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# OXIDATIVE STRESS IN EXPERIMENTAL TRAUMATIC BRAIN INJURY

Mattias Günther
M.D.


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Cover image illustrates COX-2 positive cytosols stained in green and nuclei stained in blue, in a male rat brain after traumatic brain injury.

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# OXIDATIVE STRESS IN EXPERIMENTAL TRAUMATIC BRAIN INJURY

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by


## Mattias Günther

*Principal Supervisor:*
Prof. Tiit Mathiesen
Karolinska Institutet
Department of Clinical Neuroscience
Division of Neurosurgery

*Co-supervisors:*
Prof. Mårten Risling
Karolinska Institutet
Department of Neuroscience
Division of Experimental Traumatology

Dr. Caroline Gahm
Karolinska Institutet
Department of CLINTEC
Division of ENT

Dr. Stefan Plantman
Karolinska Institutet
Department of Neuroscience
Division of Experimental Traumatology

*Opponent:*
Associate Prof. Jonathan Lifshitz
University of Arizona
Department of Child health
Division of Translational Neurotrauma Research

*Examination Board:*
Prof. Lars-Owe Koskinen
Umeå University
Department of Pharm & Clinical Neuroscience
Division of Neurosurgery

Associate Prof. Cesare Patrone
Karolinska Institutet
Department of Clinical Science and Education
Division of Stroke, Neurodegener. & Diabetes

Prof. Marianne Schultzberg
Karolinska Institutet
Department of Neurobiology
Division of Neurodegeneration

# ABSTRACT

Traumatic brain injury (TBI) is a leading cause of death and disability among the young population in the industrialized world. The injury consists of immediate damage to the brain tissue, followed by a secondary response involving inflammation and oxidative stress. No pharmacological treatment is effective and the physical and inflammatory mechanisms are insufficiently understood. Considerable variability exists in the clinical outcome after TBI. Genetic factors have been implicated to affect the posttraumatic inflammatory response. This study was undertaken to explore a possible impact of genetic polymorphism in oxidative stress reactions after experimental TBI, and to determine possible effects of direct physical forces on inflammatory cell activation. TBI was induced using mild focal and penetrating focal brain injury models, in inbred and outbred rat strains and male and female rats. Genetic susceptibility to inflammation in the central nervous system (CNS) was found to be associated to the redox active enzymes iNOS and MnSOD in inflammatory cells, but was not associated with increased neuronal degeneration at 24h. The genetic regulation of oxidative stress vulnerability was corroborated in primary neuronal cultures, where neurons primed in an environment of high susceptibility to inflammatory activity had increased compensatory antioxidative enzymes MnSOD and PRDX5, leading to reduced lipid peroxidation, nitrosylation and degeneration. Humoral stimulation was necessary for iNOS induction in neurons. Gender also affected the inflammatory response. The inflammatory enzyme COX-2 was increased in males compared to females at 24h and 72h and correlated with increased apoptosis at 24h in males, but not neuronal degeneration, astrogliosis, microgliosis or nitrosylation. Direct physical force by shock wave trauma caused an inflammatory activation in two different macrophage cell lines, which did not include iNOS or NO increase. Energy transfer by trauma activated the macrophages directly without humoral mediators, comprising a novel activation mechanism of macrophages. Posttraumatic treatment with the antioxidative compound N-acetylcysteine amide reduced neuronal degeneration, increased MnSOD at 24h and reduced apoptosis at 2h. Levels of migrating macrophages/activated microglia, iNOS, nitrosylation or NFkB were not affected. In summary, our findings demonstrated that genetic factors regulated oxidative stress related inflammation after TBI, macrophages were activated by direct physical forces and an antioxidative drug provided neuroprotection after TBI. Susceptibility to CNS inflammation and oxidative stress are interrelated and should be considered when evaluating novel antioxidative treatments.

# LIST OF PUBLICATIONS

I. **Mattias Günther**, Faiez Al Nimer, Caroline Gahm, Fredrik Piehl, Tiit Mathiesen. iNOS-mediated secondary inflammatory response differs between rat strains following experimental brain contusion. Acta Neurochir (Wien). 2012 Apr;154(4):689-97

II. **Mattias Günther**, Faiez Al Nimer, Fredrik Piehl, Stefan Plantman, Mårten Risling and Tiit Mathiesen. Neuronal vulnerability to oxidative stress is affected by genetic polymorphism and related to susceptibility to inflammation in the central nervous system. Submitted.

III. **Mattias Günther**, Stefan Plantman, Johan Davidsson, Maria Angéria, Tiit Mathiesen, Mårten Risling. COX-2 regulation and TUNEL-positive cell death differ between genders in the secondary inflammatory response following experimental penetrating focal brain injury in rats. Acta Neurochir (Wien). 2015 Apr;157(4):649-59

IV. **Mattias Günther**, Stefan Plantman, Caroline Gahm, Anders Sondén, Mårten Risling, Tiit Mathiesen. Shock wave trauma leads to inflammatory response and morphological activation in macrophage cell lines, but does not induce iNOS or NO synthesis. Acta Neurochir (Wien). 2014 Dec;156(12):2365-78

V. **Mattias Günther**, Johan Davidsson, Stefan Plantman, Svante Norgren, Tiit Mathiesen, Mårten Risling. Neuroprotective Effects of N-acetylcysteine Amide on Experimental Focal Penetrating Brain Injury in rats. Accepted J Clinical Neuroscience, 2015, March 3

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| 3-NT | 3-Nitrotyrosine |
| 4-HNE | 4-Hydroxynonenal |
| Bcl-2 | B-cell lymphoma 2 |
| CNS | Central nervous system |
| COX-2 | Cyclooxygenase 2 |
| DA | Dark Agouti |
| eNOS | Endothelial nitric oxide synthase |
| GST | Glutathione S-transferase |
| $H_2O_2$ | Hydrogen peroxide |
| ICP | Intracerebral pressure |
| IFN | Interferon |
| IL | Interleukin |
| iNOS | Inducible nitric oxide synthase |
| LDH | Lactate dehydrogenase |
| LPS | Lipopolysaccharide |
| MnSOD | Manganese superoxide dismutase |
| NAC | N-acetylcysteine |
| NACA | N-acetylcysteine amide |
| NFkB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NMDA | N-Methyl-D-aspartate |
| nNOS | Neuronal nitric oxide synthase |
| NO | Nitric oxide |
| NOX2 | Nicotinamide adenine dinucleotide phosphate oxidase |
| $O_2^-$ | Superoxide |
| $ONOO^-$ | Peroxynitrite |
| $PGE_2$ | Prostaglandin E2 |
| PRDX5 | Peroxiredoxin 5 |
| PVG | Piebald Virol Glaxo |
| ROI | Region of interest |
| ROS | Reactive oxygen species |
| TBI | Traumatic brain injury |
| TGF-β | Transforming growth factor beta |
| TNF | Tumor necrosis factor |
| TUNEL | Terminal deoxynucleotidyl transferase dUTP nick end labeling |
| VCAM-1 | Vascular cell adhesion protein 1 |
| VEGF | Vascular endothelial growth factor |

# 1 INTRODUCTION

The earliest available medical document, the Edwin Smith Surgical Papyrus (c. 26th century BC), describes several neurological disorders following wartime head injuries. Traumatic brain injury (TBI) is thus among the earliest described illnesses in the history of man (Aminoff et al. 2015). TBI is today a leading cause of disability and death among young people (Injury 1999). Despite vast improvements in general healthcare during the last century, modern healthcare still has no effective pharmacological treatment (Diaz-Arrastia et al. 2014). Outcome largely reflects the natural course of the disease.

