# *Effect of Toll-like receptor 2 activation on mitochondrial respiration and hypoxic-ischemic injury in the developing brain*

Degree Project in Medicine, 30 ECTS

Carl-Johan Mohn

Supervisors:
Carina Mallard, Joakim Ek, Amin Mottahedin

Institute of Neuroscience and Physiology

UNIVERSITY OF GOTHENBURG

**Programme in Medicine**
**Spring 2017**

**Sahlgrenska Akademin**
**University of Gothenburg**

## Table of Contents

## 1. ABBREVIATIONS

| | |
|---|---|
| AD | Alzheimer's Disease |
| ANOVA | Analysis Of Variance |
| ATP | Adenosine triphosphate |
| BBB | Blood Brain Barrier |
| CNS | Central Nervous System |
| DAMPs | Damage Associated Molecular Patterns |
| ETS | Electron Transport System |
| HI | Hypoxic-ischemic |
| IBD | Inflammatory Bowel Disease |
| LAM | Lipoarabinomannan |
| LPS | Lipopolysaccharide |
| LTA | Lipoteichoic acid |
| MS | Multiple Sclerosis |
| NF-κB | nuclear factor kappa-light-chain-enhancer of activated B-Cells |
| OXPHOS | Oxidative phosphorylation |
| PAMPs | Pathogen associated molecular patterns |
| PNS | Peripheral Nervous System |
| PRRs | Pathogen recognition receptors |
| RA | Rheumatoid Arthritis |
| SEM | Standard Error of the Mean |
| TLRs | Toll Like Receptors |

## 2. ABSTRACT

**Introduction:** Toll Like Receptors (TLR) are pathogen recognition receptors (PRRs), which play major roles in recognition and provoking inflammatory responses upon activation by pathogens such as bacteria, viruses and fungi. TLR2 is activated by binding to Pathogen associated molecular patterns (PAMPs) such as *Lipoteichoic acid* (LTA) and *Lipoarabinomannan* (LAM), which are found in Gram-positive bacteria respective Mycoplasma. As well as infections, Hypoxic-Ischemic (HI) events are a known risk factors for developing neonatal brain injury. Neonatal brain injury can result in conditions like Cerebral palsy, learning difficulties and even death.

**Aim of the study:** The pathology behind neonatal brain injury is still not fully understood. By studying some specific cellular mechanisms such as cellular respiration, myelinisation, development of neurons and microglia in a Toll like receptor dependent manner, we could contribute to the understanding of this complex issue.

**Methods:** $TLR2^{-/-}$ mice and $TLR2^{+/+}$ mice were injected intraperitoneally (ip.) with a TLR2 ligand (P3C) or saline 14h before exposed to Hypoxia-Ischemia (HI). The HI procedure were executed by ligating one of the common carotid arteries followed by putting the pups in an Oxygen deficient chamber for 50min. Brains were collected and stained immunohistochemically using different antibodies for neurons (MAP2), myelinisation (MBP) and Microglia (Iba1). Evaluation of the cellular respiration were made by using a high-resolution respirometer.

**Results:** Here we present data to show that systemic activation of TLR2, with a synthetic ligand, increases subsequent HI brain injury in neonatal mice as demonstrated by increased neural tissue loss as well as reduced myelination. Using high-resolution respirometry, we show that systemic activation of TLR2 suppresses respiration in brain-isolated mitochondria.

**Conclusion:** The results suggest that infection and inflammation might exacerbate hypoxic-ischemic injury through mitochondrial energy failure.

**Key words:** inflammation, Toll like receptor 2, neuroinflammation, mitochondrial respiration, neonatal brain injury, cerebral palsy

## 3. BACKGROUND

### 3.1 Inflammation

The word inflammation originates from the Latin word *Inflammare*, which means "to set on fire". The basic symptoms of inflammation; redness, swelling, heat and pain, were described already by the ancient Roman encyclopaedists, by the name Cornelius Celsus [1]. Rudolf Virchow modified the Celsus description of inflammation by adding "loss of function" to one of the cardinal symptoms. Some sources claim that it was Galen who added the fifth symptom [2]. Galen was the first one to describe inflammation as beneficial to the injury response. Centuries later, inflammation is still a hot topic by researchers worldwide, and is much more complex than Celsus thought.

Today inflammation is considered to be a non-specific response to *any* type of bodily injury. The five symptoms can nowadays all be explained by basic human physiology such as increased blood flow, increased extracellular fluid and release of chemicals that stimulate nerve endings[3].

Inflammation is present whether it serves as a response to infections by bacteria, viruses, fungi's or prions. It is also present when the body is affected with trauma; burns, hypoxia or ionizing radiation just to name a few. For example, it serves a pivotal role during many autoimmune diseases such as Type 1 diabetes mellitus (T1DM), Inflammatory Bowel Disease (IBD), celiac disease, Rheumatoid arthritis (RA)[4].

It is nowadays accepted to divide inflammation in "acute" or "chronic" depending on the molecular mechanism and immunological response behind these two phenomena.

### 3.2 Immune cells and Pattern recognition receptors

Immune cells, known as leukocytes, involved in the acute response are phagocytic cells such as neutrophils and macrophages. Macrophages are innate immune cells that differ in morphology and specificity regarding which tissue they reside. For instance, brain macrophages are called microglia and liver macrophages are called *Kuppfer* cells. Macrophages are differentiated monocytes, and like the majority of immune

system, products of the *Myelopoesis*. It is known that microglia differ in differentiation in comparison to some other macrophages [41].

Macrophages express a wide range of receptors on the cell surface[6]. The receptors of interest during acute inflammation are called Pattern Recognition Receptors (PRRs). PRRs can be divided into subgroups of receptors whether they recognize molecules such as Pathogen Associated Molecular Patterns (PAMPs) or Damage Associated Molecular Patterns (DAMPs). The PRRs differ in function whether they are activated by invading pathogens coming outside of the body (microbes, viruses), or as a response to endogenous molecules released during cell damage or cell death. The Toll Like Receptor family is today the most widely studied group of PPRs.

Activation of macrophage PRRs results in various intracellular pathways that result in e.g. leukocyte extravasation, production of various cell derived mediators (e.g. cytokines), phagocytosis, vasodilatation and increased permeability and activation of the adaptive immune system. Every step in the activation of the immune system is to help eliminate the pathogen/danger.

There are major differences in immune cells and mediators involved, duration, onsets and outcomes between acute and chronic inflammation. The immune cell types in chronic inflammation are dominated by monocytes, lymphocytes and plasma cells, while in acute inflammation the majority of cells involved are macrophages and granulocytes.

### 3.3 Toll Like receptors

The discovery of TLRs goes back to 1985 when scientist Christiane Nüsslein-Volhard analyzed *Drosophila* (fruit flies), and found a fly with abnormal dorso-ventral proportions. This defected *Drosophila* discovery resulted in excitement among the German scientist who reportedly shouted “Das war ja Toll” meaning *That was amazing*. The mutated gene behind this embryonic anomaly was identified, and received the iconic name the Toll gene. A decade later scientists discovered that without the Toll gene, the *Drosophila* would not survive a fungal infection[10]. Nowadays thirteen Toll genes have been identified.

Early experiments showed that activation of Toll in *Drosophila* led to an intracellular activation of *nuclear factor kappa-light-chain-enhancer of activated B-Cells* (NF-κB) [11] that resulted in various defense mechanisms. Medzhiov et al identified the first TLR in mammals, it is known today as TLR4[12].

TLRs are a family of receptors that plays a key role in the activation of the innate immune system. They recognize different PAMPs depending on which subtype of TLR is involved. PAMPs are structures on pathogens primary consisting of lipids, proteins, sugar molecules and nitrogen bases, which are highly specific for pathogens and does not exist in mammals. Other well-described proficiency of the PAMPs is that they are essential for the survival of the pathogen[13, 14], which from an evolutionary perspective makes mutations in PAMPs unfortunate for the pathogen. Sugar Complexed PAMPs (SCPs) are the largest group of PAMPs.

