the reason for the detection of TLRs 7 and 9. Otherwise there could have been some mistake during the experimental procedure or individual differences between the mice in the two experiments.

Although I found all TLRs in the mixed cell-type samples I could not find all of them in the samples with either neurons or glial cells. In the experiments with mixed cell-types I had a much higher RNA concentration than in the two experiments with either neuron or glial cell areas. In addition nerve cells contain more RNA than glial cells. Even though I cut out areas of similar sizes of neurons and glial cells the RNA content was higher in the neuron areas. In particular TLRs present in glial cells could have failed detection for this reason.

Another pattern recognition receptor recognizing dsRNA (Yoneyama et al. 2004) and ssRNA bearing 5’ phosphates (Pichlmair et al. 2006) is RIG-1. In contrast to TLRs 3 and 7 which recognize viruses and are localized in endosomes, RIG-1 is localized in the cytoplasm. Therefore it could act as a complement to TLRs 3 and 7. I found RIG-1 in both nerve cell areas and glial cell areas in trigeminal ganglia and hippocampus. The presence of RIG-1 in nerve cells from CNS is in accordance with a previous study in humans (Préhaud et al. 2005).
Expression of TLRs in CNS versus PNS

The hypothesis for my project was that there should be more TLRs in nerve cells in the PNS than in the CNS. This could not be judged from the literature overview since there are not enough studies concerning TLR expression the PNS. However, when analyzing my experiments it is likely that there are TLRs expressed in nerve cells from PNS which are not present in nerve cells from CNS. At least there is a difference between the systems.

Future perspectives

Since the TLRs were discovered quite recently there is still much not fully known concerning them, like their signaling, recognition of pathogens, expression patterns and their role in diseases. To verify my results I would make further studies on TLRs 7 and 9 expressions in nerve cells in ganglia using quantitative PCR and immunohistochemistry. It would be interesting to do more studies on TLR expression in the PNS to obtain more information on their role in infection. In addition the presence of TLRs 11, 12 and 13 have not been tested extensively yet.
Materials and Methods

Mice

Male C57/B6 mice were used in this study. All experiments were conducted according to institutional guidelines and ethics committee approval.

RNA purification and Reverse Transcriptase - PCR

Tissue samples containing mixed cell-types

The mice were anesthetized with CO₂ and sacrificed and tissue from hippocampus and trigeminal ganglia were taken out and directly put on dry ice. Total RNA was purified using RNeasy™ Mini Kit (Qiagen, GmbH, Hilden, Germany). The manufacturer’s protocol was followed. Rotor-stator homogenization was chosen as a homogenization method. The centrifugation step 5 was omitted and in step 8 the sample was allowed to incubate for 5 min before centrifugation. A final volume of 40 μl was obtained.

All RNA samples were diluted with RNase free water to contain 50.4 ng/μl of RNA. TLR3 and IRF7 PCR were run on undiluted templates with the trigeminal ganglia sample 1 containing 179.04 ng/μl, sample 2 containing 50.4 ng/μl, hippocampus sample 1 containing 348 ng/μl and sample 2 containing 306.24 ng/μl. DNA was digested using deoxyribonuclease 1, amplification grade (Invitrogen, Groningen, The Netherlands) and the manufacturers protocol was followed.

RNA was converted to cDNA using SuperScript II Reverse Transcriptase (Invitrogen, Groningen, The Netherlands) and the manufacturers protocol was followed. The mixture was not heated in step 2, instead all compounds were mixed at the same time. In addition, only 0.5 μl of SuperScript II RT was added and RNaseOUT was not used.

Prior to the PCR, the samples were diluted five times in RNase free water. A PCR-mix was made following the protocol provided with the Titanium Taq Polymerase PCR kit (Clontech Laboratories Inc., Mountain View, California, USA) to a final volume of 25 μl. (Table 10)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Length (bp)</th>
</tr>
</thead>
</table>
| TLR3 | Forward-ATATGC GCTTCAATCCGTTC  
      | Reverse-CAGGAGCATACTGGTGCTGA | 205 |
| TLR4 | F – GCAGAAAAATGCCAGGATGAT  
      | R – AGAGGTGGTGTAAAGCCCATGC | 325 |
| TLR7 | F – TCTGCGAGTCTCGGTTTTCT  
      | R – TTGTCCTGTCAGTCCACGAT | 338 |
| TLR9 | F – ACCCTGGTGTGGAAACATCAT  
<pre><code>  | R – GTTGGACAGGTGGACGAAG | 339 |
</code></pre>
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>MyD88</td>
<td>F – CCCACAAACAAGGAAGCTGG</td>
<td>R – AGGCTGAGTGCAAACCTTGT</td>
<td>315</td>
</tr>
<tr>
<td>TRAF-6</td>
<td>F – TTAGCTGGCGGGGATGCAGTCTCT</td>
<td>R – ATCGCTGCCCTGAGATCCTCT</td>
<td>241</td>
</tr>
<tr>
<td>RIG-1</td>
<td>F – GATTCTGGACCCCACCTACA</td>
<td>R – TGGGCTCAAAAGTCCACAG</td>
<td>212</td>
</tr>
<tr>
<td>IRF3</td>
<td>F – CTGGGCTAGAGCTGGAAACC</td>
<td>R – GACAGCTAACCGCAACACTT</td>
<td>293</td>
</tr>
<tr>
<td>IRF5</td>
<td>F – CAGGTGAACAGCTGCAGTA</td>
<td>R – GCTTTTGTTAAGGGCAGAC</td>
<td>222</td>
</tr>
<tr>
<td>IRF7</td>
<td>F – AGCTCCCAGATCAGAACGAG</td>
<td>R – TCACCAGGATCAGGGTCTTC</td>
<td>336</td>
</tr>
<tr>
<td>β-actin</td>
<td>F – AAAAACTGGAGAAGTGCTGTCACTT</td>
<td>R – CAGAAGCAATGCTGTACCT</td>
<td>263</td>
</tr>
</tbody>
</table>