Acute TBI is characterized by two injury phases, the primary and the secondary. The primary injury phase signifies the direct injury to the brain cells at the time of the initial impact. The secondary phase includes a neuroinflammatory response which involves blood-brain barrier damage and starts immediately after and may persist for months. It is characterized by activation of resident cells astrocytes and microglia, migration of leucocytes and phagocytes from the blood into the brain, and the production of cytokines and chemokines (Morganti-Kossmann et al. 2007) (Lenzlinger et al. 2001). This secondary inflammatory response has both detrimental and beneficial effects (Morganti-Kossmann et al. 2002). The magnitude and duration affects clinical outcome, in short term by promoting neuronal death, edema and pyrexia, and in the long term by influencing repair processes (Kadhim et al. 2008).

## 1.1 OXIDATIVE STRESS AFTER TBI

Within minutes of the initial trauma, oxidative stress occurs by the overproduction of reactive oxygen species (ROS) which overwhelm the antioxidative response (Woodcock and Morganti-Kossmann 2013) (Bains and Hall 2011). ROS reacts with proteins, lipids, carbohydrates and nucleic acids, ultimately leading to irreversible cellular damage.

Nitric oxide (NO) is an unstable diatomic radical and a key mediator in the oxidative process (Schouten 2007) (Wada et al. 1998). In low concentrations, NO controls physiological reactions such as immune cell-mediated cytotoxicity, cytostasis, regulation of vascular tone, relaxation, inhibition of platelet aggregation and neurotransmission. NO is lipophilic and diffuses freely through membranes which is why its activity is not restricted to the site of production, and the half life depends on the availability of intracellular reactants (Trackey et al. 2001). In high concentrations, NO together with superoxide ($O_2^-$) forms peroxynitrite ($ONOO^-$) at a diffusion limited rate.

Peroxynitrite is a main cause of NO mediated toxicity, and although not a free radical, a relatively stable and highly diffusible powerful oxidant (Floyd 1999) (Lu et al. 2009) (Beckman et al. 1990) (Szabo et al. 2007) (Lambert and Brand 2009). Peroxynitrite oxidizes proteins, induces membrane lipid peroxidation and inhibits mitochondrial electron transport, leading to rapid, necrotic cell death (Floyd 1999) (Lu et al. 2009). Elevation of peroxynitrite occurs within the first hour of TBI and lasts for days (Wada et al. 1998) (Hall et al. 2004) (Bayir et al. 2005) (Deng et al. 2007). Peroxynitrite is difficult to detect which is why the stabile surrogate measure 3-Nitrothyrosine (3-NT), derived from $NO_2$ nitration of tyrosine residues in proteins is often used as a stabile surrogate measure (Beckman et al. 1990).

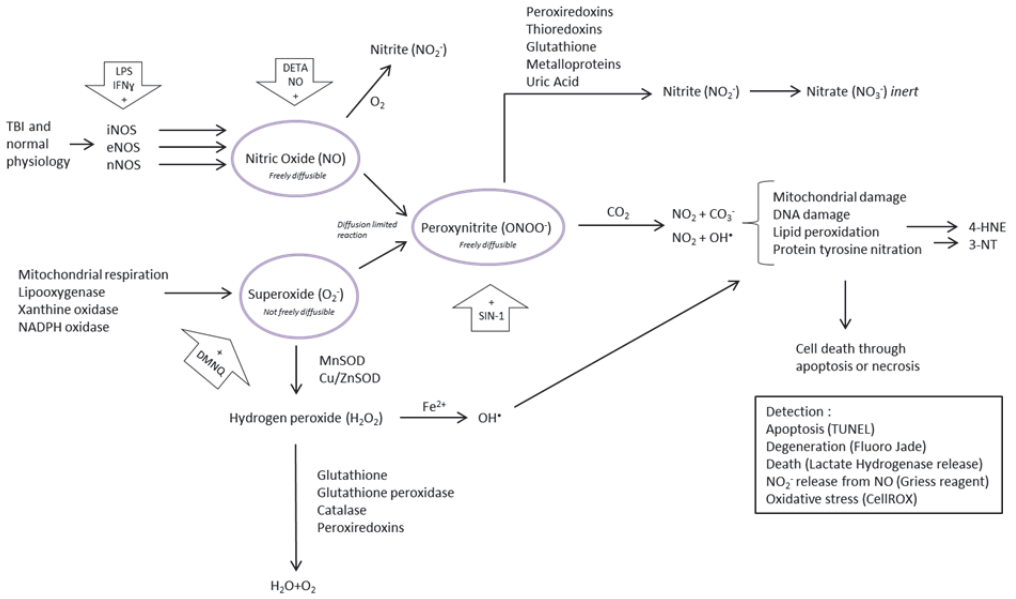


Figure 1 - Schematic overview of main oxidative reaction routes after TBI studied in the thesis. Nitric Oxide is produced in dormant conditions by eNOS and nNOS and accounts for a variety of physiological reactions. iNOS is induced in inflammation and accounts for a surge of NO. NO is freely diffusible across cell membranes and organelles, and reacts with superoxide at a diffusion limited rate, at the site of superoxide formation. Superoxide is not freely diffusible, and mainly produced as a byproduct of mitochondrial oxidative phosphorylation. NO and superoxide forms peroxynitrite, a highly reactive free radical which reacts with carbon dioxide to create deleterious reaction products. This leads to mitochondrial damage, DNA damage, lipid peroxidation and protein nitration, all damaging the cell, ultimately leading to necrotic or apoptotic cell death. A number of antioxidative systems are in place to protect the cell. MnSOD dismutates superoxide to hydrogen peroxide which is then converted to oxygen and water by antioxidative enzymes such as glutathione, catalase and peroxiredoxins. Hydrogen peroxide may also react with iron in a fenton reaction to create the deleterious hydroxyl radical, damaging the cell. Peroxynitrite is scavenged by several enzymes. Peroxiredoxin 5, thioredoxins and glutathione convert peroxynitrite to nitrite, which is then converted to nitrate, an inert molecule. The redox system was manipulated by the addition of NO (DETA NO), superoxide (DMNQ), peroxynitrite (SIN-1) and 4-HNE. The overview is not comprehensive but enhances the main reaction routes studied and manipulated in the current thesis.

Superoxide is formed by the acceptance of an electron from dioxygen, resulting in a highly reactive radical (Rigoulet et al. 2011). Superoxide does not cross membranes; peroxynitrite formation occurs primarily depending on superoxide availability (Szabo et al. 2007). Superoxide is mainly produced by NADPH oxidases and as a byproduct from mitochondrial oxidative phosphorylation, why powerful protective enzymatic systems are needed to eliminate superoxide and ensure cell survival. Elevated levels of superoxide are found after TBI in cats (Kontos and Wei 1986).