Up to this day, 13 TLRs have been found in mammals TLR1-TLR13, although only TLR1-TLR10 has been found in humans. They vary depending on their intracellular location, ligands, and adapter molecules and in which cells they are found.

TLRs can be separated in subgroups whether they are found on the cell surface or in intracellular vesicles. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are all expressed on the surface of the cell. Except their similarities in location they also mainly recognize microbial lipids, proteins and lipoproteins found in the cell membrane. TLR3, TLR7, TLR8, TLR9 are found mainly in intracellular vesicles and recognizes microbial nucleic acids.

### 3.4 TLR 2

It is known that TLR2 is involved in recognition of a wide range of PAMPs (found in bacteria, fungi, viruses and parasites) [15]. Specifically including lipopeptides from bacteria, lipoteichoic acid from Gram- positive bacteria, and lipoarbinonnan from Mycobacteria. Upon activation TLR2 forms heterodimers with TLR1 or TLR6[16]. Next step in the cascade reaction after the dimerization is recruitment of adapter molecules. MyD88, Tirap (even known as Mal), Trif and Tram are all adapter molecules known to be involved in TLR signaling process[23]. For TLR2 the adapter protein is MyD88. This results in activation of NFκ-B, which is a protein complex

that enters the cell nucleus and controls transcription of DNA[22]. Resulting in transcription of inflammatory cytokines such as Tumor Necrosis Factor-alpha (TNF-α) together with a wide range of interleukins (IL-1α/β, IL-6, IL-8) [24]. TLR2 are found in microglia, *Schwann cells*, macrophages, monocytes, dendritic cells, B-cells and T cells just to name a few.

### 3.5 Cellular respiration

The cellular respiration which results in ATP production is a well conserved series of metabolic reactions that can be found in both prokaryotes and eukaryotes. In short, the cellular respiration is process where nutrients like sugar, amino acids and fatty acids combined with the presence of oxygen results in ATP production. The respiration consists of four well described steps [43,44]. It starts with glycolysis, followed up by the citric acid cycle (even known as the Krebs cycle) and ends with the electron transport chain and oxidative phosphorylation (in some literature the last two steps are considered as one step). The different steps occur in two different intracellular locations, the cell cytoplasm and the mitochondria. The glycolysis is the only step of the respiration that can occur without oxygen and independent of mitochondria. All other steps, in most eukaryotes, include different parts of the mitochondria.

Throughout the last two steps of the cellular respiration electrons are transferred from electron donors to electron acceptors. Oxygen serves as an electron acceptor. This redox reaction produces energy that is used to form ATP. ATP synthase, which is found in the mitochondrial inner membrane is an enzyme that binds together ADP + Pi to form ATP. This unique enzyme consists of two subunits called F0 and F1, where the F0 subunit is embedded in the inner membrane of the mitochondria and the F1 in the mitochondrial matrix. The F0 subunit rotates as protons (H+), supplied from the electron transport chain, passes through the ATP synthase catalyzing the ATP reaction[45].

### 3.6 Neuroinflammation

Neuroinflammation is inflammation of the nervous tissues. Inflammation in the Central Nervous System (CNS) has been of interest among researchers for some time already. Inflammation in the Peripheral Nervous System (PNS) is also a hot topic in modern medicine, but unfortunately not in this study. CNS inflammation occurs as a

response to different crises such as bacterial infections, traumatic injury of the brain, neurodegenerative disorders, autoimmune diseases and stroke[25]. Inflammation in the brain can be acute as well as chronic. Brain inflammation can be restricted to a local region as a result of a focal stroke, or involve major parts of the brain subsequently to traumatic injury. Chronic inflammation is present in disorders like Alzheimer's disease (AD), Multiple sclerosis (MS) and Parkinson's disease[26, 27, 28]. Recent reports even suggest that inflammation is present in conditions like depression and schizophrenia[29]. The CNS is considered to be immune privileged site because of exclusive defense mechanisms like the Blood Brain Barrier (BBB) and the lack of conventional lymphatic drainage system. The BBB is a uniquely designed membrane, that separates the brain from the rest of the circulation, with both good and bad properties.

For example, a good function is that it protects the brain magnificently well form harmful substances which unfortunately leads to another well-known problem in targeting the brain with drugs. Today it is quite impossible to get the desired drug and dosage to the brain through normal drug administration. This has led to more research in drug design and understanding of the BBB. The BBB is a membrane that consists of endothelial cells which are supported by astroglia, perivascular macrophages, pericytes and a basal lamina[5]. The BBB is affected in many pathological conditions which leads to permeability changes in the BBB. Easier passage of cells, molecules, fluids and proteins can be the reason for the condition or the outcome[7]. Changes in the BBB has been observed in conditions like Bacterial Meningitis, Brain Abcesses, Epilepsy, Multiple Sclerosis and Alzeimers[8,9].

### 3.7 Neonatal Brain Injury

Neonatal brain injury was once believed to depend primarily on events of birth asphyxia but is nowadays modified and redefined, by the scientific community, to be a much more multifactorial condition. Neonatal brain injury that results in encephalopathy, clinically starts with lethargy followed by hyper excitability and ends in stupor[33,35], affects 1-3/1000 newborns in high-income countries and the incidence rate is significantly higher in low-income countries where up to 20/1000 newborns are affected[32]. Known risk factors for developing neonatal brain injuries are maternal infections as well as inflammation, due to HI, and preterm birth[36]. Neonatal

encephalopathy is a serious condition where 15-20 % die during the newborn period, and 25% suffers permanent neurological deficits [34, 35]. Acute neurological disorders after birth like *status epilepticus* and stroke that results in fatal outcomes is highest in the child's first year[30, 35]. It is reported that some forms of neonatal stroke have an incidence of 1/4000 live births[31]. Different types of learning disabilities, behavioral syndromes and cerebral palsy are unfortunate possible outcomes after neonatal encephalopathy[35]. Today, recent reports and understandings, Hypoxic-Ischemic Encephalopathy (HIE) is still the most described clinical condition. Underlying mechanisms of neonatal brain injuries are still not fully understood and the only treatment for HIE today is moderate hypothermia[37]. However, therapeutic hypothermia has limitations and many infants continue to survive with disability, and hypothermia is unsafe to use in preterm infants. Thus, there is an urgent need to better understand how neonatal brain injury develops and identify new therapeutic targets.

## 4. AIM OF THE STUDY

The role of TLR2 in response to infections in general is well determined, but the role in neonatal brain injury and the underlying mechanisms has not been fully mapped out. Even though perinatal infections usually are caused by pathogens that involves TLR2 signaling. To gather knowledge behind the complexity of neonatal brain injuries is of great interest for development of possible future treatments. Here we focus on a couple of interesting questions and hypotheses: The role of TLR2 activation/suppression prior to HI, how is the cellular respiration affected, how severe is the neural loss, how is the myelinisation effected as well as the possible effects on Microglia.

## 5. MATERIAL AND METHODS

### 5.1 Animals

To study the role of TLR2 in neonatal brain injury we used TLR2 $^{-/-}$ and TLR2 $^{+/+}$ mice. In order to acquire the mice of interest we purchased wild type mice (C57BL/6J) that are TLR2$^{+/+}$ from the Charles River Laboratories (Wilmington, MA, USA), and bred them with homozygote TLR2$^{-/-}$ (B6.129-Tlr2tm1Kir/J) mice, which were bought from the Jackson Laboratory (Bar Harbor, ME, USA). The C57BL/6J mice are a widely used inbred strain and even the first ones to have its genome sequenced. From cross-breeding these homozygote mice we obtained generations of heterozygote TLR2$^{+/-}$ mice, which were then crossed to give mixed genotypes in each litter consisting of heterozygote TLR2$^{+/-}$, TLR2$^{+/+}$ and TLR2$^{-/-}$ pups, which were used in this study. Mice of both genders were studied. Genotyping was performed following the protocol from the Jackson Laboratory (for full protocol please see Appendix, AP.1). Heterozygote pups were not analyzed in the study. We used a total number of 33 mice for the immunohistochemistry, 13 TLR2 $^{+/+}$ and 20 TLR2 $^{-/-}$. And for the respiration analysis 13 mice per group.