Only 0.25 μl of Titanium Taq Polymerase was used. Cycler conditions were 94°C for 2 min and 35 or 40 cycles of 94°C for 30 s and 68°C for 1 min and then 72°C for 7 min and 4°C for ∞. TLRs 4, 7, 9, MyD88 and RIG-1 were run for 35 cycles whereas TLR3, TRAF-6, IRF3, IRF5 and IRF7 were run for 40 cycles.

**Tissue samples containing either neurons or glial cells**

The mice were anesthetized with CO₂ and sacrificed and brain and trigeminal ganglia were taken out and directly put on dry ice. They were cut in 12 μm sections which were put on membrane slides (PEN-Membrane 2.0 μm, 50 pcs, Leica Microdissect GmbH). The tissues were stained with Toluidine Blue. Later areas from hippocampus and trigeminal ganglia containing either neurons or glial cells were cut out using Laser Microdissection (Figure 11 and 12).

![Figure 11: Picture of hippocampus. The white lines are areas taken out, the lower containing nerve cells while the higher containing glial cells.](image-url)
Immediately after the areas had been cut out 75 μl RLT buffer (Qiagen, GmbH, Hilden, Germany) was added and the tubes were vortexed to homogenize the contents. Then the RNeasy™ Micro protocol for total RNA isolation from microdissected cryosections was followed. Final volumes of 8 μl in the first experiment and 14 μl in the second experiment were obtained.

The amount of RNA could not be determined since the concentrations were too low to detect with a spectrophotometer. Instead all purified RNA was converted to cDNA, which was run in a PCR. Both procedures were performed in the same way as previously described for the mixed cell-types. Only the number of cycles in the PCR differed. The whole procedure was done twice. In the first experiment β-actin was run for 33 cycles and TLR4 and RIG-1 for 38 cycles. In the second experiment β-actin and TLRs 4, 7 and 9 were run for 43 cycles.

**Agarose gel electrophoresis**

2% agarose (Sigma) gels were made with TAE-buffer (40mM Tris, 20mM acetic acid and 1mM EDTA at pH = 8.3 (Biorad)). 5 μl 100 bp ladder (Invitrogen) was used. 10x Blue Juice (Invitrogen) and sample were mixed before being added to the vales. TAE-buffer was covering the gels which were run at 110 V. Afterwards gels in Figure 6-9 were stained with 10 μl CyberGold (Invitrogen) in 100 ml TAE-buffer for 20 minutes and the gel in Figure 10 was stained with 10 μl GelRed™ nucleic acid stain (Biotium Inc) in 100 ml TAE-buffer for 20 minutes and visualized under UV-light.

**Acknowledgements**

I would like to thank my supervisor Krister Kristensson for letting me do this project in his lab and his valuable help and comments on my work. I am also grateful to Mikael Nyhage for showing me everything in the lab and answering my questions. Special thanks to Margareta Widing for teaching how to use the cryostat. Finally, I would like to thank everyone else in the lab for their help.

Figure 12: Picture on ganglion before (a) and after (b) nerve cells have been cut out. The black square in b shows where the glial cells could be cut out.
References


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Olson J. and Miller S. 2004. Microglia Initiate Central Nervous System Innate and Adaptive Immune Responses through Multiple TLRs. The Journal of Immunology. 173:3916-3924


Rivieccio M., Suh H., Zhao Y., Zhao M., Chin K., Lee S. and Brosnan C. 2006. TLR3 Ligation activates antiviral in human fetal astrocytes: a role for viperin/cig5. the Journal of Immunology. 177: 4735-4741


Zekki H., Feinstein D. and Rivest S. 2002. The clinical course of experimental autoimmune encephalomyelitis is associated with a profound and sustained transcriptional activation of the genes encoding toll-like receptor 2 and CD14 in the mouse CNS. Brain Pathology. 12: 308-319