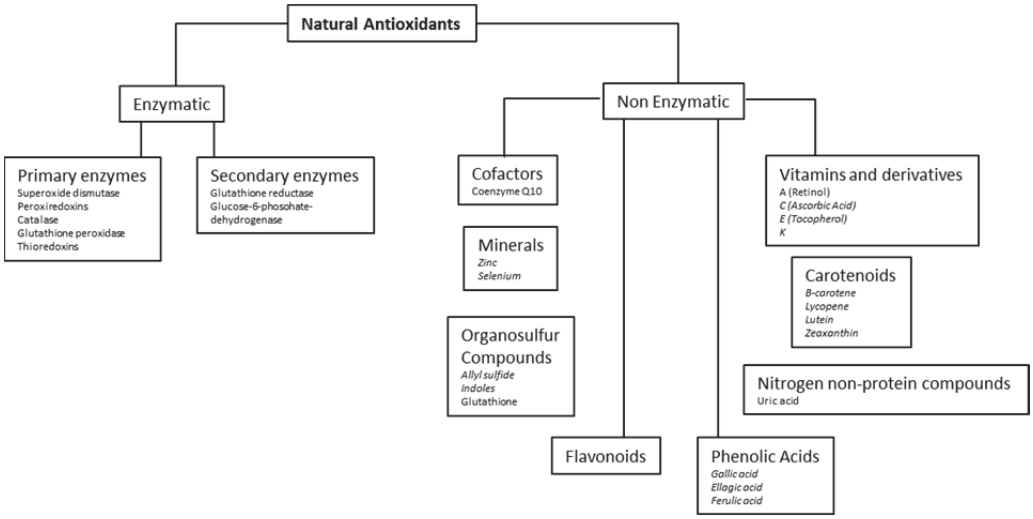


Figure 2 - Natural antioxidants separated in classes. Modified from Carocho et al. Exogenous antioxidants are stated in italic. Enzymes studied belong to primary enzymes.

The antioxidant system of the cell is divided into two major groups. Enzymatic oxidants prevent the formation of or neutralize free radicals and are divided into primary and secondary enzymes. The major primary enzymes SOD, catalase, glutathione peroxide and thioredoxins maintain cell survival in physiological conditions. In an inflammatory milieu, antioxidative enzymes are elevated to meet an increased reductive demand. The secondary enzymatic defense includes glutathione reductase which reduces glutathione from its oxidized to its reduced form. The secondary enzymes do not neutralize free radicals directly, but have supporting roles to the other endogenous antioxidants (Carocho and Ferreira 2013). Despite the efficiency of the enzymatic systems, the organism still depends on dietary antioxidants for efficient free radical detoxification. These include Vitamins A, C, E, K, and may be important to for supplementation in the posttraumatic inflammation (Pietta 2000).

After TBI, manganese superoxide dismutase (MnSOD), peroxiredoxin 5 (PRDX5), glutathione peroxidase and selenium containing amino acids account for the oxidative defense (Arteel et al. 1999) (Szabo et al. 2007) (Fukai and Ushio-Fukai 2011). MnSOD is important in the first line defense against superoxide generated in mitochondria, converting superoxide to hydrogen peroxide at a diffusion limited rate (Flynn and Melov 2013). Over-expression of MnSOD causes reduced lipid peroxidation, protein nitration and neuronal death after experimental TBI (Keller et al. 1998) and MnSOD deficient mice die at an early stage (Li et al. 1995) (Lebovitz et al. 1996). Superoxide reacts with NO three times faster than with MnSOD, leading to the irreversible formation of peroxynitrite if superoxide levels exceed the MnSOD activity (Faraci 2006). MnSOD is induced in neurons and activated microglia after TBI by increased superoxide formation, decreasing oxidative stress (Noack et al. 1998) (Keller et al. 1998).

Peroxiredoxins are present in all organisms, and mammalian cells express six isoforms. The enzymes are abundant and protect the cell by removing low levels of peroxides produced by normal metabolism. PRDX5 reduces peroxynitrite with a high rate constant of $7x10^7$ $M^{-1}s^{-1}$, and is therefore important to decrease the toxic impact of excessive formation of peroxynitrite (Dubuisson et al. 2004) (Rhee et al. 2005). PRDX5 lowers nitro-oxidative stress and cell death (Szabo et al. 2007), and PRDX5 gene silencing makes cells more vulnerable (Knoops et al. 2011). While glutathione also reduces peroxynitrite, it occurs with a lower rate constant of $8x10^6$ $M^{-1}s^{-1}$ (Briviba et al. 1998) (Sies et al. 1997), and glutathione does not react sufficiently fast with peroxynitrite in vivo. Glutathione primarily inhibits peroxynitrite dependent processes by reactions with secondary radicals (Carballal et al. 2014).

NO is formed by three NO synthases. Endothelial (eNOS) and neuronal (nNOS) isoforms are calcium dependent and endogenously expressed in the brain, while the inducible (iNOS) isoform is expressed primarily in macrophages, microglia, infiltrating neutrophils and neurons. Independent of calcium, iNOS increases following TBI and has been regarded as primarily detrimental (Deng et al. 2007). iNOS is induced by the transcription factors NFkB, STAT-1, IRF-1 and AP-1 and thus highly regulated by inflammation (Miljkovic and Trajkovic 2004) (O'Connell and Littleton-Kearney 2013). Inhibition of iNOS has resulted either in decreased lesion volumes and improved sensorimotor-outcomes, (Wada et al. 1998) (Gahm et al. 2000) (Gahm et al. 2006) but also worse functional outcome and increased mortality (Sinz et al. 1999) (Hlatky et al. 2003) (Jafarian-Tehrani et al. 2005) (Lu et al. 2009).

The concentrations and kinetics of NO, superoxide and peroxynitrite are essential for cell signaling, metabolism, and the commitment of the cell to survival or death (Dranka et al. 2010). Antioxidant treatment ameliorates these effects and provides neuroprotection in experimental models, thus confirming a deleterious effect of the oxidative cascade after TBI (Bains and Hall 2011).

## 1.2 GENETIC POLYMORPHISM AFTER TBI

Considerable variability exists in the clinical outcome after TBI, which is only partially explained by known factors. Accumulating evidence implicates genetic elements in the pathophysiology of brain trauma in humans (Dardiotis et al. 2010), and several genes influence outcome (Jordan 2007). The natural heterogeneity of humans suffering from TBI makes studies of genetic impact on outcome and possible intervention challenging, but inbred rodent strains react reproducibly regarding inflammation in the central nervous system (CNS), thus providing an investigative platform (Reid et al. 2010) (Dardiotis et al. 2010) (Maas et al. 2010) (McAllister 2010). The rat strain Dark Agouti (DA) has high susceptibility while Piebald Virol Glaxo (PVG) is reported to be protected from effects of TBI, experimental autoimmune encephalomyelitis, nerve axotomy and spinal cord injury (Al Nimer et al. 2011) (Bellander et al. 2010) (Dominguez et al. 2012) (Lidman et al. 2003) (Braden 1958) (Wilson 1965) (Lorentzen et al. 1997) (Weissert et al. 1998) (Lundberg et al. 2001). These differences are likely to be a combined effect of a number of genetic variations rather than a few spontaneous mutations.