### 5.2 Ethics

Animal experimental procedures conformed to guidelines established by the Swedish Board of Agriculture (SJVFS 2015: 38), were approved by the Gothenburg Animal Ethics Committee (18–2015), and are reported in a manner consistent with the ARRIVE (Animal Research: Reporting in Vivo Experiments) guidelines.

### 5.3 P3C administration

We injected postnatal-day 8 (P8) mice with Pam3CSK4 (P3C, Invivogen, France) or Saline (0.9% NaCl) 14h before the HI events. P3C was injected intraperitoneally (i.p.) with a dosage of 5mg/kg. The P3C dosage was based on previous studies[17, 18, 19].

### 5.4 Hypoxia-Ischemia

To induce neonatal hypoxic-ischemic injury we used a well-established technique. The surgical procedure is as follows, and has been used in previous studies[19, 20]. At P9 the mice were anesthetized using isoflurane, 3.0 % isoflurane for induction and 1.0-1.5% for maintenance, in a mixture of Nitrous oxide and oxygen (1:1). The left

common carotid artery was exposed and then ligated using non-absorbable prolene sutures; the surgical procedure is done with microscope guidance and does not last more than five minutes. After the surgical operation, the mice pups were returned to their home cage with the dam to recover for 1h.

After the recovery, the mice were placed in an incubator for the hypoxic-ischemic insult. Rectal temperature of each mice was measured. The incubator contains a humidified gas mixture (36°C) with 10.00 ± 0.01 % oxygen in nitrogen. The mice are in the incubator for 50min. After the hypoxic insult the mice were returned to their cage.

The combination of artery ligation and exposure to hypoxia induce cerebral hypoxia-ischemia in the hemisphere ipsilateral to the artery ligation and thus result in unilateral brain damage.

**5.5 Immunohistochemistry**

Five days after the insult the mice were killed with an overdose of thiopental. Five days have been shown in previous studies to be enough time to establish an adequate injury[19]. The mice were perfused with saline and 5% buffered paraformaldehyde (Histofix;Histolab, Sweden). The brains were quickly removed and further fixed in formaldehyde 4 $^{o}$C overnight. Dehydration was done with graded series of ethanol and X-tra solv (Medite), and then the brains were embedded in paraffin. Ten μm thick coronal sections were cut at five levels at 40 sections apart (Fig 1.) for immunohistochemical staining. Antigen retrieval was achieved by boiling gently the sections in 10 mM sodium citrate buffer (pH 6.0) for 10min. Then placed in $H_2O_2$ (3% in PBS) for 10min. For blocking unspecific binding activity, the sections were immersed in a blocking solution (4% horse serum, 3% bovine serum albumin in phosphate-buffered saline, PBS) for 30min. Then the sections were incubated with the primary antibodies at 4 $^{o}$C overnight: mouse monoclonal anti microtubule-associated protein 2 (MAP-2; clone HM-2, 1:1000; Sigma-Aldrich) or mouse monoclonal anti myelin basic protein (MBP, 1:10,000, SMI-94R; Covance) or polyclonal rabbit anti ionized calcium binding adaptor molecule 1 (IBA1, Wako). For the secondary antibody we used corresponding biotinylated antibodies (Vector Laboratories). The sections were coated with the secondary antibodies for 1h in room temperature (RT).

This was followed by treatment of sections by the Vectastain ABC Elite kit (Vector) as outlined by the manufacturer. To visualize the target proteins (MAP2/MBP) enzyme substrate DAB was used.
(For full staining protocol please see the appendix, AP.2).

**5.6 Assessment of brain injury**

A Nikon Optiphot-2 microscope equipped with AVT dolphin F145B camera (Allied Vision Technologies) was used for capturing images. Positively stained areas were measured using Olympus Micro Image analysis software system (V4.0, Olympus Optical). The MAP-2 stained area in the ipsilateral hemisphere was subtracted from the contralateral hemisphere and the result was divided by MAP-2 area in contralateral hemisphere to obtain % MAP-2 loss. MBP stained area was measured in subcortical region and the % loss was calculated as for MAP-2. One section at each brain level was analyzed. Data were analyzed by two-way analysis of variance (ANOVA) followed by *Bonferroni* post hoc test when analyzing brain injury at different levels, and by two-tailed unpaired *t*-test when comparing total brain injury. Graphpad Prism V 6.01 was used for data analysis, and data are presented as Mean ± SEM.

**5.7 Microglia Stereology**

A Leica DM 6000 B Microsystem microscope integrated with MBF Bioscience Neurolucida and Stereoinvestigator 64 software was used for the Stereology analysis. The third coronal section level (L3) of the Iba-1 stained sections was used for the analysis. Areas from each hemisphere, the contralateral and the injured ipsilateral, on each brain were mapped out using the Freehand tool at a 5x zoom. Microglia cell counting was performed using the Fractionator Probe with a grid size of 1000 μm x 1000 μm. Approximately 20 grids from each hemisphere were counted. The estimated cell population from each hemisphere was divided by the area of the hemisphere. The cell counting was carried out by a person blind to the samples. An unpaired *t-test* was performed to analyze the data. (For full Stereology protocol please see appendix, AP. 3).

**5.8 Mitochondrial isolation**

To isolate the mitochondria in the brain we used a method described in detail in a previous study[21]. Briefly, one hemisphere from P9 mice was removed and then homogenized using a 2-mL all-glass Dounce tissue grinder (Kontes Glass Co., Vineland, NJ, USA) in 1.9mL isolation buffer (IB) and then centrifuged at 1100 *g* for 2 min (4 $^{o}$C). The supernatant was collected and divided into two Eppendorf tubes with a final volume of 700 µL each and mixed with 75 µL of 80% Percoll Soltuion (GE Healthcare) then carefully layered on the top of freshly made 10% Percoll Solution (750 µL, 80% Percoll was diluted in IB to prepare the 10% solution) in a 2 mL Eppendorf tube and centrifuged at 18 500 *g* for 10 min (4°C).

The myelin-containing top fraction was removed leaving the mitochondria-enriched pellet in the bottom of the Eppendorf tube. One microliter sucrose washing buffer was added to the pellet then mixed and finally centrifuged at 10 000 *g* for 5 min at 4°C. The final mitochondrial pellet was resuspended with 1 mL MIR05 buffer and placed on ice until the next step, the respiration analysis [55].

**5.9 Mitochondria respiration analysis**

Mitochondrial respiration was measured using a modified protocol from previous studies[38, 39, 40, 41]. The full protocol has previously been described[21].
Briefly, we used a high-resolution *respirometer,* model Oroboros Oxygraph-2 k (Oroboros Instruments, Innsbruck, Austria). Samples were run in pairs of saline and P3C in two chambers of O2K devices. All measurements were performed at 25℃ in 2.1 mL MIR05 buffer. The O2 flow was monitored in real-time using DatLab V 6.0 software. After injecting 50 µL of isolated mitochondria into the chambers, saturating dosages of substrates were added to obtain final concentration of 4.8, 2.5, 0.12 mM for pyruvate, malate, and ADP respectively and 1 µmol/L of carbonyl cyanide 4-trifluoromethoxy phenylhydrazone (FCCP). Pyruvate and malate initiate leak state respiration, ADP induces complex I-mediated oxidative phosphorylation (OXPHOS capacity), and FCCP induces uncoupled respiration that corresponds to electron transport system (ETS) capacity. Respiratory control ratio (RCR) was calculated by dividing OXPHOS capacity by leak state respiration. OXPHOS capacity was

corrected for leak state respiration by subtracting leak state value from max OXPHOS to obtain Free OXPHOS capacity. Free ETS capacity was also calculated by subtracting leak state value from ETS capacity. All obtained values were normalized to total protein content, and two-tailed paired *t*-test was performed to analyze the data.