DA and PVG strains have fundamentally different inflammatory responses after TBI. DA expresses higher levels of macrophages (Bellander et al. 2010), granulocytes, natural killer cells and microglia compared to PVG (Al Nimer et al. 2013a). DA also has a higher activation of the complement system factors C3, C1q and CD11b, but not CD55 and CD59 (Bellander et al. 2010) (Al Nimer et al. 2013a). Both inflammatory cells and complement activation are linked to increased oxidative stress. Activated microglia is the main endogenous source of ROS in several major CNS disorders (Block et al. 2007). C3-/- mice had smaller infarct volumes, improved neurological deficits, reduced granulocyte infiltration and oxidative stress after brain ischemia than wild type mice (Mocco et al. 2006). C1q -/- mice neurons had less oxidative stress after hypoxia/ischemia compared to wild type (Ten et al. 2010). Most of the genetic polymorphisms that have been identified regulate disease susceptibility affect immune related molecules and pathways (Al Nimer et al. 2013a). However, genetic influence on oxidative stress is insufficiently elucidated and conflicting results in TBI research may partly be related to unknown genetic variability (Teasdale et al. 1997) (Popovich et al. 1997) (Steward et al. 1999) (Friedman et al. 1999) (Inman et al. 2002).

## 1.3 GENDER INFLUENCE AFTER TBI

In experimental TBI, females are more resistant to TBI than males (McCullough and Hurn 2003) (Roof and Hall 2000). In humans, epidemiological studies show contradictory results on gender associated morbidity. Female gender correlated with reduced mortality and lower complication rates after TBI in some studies (Ley et al. 2013) (Groswasser et al. 1998) (Berry et al. 2009), while others found no gender association (Renner et al. 2012) (Leitgeb et al. 2011), or even

higher mortality in in females (Farace and Alves 2000) (Ottochian et al. 2009). The reasons for the relative protection of females in animal models are unclear. In a brain ischemia-reperfusion model, male mice showed increased levels of proinflammatory enzymes COX-2, NOX2 and VCAM-1 compared to females (Brait et al. 2010), possibly related to female sex hormones. Progesterone inhibits COX-2, $PGE_2$ and TNFα expression in male rats after TBI (Si et al. 2014). Edema formation and intracranial pressure after TBI vary according to the estrus cycle (Maghool et al. 2012). Progesterone reduces lipid peroxidation and suppresses neuronal hyperexcitability, leading to membrane stabilization (Roof and Hall 2000). Further knowledge of underlying mechanisms for different inflammatory traits is therefore warranted.

| Marker | Time | DA | PVG | Reference |
|---|---|---|---|---|
| Ox-42 (macrophages) | 7h | up | down | Bellander 2010 |
| w3/13 (leucocytes) | 3h | down | up | |
| ED-1 (microglia/macrophages) | 7-13h | same | same | |
| C3 (complement system) | 7h | up | down | |
| Fluoro Jade | 30d | up | down | |
| Granulocytes | 24h | up | down | Al Nimer 2012 |
| leucocytes | 1-6d | same | same | |
| T-cells | 1-6d | same | same | |
| NK cells | 1-6d | up | down | |
| Macrophages | 6d | up | down | |
| Microglia | 1-6d | up | down | |
| Lymphocytes | 1-6d | same | same | |
| C1q (complement system) | 6d | up | down | |
| CD55 (complement system) | 6d | same | same | |
| CD59 (complement system) | 6d | same | same | |
| CSF-NFL (neurofilament light) | 1-6d | up | down | |
| GFAP (glia cells, gliosis) | 6d | same | same | Al Nimer 2010 |
| CD11b (complement system) | 6d | up | down | |
| Gsta4 | 1d | down | up | Al Nimer 2012 |
| Neuronal survival | 30d | down | up | |
| iNOS | 24h | down | up | Günther 2011 |
| eNOS | 24h | same | same | |
| nNOS | 24h | same | same | |
| w3/13 (leucocytes) | 24h | same | same | |
| MnSOD | 24h | down | up | |
| 3-NT | 24h | same | same | |
| Fluoro Jade | 24h | same | same | |

Figure 3 – Inflammatory responses by DA and PVG rats after trauma to the CNS. DA is susceptible to CNS inflammation and DA is reported to be protected. The strains display fundamentally different response patterns in CNS inflammation. A higher response than the other strain is termed “up” while a lower response is termed “down”. All quantifications are made in the CNS after trauma.

## 1.4 TREATMENT OF TBI

No pharmacological treatment that specifically targets the CNS has led to better neurological outcomes in phase III clinical trials (Diaz-Arrastia et al. 2014). The most important barrier to finding effective therapeutic interventions includes insufficient understanding of fundamental pathophysiological reactions in the brain. Confounding factors such as genetic polymorphisms and gender are not sufficiently appreciated and complicate clinical trials. The heterogeneity of TBI ranges from mild concussion to severe penetrating brain injury and it is likely that different trauma mechanisms comprise different pathophysiology and varying inflammatory activation, requiring specific treatment (Saatman et al. 2008) (Marklund et al. 2006). Treatment of TBI today comprises surgery combined with neurocritical care to treat mass effects and intracerebral pressure (ICP) and to prevent secondary insults. However, there is no pharmacological intervention to ameliorate the secondary processes or attenuate CNS inflammation.

### 1.4.1 Surgery

Surgical management of TBI depends on the underlying pathology. Operative indications include acute subdural hematomas, chronic subdural hematomas, epidural hematomas and intraparenchymal lesions (contusions and hematomas) (Feinberg et al. 2015). Standard techniques include craniotomy for evacuation of mass lesions and decompressive craniectomy for reducing ICP due to diffuse cerebral swelling. Early surgical intervention, when indicated, is effective to reduce ICP and improve survival and neurological outcomes (Foundation 2007). If elevated, the primary goal of surgery is to reduce ICP to levels <20 mm Hg (Feinberg et al. 2015). However, the surgical intervention does not directly affect neuroinflammatory events. Medical interventions to lower ICP and lower diffuse swelling would therefore decrease the need for surgical interventions in some TBI cases (Feinberg et al. 2015).

### 1.4.2 Neurocritical care

Advances in neurocritical management of TBI have reduced mortality by nearly half since the 1970s (Feinberg et al. 2015). The primary aim is to limit the secondary injury and to avoid secondary insults to the brain. Secondary insults include fever, seziures, hypoxia, coagulopathy, hyperglycemia, endocrine derangements, vasospasm, nutritional derangements and paroxysmal sympathetic hyperactivity (Sheriff and Hinson 2015). The inflammatory response resulting from acute TBI is not limited to the brain. Multiple organ dysfunction syndromes are commonly seen, triggered by catecholamines, neurokinins, cytokines, growth factors, and chemokines (Ghirnikar et al. 1998). Oxidative stress is a major cause of morbidity and mortality in critically ill patients

(Hanafy and Selim 2012). It is likely that oxidative stress also affects the brain, as a result of the direct impact and as part of the global critical illness. The levels of nutrients with antioxidative properties are decreased in neurological critical illness. Patients with intracerebral hematomas have decreased plasma levels of vitamin C (Polidori et al. 2001), and low endogenous stores on antioxidant nutrients are associated with increased free radical generation (Hanafy and Selim 2012). The restoration of endogenous antioxidants combined with administration of oxidative stress reducing compounds is therefore a likely component of future TBI care. The implementation of antioxidant-therapy in neurocritical care needs multiple considerations. The lack of blood brain barrier penetration, suboptimal drug dosages, poor target specificity, low bioavailability at desired sites and a narrow therapeutic time windows remain unsolved challenges (Hanafy and Selim 2012). The monitoring of oxidative stress in the CNS would also be crucial. Experimental methods to study include microdialysis to measure free radicals and oxidative stress in interstitial brain fluid (Clausen et al. 2012) and MRI evaluation of oxidative stress by measuring the pro-oxidant ferric form of hemoglobin on T1-weighted imaging (Leung and Moody 2010).

### 1.4.3 Pharmacotherapy

Pharmacotherapy for TBI is a highly prioritized research field. The U.S. department of defense alone currently funds more than 500 projects. Critical areas to be addressed involve the standardization of pre-clinical models of TBI, identification of predictive biomarkers, pharmacotherapy aimed at neurorepair, regeneration and protection, and combination therapies for multiple injury mechanisms. So far, all clinical trials in TBI treatment have failed, but more than 50 pharmacotherapies are currently undergoing phase II or III evaluations. The efficacy of neuroprotective drugs relates to the study cohort and study size. Nimodipine, a calcium channel blocker in clinical use for preventing vasospasm after subarachnoid bleedings, significantly reduced unfavorable outcome (death, vegetative survival, or severe disability) at 6 months (Harders et al. 1996) This was later disputed in a systematic review (Vergouwen et al. 2006), and considerable uncertainty still remains over its effect (Langham et al. 2003).

The US department of defense neurotrauma pharmacology workgroup has selected a few leading drug candidates based on mechanism of action, pre-clinical evidence in TBI and other related models and clinical development to date. These include Amantadine, Cyklosporine A, Donepezil, Erythropoetin, FK-506, Glyburide, Growth hormone, Huperzine A, Lithium, Methylphenidate, Minocycline, NAC, Rivastigmine and Simvastatin (Diaz-Arrastia et al. 2014). The pharmacological candidates have a range of targets, including apoptosis, excitatory amino acids, inflammation, free radical production, hyperdepolarization and effect of altered levels of $Ca^{2+}$ (Marklund et al. 2006).

With increasing knowledge of oxidative stress, antioxidative therapies are again gaining interest. Several antioxidants, including N-acetylcysteine (NAC), lipoic acid, tocopherol, probucol, Tirilazad and Coenzyme Q10 have been evaluated in TBI but failed to produce lasting effects (Sunitha et al. 2013). Drug administration to the brain requires crossing the blood brain barrier which is a recurring problem in CNS pharmacotherapy. The well-known antioxidative compound, NAC, with neuroprotective effects in experimental TBI and brain/spinal cord ischemia (Hicdonmez et al. 2006), has a limited CNS bioavailability of 6-10% (Sunitha et al. 2013). Drugs with increased blood brain barrier penetrance would have a higher potential to be effective treatments. *N*-acetylcysteine amide (NACA) is a drug designed to overcome CNS penetrance deficits. Novel drugs need to show robust neuroprotection in experimental TBI and a pharmacological mechanism of action in the CNS that needs to be established before clinical testing. Thorough preclinical trials lower the risk of failure in clinical trials.

# 2 AIMS OF THE THESIS

I. To study the impact of genetic polymorphism on NOS isoenzyme expression, antioxidative enzyme MnSOD and markers of protein nitrosylation following experimental brain contusion in the rat.

II. To investigate the impact of genetic polymorphism on oxidative stress in isolated rat neurons by measuring iNOS expression, antioxidative enzymes MnSOD, PRDX5 and markers of protein nitrosylation, lipid peroxidation and neuronal degeneration.

III. To study the gender influence on the posttraumatic inflammation by measuring COX-2, iNOS, markers of nitrosylation, astrogliosis, microgliosis, apoptosis and neuronal degeneration following experimental brain contusion in the rat.

IV. To investigate whether shock wave trauma caused inflammatory activation and iNOS induction in rodent macrophage cell lines.

V. To investigate the neuroprotective capacity of *N*-acetylcysteine amide by analysis of neuronal degeneration, apoptosis, iNOS expression and markers of inflammation and oxidative stress, following experimental focal penetrating brain injury in the rat.

# 3 MATERIALS AND METHODS

## 3.1 ETHICAL PERMITS

All animal experiments were approved by the Swedish ethics committee in Stockholm. Article I: N307/06, Article II: N176/12, Article III: N255/09 and N81/13, Article IV: N/A, Article V: N530/11.

## 3.2 RATS

The DA strain was originally obtained from Medizinische Hochschule, Hannover, Germany. The PVG and Sprague Dawley strains were obtained from Harlan UK Ltd and NOVA-SCB. All animals were bred in an in-house breeding facility in polystyrene cages containing aspen wood shavings with 12h light/dark cycles and fed standard rodent chow and water ad libitum.

## 3.3 CELL CULTURES

### 3.3.1 Immortalized cell lines

Cell lines were grown and sub-cultured in T-25 and T-75 flasks (Nunc). NR8383 macrophages (rat) were grown in F-12K medium with 15% FBS and RAW264.7 macrophages (mouse) were grown in DMEM with 10% FBS. All serum was heat inactivated. The cells were harvested and re-suspended on glass cover slips with a 13 mm diameter and 2 mm thickness (Labora Chemicon), coated with Poly-D-lysine (Sigma-Aldrich), and placed in Nunclon 24-well plates (Nunc).

### 3.3.2 Primary neuronal cultures

Female DA and PVG rats were simultaneously pared with respective males for 72h. Pregnant rats were sacrificed by $CO_2$ 18 days later, ensuring an embryonic post gestation age between E18-E21. Hippocampal neuronal cultures from DA and PVG were prepared simultaneously by dissecting the embryonic hippocampi before dissociation by trypsin (Life Technologies) in 37° C for 15 min followed by mechanical dissociation by a Pasteur pipette. The cell concentration was determined in the suspension by Countess automated cell counter (Life Technologies) and cells were seeded at $3x10^5$ cells/well and placed in Nunclon 24- or 48-well plates (Thermo Scientific), coated with Poly-L-Lysine (Sigma-Aldrich). The cells were kept in Neurobasal medium

supplemented with B-27, L-Glutamine 200 mM and gentamicin 15 µg/ml (Life Technologies). The B-27 supplement contained antioxidants Vit E, Vit E acetate, SOD, Catalase and Glutathione. The neuronal-glial ratio was >98% determined by immunofluorescence double staining with NeuN and GFAP.

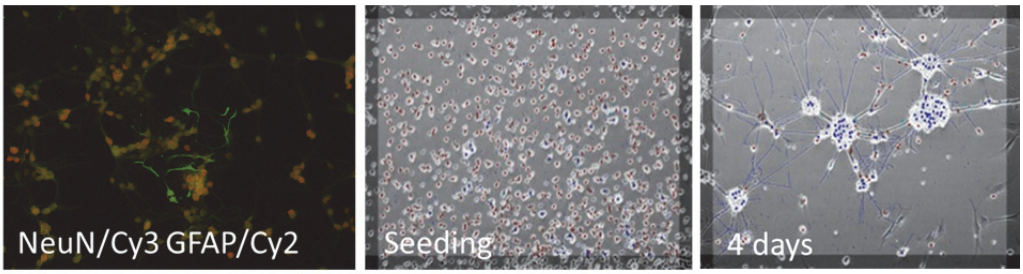


Figure 4 – left: double staining immunofluorescence with NeuN (neurons) and GFAP (glia cells). Secondary detection by Cy3 (red) and Cy2 (green). The The neuronal-glial ratio was >98% in the primary neuronal cultures. Middle: neuronal cultures at seeding, with red marks for Cell-IQ counting. Right: After 4 days the cells form neurospheres and neurites. Oxidative measurements were made at day 2, before the formation of neurospheres.