### 5.10 Statistics

As mentioned before, we analyzed the brain injuries (MBP/MAP2) using a two-way analysis of variance (ANOVA) followed by *Bonferroni* post hoc test when comparing different levels, and a two-tailed unpaired *t*-test when comparing total brain injury. For the respiration analysis, we used a two-tailed paired *t*-test. We did not perform specific power calculations but used similar numbers of mice as our previous studies using the same brain injury model.

## 6. RESULTS

Here we present the results that systemic activation of TLR2 using P3C, prior to HI insults, in neonatal mice exacerbates neural tissue loss as well as affects the mitochondrial respiration, seen as MAP-2 and MBP stained area loss respective suppression on mitochondrial respiration.

### 6.1 MAP-2 Stained Neuron loss

To explore the effect of TLR2 activation on HI injury, P3C or Saline were injected 14h before the HI insult. To analyze the size of the damage we collected the brains five days after the HI insult, i.e. postnatal day 14, and then stained the tissue with MAP-2. The MAP-2 protein stabilizes microtubule growth, and it is believed to have an important role in adequate neuron development. Samples from five different coronal section levels (L1-L5) of the cerebrum was used (Figure 1 (a) and (b)). P3C administration significantly increased the neural loss in two of the five levels (Figure 1(a)). The average loss of neural tissue in all five levels increased as well, by 14.0% ± 2.4 ($p < 0.001$, Figure 1(e)). As mentioned above we used pups of both gender, no difference in sensitization between the two genders was observed (data not shown). To rule out a possible temperature depended effect on the injury size we measured rectal temperatures at the time of HI induction. Prior injection with P3C did not affect the temperature (Saline: 35.2 ± 0.2℃; P3C: 35.4 ±0.1℃, n = 8/group, $p = 0.51$).

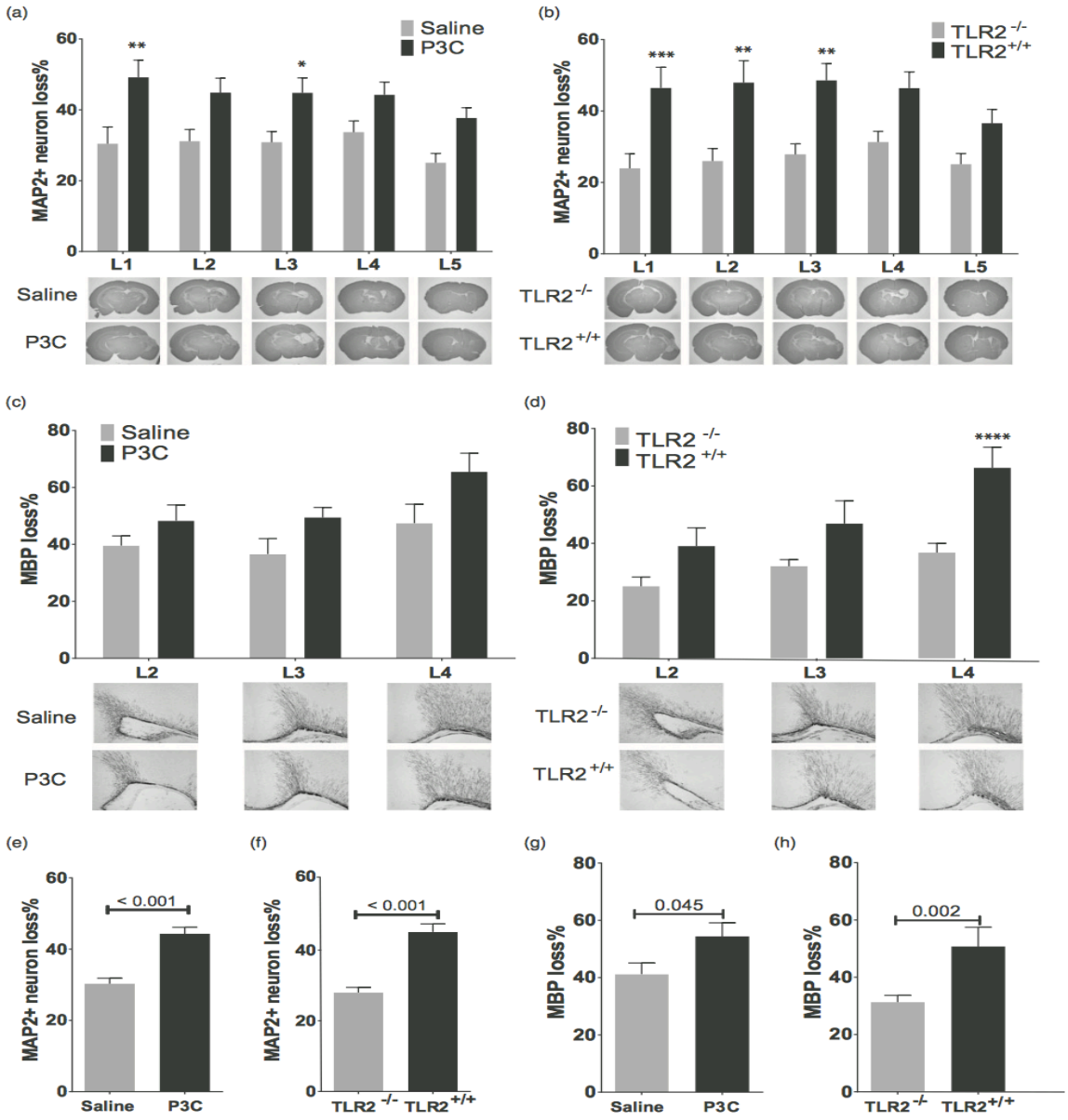
To answer the question whether P3C exposure is TLR2 dependent, we used TLR2 $^{-/-}$ and TLR2 $^{+/+}$ mice. In TLR2 $^{+/+}$ mice (n = 13) the MAP-2 stained neuron loss was more extensive than in TLR2$^{-/-}$ (n=20) (Figure 1(b)). And in general, the TLR2$^{-/-}$ mice were significantly protected against the injury by 17.1% ± 2.5 ($p < 0.001$, Figure 1(f)).

### 6.2 MBP Stained Myelin loss

To investigate the potential myelin loss caused by TLR2 activation prior to HI, we stained three levels (L2-L4) of coronal sections from the cerebrum with MBP. P3C caused a 13.2% ± 6.2 overall increase in HI-induced loss of MBP (Figure 1(g)) compared to saline injected controls. However, regarding the individual levels of the brain no statistically significant difference could be proven (Figure 1(c)). Similar to

MAP2 results, no difference was observed between the genders (data not shown). However, the TLR2 $^{-/-}$ mice were significantly protected from myelin loss at the most anterior level (Figure 1 (d)) compared to the TLR2 $^{+/+}$. Regarding overall comparison in percentage loss of myelin the TLR2 $^{-/-}$ mice were significantly protected by 14% (Figure 1 (h)).

### 6.3 Figure 1




*(Figure1 (a)) Comparing MAP2 (neuronal marker) loss in saline (n= 14) injected and P3C (n=15) injected neonatal mice, 14-hours prior to HI, from five different levels (L1-L5). MAP2 stained areas calculated as loss in %-stained area.*

*(Figure1 (b)) Comparison of MAP2 neuronal loss in P3C injected TLR2 +/+ (n=13) and TLR2 -/- (n=20) mice, 14-hours prior to HI, from five different levels (L1-L5). MAP2 stained areas calculated as loss in %-stained area.*

*(Figure1 (c)) Comparison of MBP loss in subcortical white matter, in saline (n= 14) and P3C (n=15) injected neonatal mice, 14-hours prior to HI, from three different levels (L2-L4). MBP stained areas calculated as loss in %-stained area.*

*(Figure1 (d)) Comparing MBP loss in P3C injected TLR2 +/+ (n=13) and TLR2 -/- (n=20) mice, prior to HI, from three different levels (L2-L4). MBP stained areas calculated as loss in %-stained area*

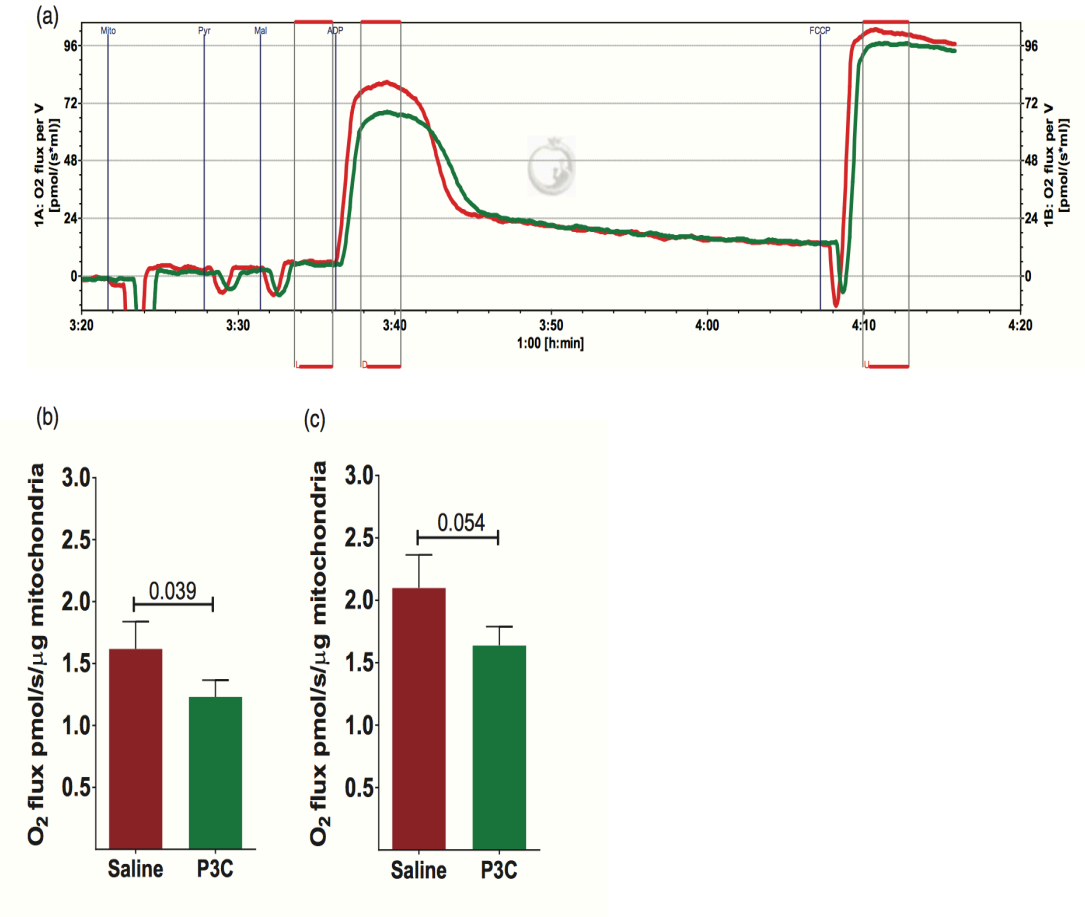
*(Figure1 (e,f)) Total MAP2 percentage loss in mice described in (a) and (b).*
*(Figure1 (g,h)) Total MBP percentage loss in mice described in (c) and (d).*

*All data are presented as Mean ± SEM. The asterisks (*) on the bars in (a-d) marks significant difference, obtained by two-way analysis of variance (ANOVA) analysis followed by Bonferroni post hoc test. The numbers on the bars in (e-h) show the p values obtained by student t-test.*

### 6.4 Suppression on brain mitochondria respiration

To evaluate if mitochondrial function is affected following TLR2 activation we used high-resolution respirometry to assess mitochondrial respiration in real-time with various substrates for complex I activity (Figure 2(a)). The system we used has a high sensitivity and low signal to noise ratio. Respiratory control ratio in the P3C ($13.53 \pm 0.56$, $n = 13$) versus the Saline ($13.81 \pm 0.51$, $n = 13$, $p = 0.544$) control mice was not significant at 14h after P3C administration. However, the ADP-induced phosphorylating respiration (OXPHOS capacity) was suppressed significantly in the P3C treated mice by 23% compared to the control ($p = 0.040$). OXPHOS corrected for leak state (Free OXPHOS capacity) was also decreased by 23.9% (Figure 2(b)). P3C diminished the electron transport system (ETS) capacity by 21%, although the difference did not reach statistical significance ($p = 0.054$). Free ETS capacity showed a 22% suppression after P3C treatment (Figure 2(c)).

## 6.5 Figure 2




*(Figure 2(a)) A representative respirogram of brain mitochondria, isolated from mice injected intraperitoneally with saline (red line) or P3C (green line) at 14 hours prior to analysis. L: leak state; D = ADP-induced OXPHOS and U = uncoupled respiration.*

*(Figure 2(b)) Free OXPHOS capacity (max OXPHOS capacity mediated by complex I activity and corrected for leak respiration) as calculated by subtracting L from D, and normalized to protein concentration. (n = 13 per group). The number above the bar indicates p value obtained by student t-test.*

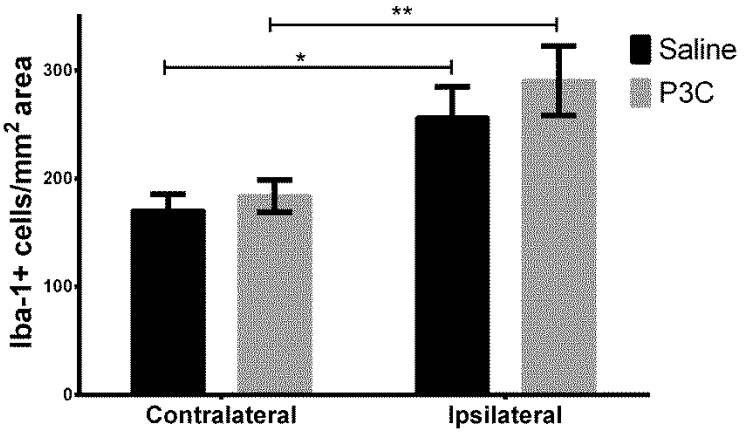
*(Figure 2(c)) Free ETS capacity (uncoupled respiration corrected for leak respiration) as calculated by subtracting L from U, and normalized to protein concentration (n = 13 per group). The data are presented as Mean ± SEM. The number above the bar indicates p value obtained by student t-test.*

### 6.6 Differences in Microglia population

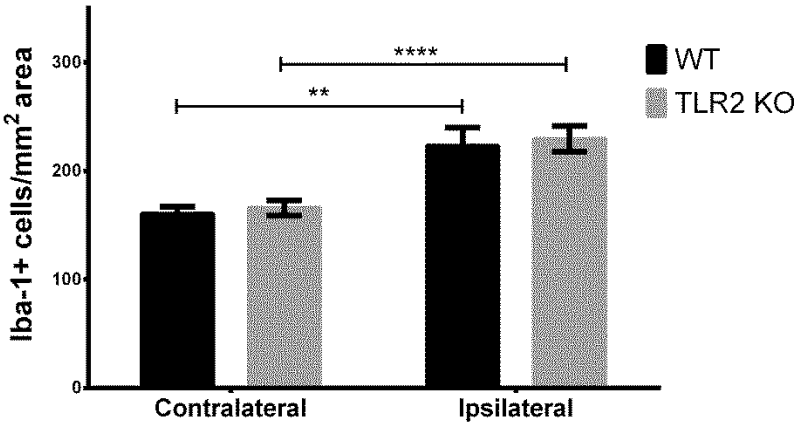
To map out the possible differences in number of microglia cells in the P3C and Saline injected mice, as well as differences in WT and TLR2 KO mice, we used Stereo Investigator 64 with Fractionator plug in software to estimate the number of cells. A statistically significant difference could be proven between the ipsilateral and contralateral hemispheres in the P3C and Saline injected mice (Figure 3(a)). Furthermore, a statistically significant difference could be shown between the ipsilateral and contralateral hemispheres in WT and TLR 2 KO mice (Figure 3 (b)). However, no significant differences could be proven between the P3C and Saline injected mice or in the WT and TLR2 KO mice.