## 3.4 TRAUMA MODELS

### 3.4.1 Experimental traumatic brain contusion

Male rats weighing approximately 230–300 g, at an age of 8–12 weeks, were anesthetized by intraperitoneal injection of 2.7 ml/kg of a mixture of Hyperoxynitriteorm (fluanisone, 10 mg/ml, fentanyl citrate, 0.315 mg/ml, Janssen), Dormicum (midazolam, 1 mg/ml, Roche) and sterile water. In addition, 0.2 ml of Marcain (bupivacaine, 5 mg/ml, AstraZeneca) was injected subcutaneously in the sagittal midline of the skull before the skin incision was made. The rats were placed in a stereotactic frame and a 2 mm craniotomy was drilled 3 mm posterior and 2.3 mm lateral to the bregma. A standardized parietal contusion was made by letting a 24 gram weight fall onto a rod with a flat end diameter of 1.8 mm from a height of 7 cm (Feeney et al. 1981) (Holmin and Mathiesen 1996). The rod was allowed to compress the tissue for a maximum of 3 mm. Sham operated animals were subjected to identical treatment except for the weight drop injury. All animals were sacrificed using $CO_2$.

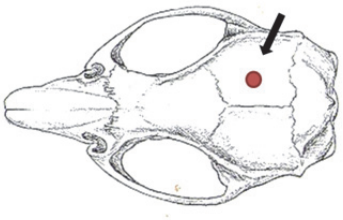

Figure 5 – Rodent skull illustrating the craniotomy (performed, marked with a red dot (image in public domain).

### 3.4.2 Focal penetrating traumatic brain injury

The penetrating model leads to cell death and cavity formation, hemorrhage, neurodegeneration, gliosis, and a deficiency in reference memory, likely due to injuries of the cortex and the hippocampus . Male and female rats weighing between 250 and 300 g were anesthetized by a 2.4 mL/kg intra-abdominal injection of a mixture of 1ml midazolam (5 mg/mL), 1mL Hypnorm (VetaPharma) and 2 mL $dH_20$. A midline incision was made through the skin and periosteum, and a burr hole 2.7 mm in diameter was drilled with its center 3 mm lateral and 3 mm posterior to the bregma. The rat was placed in a stereotactic frame and positioned with the probe directly above the dura mater. A lead pellet was accelerated by air pressure hitting a metal cylinder probe with an attached carbon fiber pin with a tip radius of 1 mm. Depth of penetration into the brain by the pin was limited to 5 mm. After the injury the craniotomy was left open and the skin was sutured. Sham operated animals were subjected to identical treatment except for the penetrating injury. All animals were sacrificed by an overdose of pentobarbital. Penetrating brain injuries are particularly prevalent in war zones (Meyer et al. 2008) and in areas with a high incidence of gun related violence (Coronado et al. 2011), although occurring in all parts of the world.

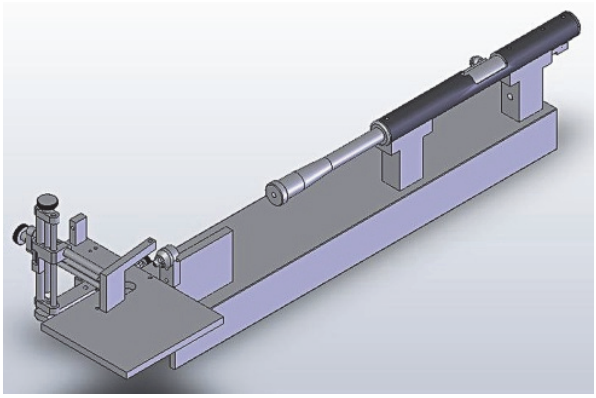

Figure 6 – Illustration of the focal penetrating traumatic brain injury model. The rat is placed in a stereotactic frame (lower left corner). A modified air rifle is accelerating a lead pellet creating the injury. Figure courtesy of Johan Davidsson, Chalmers University of Technology.

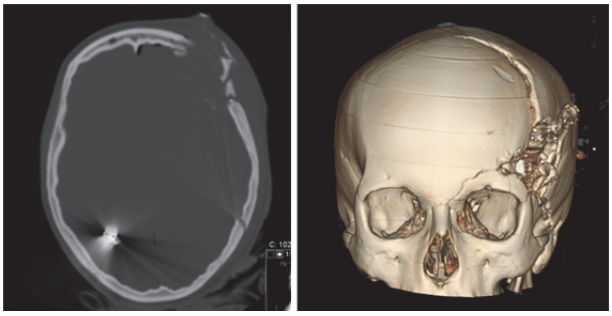

Figure 7 – Right: 3D reconstruction of a CT scan of the head showing a penetrating brain injury by a bullet entering the left temporal part of the skull. Left: CT scan of the head showing bony destruction (upper right) and a bullet remaining inside the brain (lower right). The pictures serve only to illustrate the need for encompassing the injury panorama in penetrating injuries. Picture courtesy of Dr. Ulrika Sandvik, the Neurosurgery department at Karolinska University Hospital in Stockholm.

### 3.4.3 Flyer Plate

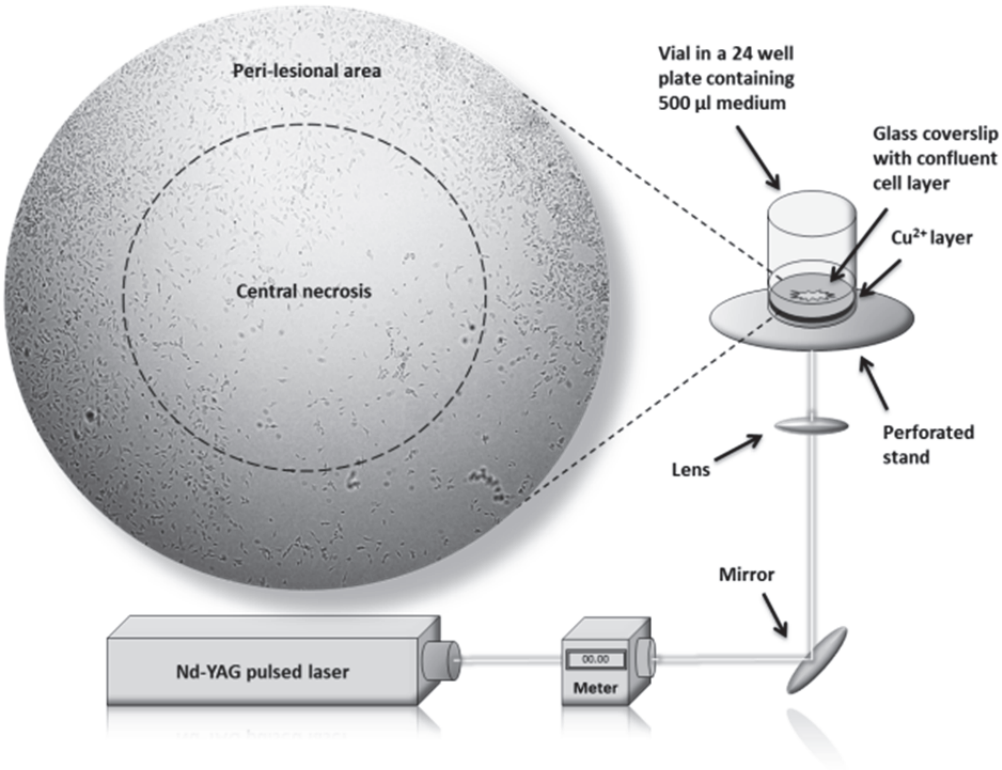


Figure 8 – illustration of the flyer plate model.