## 6.7 Figure 3

(a)




(b)




*(Figure 3(a)) A representative diagram of the estimated number of microglia, cells/mm$^2$, in the contralateral and ipsilateral hemispheres in saline and P3C injected mice at five days after HI injury at P9.*

*(Figure (3b)) A representative diagram of the estimated cell population of microglia, cells/mm$^2$, in the contralateral and ipsilateral hemispheres in wildtype (WT) and TLR 2 knockout (KO) mice.*

*All data is presented as Mean ± SEM, the asterisks (*) marks statistically significant difference.*

## 7. DISCUSSION

Our results suggest that systemic activation of TLR2 suppresses mitochondrial respiration and exacerbates HI induced brain injury.

Inflammation is present whether perinatal brain injury occurs in term or pre-term infants. Furthermore, pre-term birth is often a result of maternal infections. There are many different causes of perinatal brain injury, metabolic and ischemic, such as hypoxia-ischemia, arterial ischemic stroke and intrauterine growth retardation, just to name a few. Inflammation can occur before, during and after the brain injury, and influences the extent of the injury. Perinatal HI has been linked to neonatal encephalopathy and neurodevelopmental disorders like cerebral palsy. In addition, infections intensify the risk of developing brain injury [17,35].

TLR2 plays a key role in initiating of an inflammatory response, when infections with mycoplasma and Gram-positive are present. Recent studies have shown that repeated injections with P3C in neonatal mice harm normal brain development [46]. Another study of interest shows that TLR2 $^{-/-}$ mice are more protected to HI induced brain injuries [47]. Our results show that TLR2 activation by single dose of P3C amplifies the HI induced brain injury, seen as increased neural tissue loss and demyelination. Bacterial endotoxins, that initiates an inflammatory response through TLR4 activation, dramatically sensitizes the neonatal brain in rats to subsequent HI [48]. Similar effects have been shown following TLR3 activation [20]. However, not all evidence point towards the same conclusion and there are also reports that show a protective effect of TLR2 activation prior to HI, though in adult mice [49, 50]. This might be explained with different immune response mechanisms in the newborn compared to the adult, or different intervals between P3C administration and HI. The time interval between LPS administration and following HI insult has been shown to be critical [51]. However, the mechanisms behind this inflammation/infection increased injury vulnerability is still unknown.

Regarding the possible effects on microglial cell population, our results show no difference in the P3C or Saline injected mice. The only statistically significant difference is between the injured and uninjured hemisphere. Thus, the data suggest that it may not be the number of microglia that is responsible for increasing the

vulnerability. To further understand the role of microglia in P3C sensitization; earlier/later time points or specific phenotypes of microglia could be investigated. Iba-1 is today an adequate marker for Microglia visualization, but its similarity and possible overlap with MRF-1 (Microglia response factor) and whether it could be used as a Microglia marker is not determined[56]. Activated, or more correctly illustrated, reactive Microglia expresses more Iba-1 than ramified (resting) Microglia and this can make the cell counting procedure more difficult. The plasticity, and the different phases of Microglial "activation" would be interesting to investigate further.

We also found that systemic activation of TLR2 suppressed mitochondrial ATP production through suppression of complex I mediated oxidative phosphorylation. The high resolution respirometry results implement an overall estimation of the cellular respiration, during a state of systemic TLR2 activation. To evaluate a complete effect of how systemic TLR2 activation affects cellular respiration and ATP production, a more detailed analysis involving the rest of the complexes and enzymes is needed. Accurate evidence regarding local and systemic inflammation effects on brain mitochondria is narrow, but harmful impacts has been reported in some studies [52, 53]. Further investigation is definitely needed for more accurate statements. However, there are some potential hypotheses that have been examined, for example that suppressed respiration leads to necrosis pathways initiated by cumulative effects of ATP depletion, and the possible effects of glutamate excitotoxity [54].

In conclusion, our results show, for the first time, that systemic TLR2 activation exacerbates neonatal HI brain injury. Inflammation-induced suppression of cellular respiration may play a role and increase the vulnerability to HI injury. Therefore, targeting mitochondrial dysfunction in inflammation-related neonatal brain injury may be a novel therapeutic strategy.

## 8. POPULÄRVETENSKAPLIG TEXT PÅ SVENSKA

### Introduktion

Inflammation är kroppens egna sätt att reagera på något som kan potentiellt vara skadligt för kroppen. Inflammation kan ses som en inbyggd försvarsmekanism mot yttre hot. Vad som kan orsaka inflammation är exempelvis fysiska skador på kroppen, tillstånd som leder till syrebrist i kroppens olika organ och infektioner med bakterier och virus mm. Inflammation är inte alltid bra, det kan även skada kroppen. Exempel på detta är vissa sjukdomar där kroppens försvarsmekanismer angriper kroppens egna vävnader och organ (autoimmunitet). Detta ses till exempel i sjukdomar som Multipel Skleros (MS), Diabetes typ 1 och inflammatoriska tarmsjukdomar. Nyfödda barn som utsätts för syrebrist i hjärnan och/eller infektioner under graviditeten, kan få allvarliga skador och bestående men i form av Cerebral Pares (CP) eller svåra inlärningsproblem. I värsta fall kan även död inträffa. Inflammationsprocesserna kan vara väldigt komplexa och i sistnämnda fallet vet man inte exakt hur detta går till.

För att förstå sig på inflammation och dess konsekvenser måste man förstå sig på mekanismerna bakom detta. Toll-Like Receptorer (TLR) är receptorer som finns på många kroppens vita blodkroppar och de är involverade i igenkänning av flertalet bakterier, virus och svampar. När en TLR binder in exempelvis på en bakterie så börjar inflammationsprocessen vars uppgift är att avlägsna i detta fall bakterien. Toll-like receptor 2 (TLR2), som är en grupp av Toll-Like receptorer, känner igen bakteriella strukturer som förekommer till exempel i mycoplasma och Gram positiva bakterier. Dessa två är även en av de vanligaste bakteriella infektionerna hos gravida.

**Syfte med studien:** I denna studie tittar vi på effekten av TLR2 aktivering tillsammans med syrebrist i hjärnan. Vi kollar efter parametrar som: storlek på hjärnskada och effekten på de utsatta cellers energiproduktion.

### Metoder

Genom att använda möss som saknar TLR2 och jämföra med vanliga möss kan vi svara på frågorna vi undrar över. För att orsaka en hjärnskada så kommer vi att strypa blodflödet till hjärnan på mössen och sedan lägga dem i en syrefattig kammare. Innan detta kommer vi att stimulera TLR2 med artificiella substanser. Därefter analyseras

hjärnorna med att färga in hjärnvävnad med olika färger och sedan analysera dessa i mikroskop. Slutligen analyseras cellernas energiproduktion med högupplöst respirometri (metod för att analysera olika delar i energiproduktionen).

**Resultat**

Vi kunde påvisa att storleken på hjärnskadan hade en koppling till om mössen saknade TLR2. De möss som saknade TLR2 hade mindre storlek på hjärnskadan det vill säga mindre hjärnceller (nervceller) i den analyserade hjärnan. Även negativa effekter på elektrontransportkedjan (en del av cellens energiproduktion) sågs i de möss som hade TLR2. Detta betyder alltså att TLR2 aktivering innan en period av syrebrist gör hjärnan mera sårbar för skada, och utfallet efter syrebristen blir signifikant värre.  Detta summerat talar för att infektion och eller inflammation bidrar till förvärring av syrebrist orsakade hjärnskador.

## 9. ACKNOWLEDGEMENT

I would like to thank Docent Carina Mallard not only for excellent supervision during this degree project, but also for the support and all the valuable guidance since my early days at the lab.

I would like to thank Amin Mottahedin for a great many things. I feel beyond honoured to be a part of this project and publication.

I would like to thank Docent Joakim Ek being a trusted advisor, a coach, a mentor, for me since day one at the lab.

Finally I would like to thank Pernilla Svedin, Syam Nair, Henrik Hagberg, Peter Smith, Xiaoyang Wang, Anna-Lena Leverin, Mona Svedman and everyone who contributed to this project.

And a special thanks to the Institute of Neuroscience and Phsyiology.

## 10. REFERENCES


1. Spencer WG, editor. Celsus. *De medicina*. London: Heinemann; 1935. and trans.
2. Rather, L. J. (1971). *"Disturbance of function (functio laesa): the legendary fifth cardinal sign of inflammation, added by Galen to the four cardinal signs of Celsus"*
3. Parakrama Chandrasoma; Clive R. Taylor (c. 2005). "Part A. "General Pathology", Section II. "The Host Response to Injury", Chapter 3. "The Acute Inflammatory Response", sub-section "Cardinal Clinical Signs"". *Concise Pathology* (3rd edition (Computer file) ed.). New York, N.Y.: McGraw-Hill. ISBN 0-8385-1499-5. OCLC 150148447. Retrieved 2008-11-05
4. Cotran; Kumar, Collins (1998). *Robbins Pathologic Basis of Disease*. Philadelphia: W.B Saunders Company
5. Bradbury M. W. B. (1985) The blood-brain barrier: transport across the cerebral endothelium. Circ. Res. **57**:213–222
6. Kim E, Son YJ, Yang Y Shen T, Kim I, Aravinthan A, Kim JH, Cho JY 1-(2,3-Dibenzimidazol-2-ylpropyl)-2-methoxybenzene Is a Syk Inhibitor with Anti-Inflammatory Properties. *Molecules.* 2016 Apr 18;21(4):508.
7. Alfred H Schinkel, P-Glycoprotein, a gatekeeper in the blood–brain barrier, Advanced Drug Delivery Reviews, Vol 36, Issue 2, 1999
8. Raza, Muhammad W.; Shad, Amjad; Pedler, Steve J; Karamat, Karamat A. (2005). "Penetration and activity of antibiotics in brain abscess". Journal of the College of Physicians and Surgeons Pakistan. 15 (3): 165–7.
9. Nagele, Robert G. (2006). "Alzheimer's disease: new mechanisms for an old problem". UMDNJ Research. University of Medicine and Dentistry of New Jersey. 7 (2).
10. Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA (1996). "The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults". *Cell*. **86** (6): 973–983
11. Gonzalez-Crespo S, Levine M. Related target enhancers for dorsal and NF-kappa B signaling pathways. *Science.* 1994; **264**: 255–258
12. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature.* 1997; **388**: 394–397
13. Mahla RS, Reddy MC, Prasad DVR, Kumar H. Sweeten PAMPs: Role of Sugar Complexed PAMPs in Innate Immunity and Vaccine Biology. *Frontiers in Immunology* 2013; 4:248.
14. Lloyd DH, Viac J, Werling D, Rème CA, Gatto H Role of sugars in surface microbe-host interactions and immune reaction modulation. *Vet Dermatol.* 2007 Aug; 18(4):197-204.
15. Akira, S., Uematsu, S. & Takeuchi, O. Pathogen recognition and innate immunity. *Cell* **124**, 783–801 (2006).
16. Mi Sun Jin, Sung Eun Kim, Jin Young Heo, Mi Eun Lee, Ho Min Kim, Sang-Gi Paik, Hayyoung Lee, Jie-Oh Lee, Crystal Structure of the TLR1-TLR2 Heterodimer Induced by Binding of a Tri-Acylated Lipopeptide, Cell, Volume 130, Issue 6, 21 September 2007, Pages 1071-1082

17. Hagberg, H., Mallard, C., Ferriero, D. M., Vannucci, S. J., Levison, S. W., Vexler, Z. S., Gressens, P. (2015) The role of inflammation in perinatal brain injury. *Nat.* Rev. Neurol. 11, 192–208.

18. Hübschle, T., Mütze, J., Mühlradt, P. F., Korte, S., Gerstberger, R., Roth, J. (2006) Pyrexia, anorexia, adipsia, and depressed motor activity in rats during systemic inflammation induced by the Toll-like receptors-2 and -6 agonists MALP-2 and FSL-1. Am. *J. Physiol*. Regul. Integr. Comp. Physiol. 290, R180–R187.

19. Mottahedin A, Smith PL, Hagberg H, et al. TLR2- mediated leukocyte trafficking to the developing brain. *J Leukocyte Biol* 2016; 101: 297–305.

20. Stridh L, Mottahedin A, Johansson ME, et al. Toll-like receptor-3 activation increases the vulnerability of the neonatal brain to hypoxia–ischemia. J Neurosci 2013; 33: 12041–1251

21. Wang X, Leverin A-L, Han W, et al. Isolation of brain mitochondria from neonatal mice. J Neurochem 2011; 119: 1253–1261.

22. Albensi BC, Mattson MP (February 2000). "Evidence for the involvement of TNF and NF-kappaB in hippocampal synaptic plasticity". *Synapse*. **35** (2): 151–9

23. Shigeoka AA, Holscher TD, King AJ, et al. (May 2007). "TLR2 is constitutively expressed within the kidney and participates in ischemic renal injury through both MyD88-dependent and -independent pathways". *J. Immunol*. **178** (10): 6252–8

24. Kawai, Taro; Shizuo Akira (20 April 2010). "The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors". *Nature Immunology*.

25. Gendelman, Howard (2002). "Neural immunity: Friend or foe?". *Journal of NeuroVirology*. **8**: 474–479

26. Tansey, Malu (May 2012). "Neuroinflammation and Non-motor Symptoms: The Dark Passenger of Parkinson's Disease?". *Curr Neurol Neurosci Rep*. **12**: 350–358

27. W.S. Griffin, J.G. Sheng, G.W. Roberts, R.E. Mrak Interleukin-1 expression in different plaque types in Alzheimer's disease: significance in plaque evolution J. Neuropathol. Exp. Neurol, 54 (1995), pp. 276–281

28. Lucas S.-M., Rothwell N. J., Gibson R. M. (2006). The role of inflammation in CNS injury and disease. Br. J. Pharmacol. 147, S232–S240

29. Najjar S., Pearlman D. M., Alper K., Najjar A., Devinsky O. (2013). Neuroinflammation and psychiatric illness. J. Neuroinflammation 10:43

30. Maytal J, Shinnar S, Moshe SL, Alvarez LA. Low morbidity and mortality of status epilepticus in children. Pediatrics

31. Nelson KB, Lynch JK. Stroke in newborn infants. Lancet Neurol 2004;3:150-158

32. Pauliah SS, Shankaran S, Wade A, . therapeutic hypothermia for neonatal encephalopathy in low- and middle-income countries: A systematic review and meta-analysis. PloS One 2013; 8: e58834