A Nd–YAG laser (wavelength 1.064 nm, Quantel) creating laser pulses with a duration of 6 nanoseconds and a pulse energy of 5-6 mJ was aimed on to a fused silica window with a 7 mm thin layer of copper vapor deposit on the top side. The diameter of the beam at the target was <3 mm. When hit by a laser pulse, the inner layer of the copper vaporized accelerating a flyer-plate which hit the bottom of a cell culture well, placed in immediate contact with the fused silica window. As a result, a shock wave pulse with cavitation was generated in the well. The amount of medium used in each well was 400µl, as it resulted in visual confirmation of cavitation in the well by droplet formation on the inside of the well roof (Sonden et al. 2006).

## 3.5 PHARMACOLOGICAL INTERVENTION

### 3.5.1 LPS/IFN-ɣ induction

Cell cultures were induced by 500 ng/ml LPS from E-coli 0128:B12 (Sigma-Aldrich), and 100 ng/ml recombinant Rat IFN-ɣ (Millipore), added to the cell medium for 24h. LPS/IFN-ɣ stimulation is an established method of macrophage induction (Griscavage et al. 1993) (Stuehr and Marletta 1987).

### 3.5.2 Oxidative stress induction

Oxidative stress was induced by the following compounds. (1) Diethylenetriamine/NO adduct (DETA NO) releases 2 mol NO/mol parent compound (Sigma Aldrich). A stock was prepared (50 mM) in $dH_2O$ which was diluted in cell culture medium in concentrations according to previous studies (Dranka et al. 2011) (Dranka et al. 2010). (2) 2,3-Dimethoxy-1,4-naphthoquinone (DMNQ) releases $O_2^-$ (Sigma Aldrich). A stock was prepared (15 mM) in DMSO which was diluted in cell culture medium in concentrations according to previous studies (Dranka et al. 2011) (Dranka et al. 2010) (Tamm et al. 2008). The concentration of DMSO in cell culture medium did not exceed 0.1%. (3) 3-Morpholinosydnonimine hydrochloride (SIN-1) uses molecular oxygen to generate both $O_2^-$ and NO that spontaneously form $ONOO^-$ (Sigma Aldrich). A stock was prepared (3 mM) in $dH_2O$ which was diluted in cell culture medium in concentrations according to previous studies (Acquaviva et al. 2004; Trackey et al. 2001). (4) 4-Hydroxynonenal (4-HNE) is formed by peroxidation of fatty acids (Calbiochem). The stock was supplied at 10 mg/ml and diluted in cell culture medium in concentrations according to previous studies (Dranka et al. 2011) (Malecki et al. 2000).

### 3.5.3 *N*-acetylcysteine amide

NACA (Sentient LifeSciences) was administered in rats by intraperitoneal injections 300 mg/kg after 2 min and additional boluses of 300 mg/kg after 4h (100 mg/mL in distilled water). The dosage was chosen to deliver maximum CNS efficacy according to recent studies (Patel et al. 2014).

## 3.6 ASSAYS

### 3.6.1 Rat brain sections

Rat brains were snap frozen in 2-methylbutane and stored at -70°C. They were then cut into 14-µm coronal and horizontal sections using a Microm HM560 and a Leica CM3000 cryostat. The

sections were extending from the frontal to the dorsal border of the injury, macroscopically verified to encompass contused brain matter. The frozen sections were mounted onto Thermo Scientific Superfrost plus slides and stored at -70°C.

### 3.6.2 Immunohistochemistry

Sections were rehydrated in PBS followed by fixation in 4% formaldehyde, incubation in 0.3% $H_20_2$, incubation for 1h in bovine serum albumin (BSA) with 0.3% Triton X-100, sodium azide and avidin block solution (ABC method, Vectastain Elite ABC peroxidase kit, Vector Labs). The primary antibody was incubated overnight at 4º C. Sections were incubated for 1h with a biotinylated secondary antibody, followed by avidin-biotin complex for 1h, followed by DAB 3 min and counterstaining with Hematoxylin III nach Gill and Entellan mount (Merck) or Pertex (Histolab). All steps included washing for 3x10 min with phosphate buffer solution (PBS).

### 3.6.3 Immunofluorescence

Sections were rehydrated in PBS followed by fixation in 4% formaldehyde, incubation for 1h in BSA with 0.3% Triton X-100 and NaAzid and incubated over night at 4º C with the primary antibody diluted in 1% bovine serum albumin, 0.3% Triton X-100 and NaAzid. Following 1h incubation with the secondary antibody, sections were mounted with Mowiol (Polysciences) or ProLong Gold antifade with or without DAPI (Life Technologies).

### 3.6.4 Fluoro Jade

Fluoro Jade marks degenerating neurons (Schmued et al. 1997). Sections were, after fixation in 4% formaldehyde, incubated for 10 min in 0.06% $KMnO_4$ and 30 min incubation in Fluoro Jade B (Millipore) or Fluoro Jade (Histochem) in a working solution of 0.00002% in 0.1% acetic acid, before being washed in $dH_2O$ and dried on a 50°C hot plate (approx. 50º C) and mounted with Entellan or Pertex.

### 3.6.5 TUNEL

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is indicative of apoptosis, and was performed with TACS 2 TdT-Blue Label in situ Apoptosis Detection Kit (Trevigen) according to manufacturer's instructions.

### 3.6.6 In situ hybridization

Radioactive in situ hybridization was done by using 35S-labeled 40- to 48-mer oligonucleotides complementary to mRNA encoding respective protein (Dagerlind et al. 1992). Oligonucleotides were manufactured by Cybergene. The probes were hybridized to the sections without

pretreatment overnight at 42°C. After hybridization, the sections were washed several times in 13 SSC at 60°C, dehydrated in ethanol and dipped in NTB2 nuclear track emulsion (Kodak, Rochester, NY). After 4 weeks, the sections were developed in D-19 developer (Kodak) and coverslipped.

### 3.6.7 Western blot

Cells were washed with 4°C HBSS. RIPA lysis buffer (TBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide, PMSF, protease inhibitor cocktail, sodium orthovanadate) was added for 15 min at 4°C (Santa Cruz Biotechnology). Cells were scraped from the bottom of the wells and placed in plastic tubes (6-8 wells were combined for one sample in electrophoresis) and centrifuged for 10 min at 10.000 rpm at 4°C. The protein content was determined in the supernatant by a protein assay (Bio-Rad). Samples were denaturated (70°C, 10 min) and reduced (2.5% β-mercaptomethanol), and loaded on NuPAGE Novex Bis-Tris 10% mini gels (Life Technologies) with Odyssey protein molecular weight marker (Li-Cor).