33. Sarnat HB, Sarnat MS. Neonatal encephalopathy following fetal distress: a clinical and electroencephalographic study. Arch Neurol 1976;33:696-705

34. Volpe JJ. Neurology of the newborn. 4th ed. Philadelphia: W.B. Saunders, 2001:912.

35. Ferriero DM. Neonatal brain injury. N Engl J Med 2004; 351: 1985–1995

36. Dammann O, Kuban KC, Leviton A. Perinatal infection, fetal inflammatory response, white matter damage, and cognitive limitations in children born preterm. Ment Retard Dev Disabil Res Rev 2002;8:46-50

37. Feigin V, Anderson N, Gunn A, Rodgers A, Anderson C. The emerging role of therapeutic hypothermia in acute stroke. Lancet Neurol 2003;2:529-529

38. Puka-Sundvall M, Wallin C, Gilland E, Hallin U, Wang X, Sandberg M, Karlsson J, Blomgren K, Hagberg H. Impairment of mitochondrial respiration after cerebral hypoxia-ischemia in immature rats: relationship to activation of caspase-3 and neuronal injury. Brain Res. Dev. Brain Res. 2000;125:43–50.

39. Frezza C, Cipolat S, Scorrano L. Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts. Nat. Protoc. 2007;2:287–295

40. Hansson MJ, Mansson R, Morota S, et al. Calcium-induced generation of reactive oxygen species in brain mitochondria is mediated by permeability transition. Free Radic. Biol. Med. 2008;45:284–294.

41. Kuznetsov AV, Veksler V, Gellerich FN, Saks V, Margreiter R, Kunz WS. Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells. Nat. Protoc. 2008;3:965–976.

42. Hoeffel G, Ginhoux F. Ontogeny of Tissue-Resident Macrophages. Front Immunol. 2015 Sep 22;6:486.

43. Stryer, Lubert (1995). *Biochemistry* (fourth ed.). New York – Basingstoke: W. H. Freeman and Company.

44. Anderson, Douglas M., ed. (2003). *Dorland's Illustrated Medical Dictionary* (30th ed.). Philadelphia, PA: Saunders. pp. 35, 71

45. Dimroth P, Kaim G, Matthey U (1 January 2000). "Crucial role of the membrane potential for ATP synthesis by F(1)F(o) ATP synthases". *J. Exp. Biol*. **203** (Pt 1): 51–9.

46. Du X, Fleiss B, Li H, . Systemic stimulation of TLR2 impairs neonatal mouse brain development. PloS One 2011; 6: e19583

47. Stridh L, Smith PL, Naylor AS, . Regulation of toll-like receptor 1 and -2 in neonatal mice brains after hypoxia-ischemia. J Neuroinflam 2011; 8: 45

48. Eklind S, Mallard C, Leverin A-L, . Bacterial endotoxin sensitizes the immature brain to hypoxic–ischaemic injury. Eur J Neurosci 2001; 13: 1101–1106

49. Lu C, Liu L, Chen Y, . TLR2 ligand induces protection against cerebral ischemia/reperfusion injury via activation of phosphoinositide 3-kinase/Akt signaling. J Immunol 2011; 187: 1458–1466

50. Hua F, Ma J, Ha T, . Preconditioning with a TLR2 specific ligand increases resistance to cerebral ischemia/reperfusion injury. J Neuroimmunol 2008; 199: 75–82.

51. Eklind S, Mallard C, Arvidsson P, . Lipopolysaccharide induces both a primary and a secondary phase of sensitization in the developing rat brain. Pediat Res 2005; 58: 112–116

52. Hunter RL, Dragicevic N, Seifert K, . Inflammation induces mitochondrial dysfunction and dopaminergic neurodegeneration in the nigrostriatal system. J Neurochem 2007; 100: 1375–1386.

53. Karlsson M, Hara N, Morata S, . Diverse and tissue-specific mitochondrial respiratory response in a mouse model of sepsis-induced multiple organ failure. Shock 2016; 45: 404–410

54. Kohmura E, Yamada K, Hayakawa T, . Hippocampal neurons become more vulnerable to glutamate after subcritical hypoxia: An in vitro study. J Cereb Blood Flow Metab 1990; 10: 877–884

55. Mottahedin A et al, Systemic activation of Toll-like receptor 2 suppresses mitochondrial respiration and exacerbates hypoxic–ischemic injury in the developing brain J Cereb Blood Flow Meta Vol 37, Issue 4, pp. 1192 – 1198

56. Deininger MH, Meyermann R, Schluesener HJ (March 2002). "The allograft inflammatory factor-1 family of proteins". *FEBS Lett*. 514 (2-3): 115–21

## 11. APPENDIX 1

# DNA Extraction Using Sigma XNAT kit

Do this in Western Lab

Use filter pipette tips

1. For each sample pipette 50 µl of **Extraction Solution** into microcentrifuge tube, add 12.5 µl of **Tissue Preparation Solution** _ pipette up and down several times to mix. (4 part ES, 1 part TP)
2. Place ear/tail biopsy into the solution _ mix thoroughly via vortexing
3. Incubate in RT for 10-20 minutes.
4. Incubate at 96°C for 3 minutes. (Tissue will not be digested!)
5. Add 50 µl of **Neutralizing Solution B** and mix by vortexing
6. Store at 4°C or use immediately. For long-term remove the tissue and freeze the rest.

## 12. APPENDIX 2

# MAP-2 Immunohistochemistry

**Primary antibody**: monoclocal anti-MAP2, clone HM-2 (Sigma)
**Secondary antibody**: biotinylated Horse anti-mouse (Vector)

### Protocol

1. Melt paraffin at 65ºC for 30min
2. Deparaffinise: Xylene 10min, 100% 5min, 95% 5min, 70% 5min
3. 3x5min washes in PBS/tween20 (Heat citrate buffer for next step)
4. Boil gently section in citrate buffer (pH6) for 10 min
5. Allow to cool for 20min
6. 3x5min washes in PBS/tween20
7. Block sections in $H_2O_2$ (3% in PBS) for 10min
8. 3x5min washes in PBS/tween20
9. Block in 3% horse serum/PBS for 30min
10. Incubate in primary antibody (3% horse serum/PBS, 1:2000) overnight in fridge
11. 3x5min washes in PBS/tween20
12. Incubate in seconday antibody (PBS, 1:250) for 1h
13. 3x5min washes in PBS/tween20
14. Incubate in ABC complex for at least 30min
15. 3x5min washes in PBS
16. Develop with DAB kit (Vector) for about 5min
17. Dehydrate and mount sections

## 13. APPENDIX 3

**Protocol for Stereo Investigator 64bit**

Calibrate the lens using the Calibration Grid Silde (250 μm and 25 μm)

**Fractionator**

1. Find your desired tissue section using the Microscope, 1.25x zoom.
2. Choose the reference point in the middle of the section.
3. To draw contours, use the **Freehand** option.
4. If you want to move around, press the **Joy Track** button on the toolbar, and then use the Joystick.
5. To end the drawings, use the **Close Contour** button on the toolbar.
6. Choose **Fractionator** from the Probes menu.
7. Put in your desired Grid size (for example 1000 μm x 1000 μm)
8. Use the same Grid size during all your probe runs.
9. Zoom in to 20x, or depending on your grid size. For counting Microglia 20x works fine.
10. Use a marker from the toolbar to the left.
11. Count all the cells from the box. If a cell crosses the green line then you should count it. If it crosses the red line then do not. And if it crosses both then leave uncounted. Use Ctrl +Z to undo.
12. To get to the next counting frame press **F2**.
13. Go through all the counting frames until a message appears that says the run is complete.
14. To show the results press the **Display Probe run list** button on the toolbar.