Electrophoresis and transfer to PVDF membranes was done in XCell SureLock Mini-Cell, using buffers according to manufacturer's instructions (Life Technologies). Membranes were blocked for 1h in Odyssey blocking buffer (Li-Cor) and incubated overnight in 4°C with the primary antibody and tubulin loading control diluted in Odyssey blocking buffer. Membranes were washed 4x5 min in PBS+0.1% Tween20 and incubated in secondary antibodies diluted in Odyssey blocking buffer for 1h, followed by washing 5x5 min in PBS+0.1% Tween20 before being scanned by Odyssey infrared imaging system (Li-Cor), allowing two antibodies to be detected simultaneously in 700 nm and 800 nm.

### 3.6.8 In Cell Western

Cells were fixed in 4% formaldehyde for 10 min and permeabilized 5x5 min in PBS+0.1% Triton X-100. Cells were blocked for 1h in Odyssey blocking buffer, and incubated overnight at 4°C with the primary antibody diluted in Odyssey blocking buffer, validated for ICW specificity by western blot. Cells were washed 5x5 min in PBS+0.1% Tween20 before being scanned in 169 µm resolution by Odyssey infrared imaging system. Secondary IR antibodies used were the same as for western blot. The integrated intensity in each well was normalized to the actual cell number by DRAQ5 and Sapphire700 normalizing cell staining agents (Li-Cor), reducing any difference in protein expression as a result of varied cell amounts.

### 3.6.9 Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) is an oxidoreductase present in all cell types. LDH is released into the cell culture medium relatively to the loss of cell membrane integrity, thus providing a measure of necrotic cell damage. LDH activity in cell culture medium was measured by a

colorimetric assay (Abcam). LDH reduces NAD to NADH, which interacts with a specific probe to produce a color (λmax = 450 nm), quantified by Multiskan EX plate reader (Thermo Fisher Scientific). A standard curve was constructed. The LDH activity in the medium was normalized to the total protein amount in the wells, quantified for western blot as previously described.

### 3.6.10 CellROX oxidative stress detection

CellROX green reagent is a fluorogenic probe for measuring oxidative stress in living cells. The cell-permeant dye is weakly fluorescent while in a reduced state and exhibits bright green photostable fluorescence upon oxidation by ROS and subsequent binding to DNA, with absorption/emission maxima of ~ 485/520 nm (GFP) (Life Technologies). CellROX was added to the wells in a 5µM final concentration after 2h of oxidative stress. NucBlue reagent, a Hoechst 33342 cell-permeant nuclear counterstain was added for 15 min (Life Technologies). After 30 min, the cells were washed x 2 with 4°C HBSS. The cell culture plates were photographed in 20x magnification in a Zeiss Observer Z inverted microscope. For each view, a DAPI and a GFP picture was taken and subsequently quantified in CellProfiler (Jones et al. 2008) by measuring the integrated intensity of the GFP staining at the loci of corresponding DAPI staining, thus measuring oxidative stress level in individual cells.

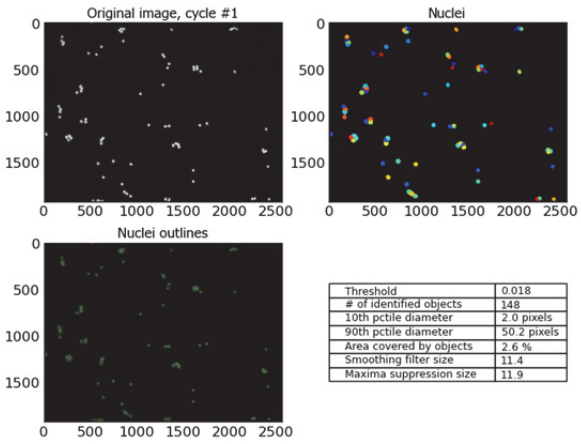


Figure 9 – Illustration of automated quantification by CellProfiler. Pictures were quantified by the nuclear DAPI staining, and the GFP response was measured for each nucleus.

### 3.6.11 Gene array analysis

Trizol (Life Technologies) was added for 3 min, and cells were removed using a cell scraper. 8 wells were pooled for each sample and kept in -70° C before being analyzed in Affymetrix RaGene-1_1-st-v1 and MoGene-1_1-st-v1 chips (n=3). Normalization and transformation of CEL-files was done in Affymetrix Expression Console software using the PLIER protocol. Gene by gene comparison and hierarchical clustering was done in Affymetrix Transcriptome Analysis

Console. Genes with significant differential regulation (fold change 1.4 and $p<0.05$) were analyzed in DAVID bioinformatics database (Huang et al. 2009). Functional annotation clusterings with enrichment scores >1.3 (corresponding to $p<0.05$) were compared for the groups.

### 3.6.12 Cell-IQ

Cell monitoring and morphological differentiation was made with the Cell-IQ 2 live cell imaging and analysis platform (Chipman Tech). Cells were continuously photographed by a 10x phase contrast microscope camera in Nunclon 24-well plates (Nunc), in an incubator setting (figure 2). Cell growth was measured and defined as Δ cell count / Δ time. Analysis of cell morphology and cell count was done with Cell-IQ analyzer software (Chipman Tech).

### 3.6.13 Griess nitrite assay

Nitrite content in the cell medium was measured by the Griess nitrite assay (Promega). Nitrite ($NO_2^-$) reacts with sulfanilic acid to form a diazonium cation which produces a red–violet colored azo dye (Tsikas 2007). Absorbance was measured at 540 nm by a Multiskan EX plate reader (Thermo Fisher Scientific).

## 3.7 QUANTIFICATION

### 3.7.1 Immunohistochemistry

The region of interest (ROI) was defined in coronal sections medially by the interhemispheric fissure and the midline; basally by the lower part of the third ventricle or corpus callosum, and laterally by the lateral border of the right hemisphere. Sections were analyzed in mid-lesion at approximate level Bregma -3.86 mm. In horizontal sections the ROI was defined medially by the interhemispheric fissure and the midline, dorsally by the dorsal cerebral border and laterally by the lateral border of the right hemisphere. Sections were analyzed in mid-lesion at approximate level Bregma -1.70 mm. The central necrotic part of the contusion was omitted from the ROI.

Sections were photographed in 4x - 400x magnification by Leica DMRB and a DM400B microscope equipped with a DFC320 camera or a Nikon Eclipse E600 microscope. Filters used were for Cy3 G-1B (ex/em 541-551/590 nm) or N2.1 (ex/em 515-560/590 nm), Alexa 488 and Fluoro-Jade filter FITC (ex/em 465-495/515-555 nm) or L4 (ex/em 450-490/515-560 nm), DAPI filter DAPI (ex/em 340-380/435-485). Sections were quantified by manual counting or computer assisted analysis by 2-30 sections per animal. For manual morphological identification and quantification all slides were blinded to the assessor. Quantification of TUNEL positive cells was done by counting positive cells, which displayed dark blue spots of DNA fragmentation in

conjunction with pyknotic nuclei. Quantification of 3-NT staining was performed in 400x magnification according to Hooper et al. (Hooper et al. 2000), with a slight modification to fit the ROI: 0 = none, 1 = 1-20 positive loci of staining in ROI, 2 = 20-40 scattered discrete loci of staining or areas of weak staining, 3 = Extensive areas of strong staining. Computer assisted analysis was done in CellProfiler (Jones et al. 2008) or ImageJ (Schneider et al. 2012) using specified scripts.

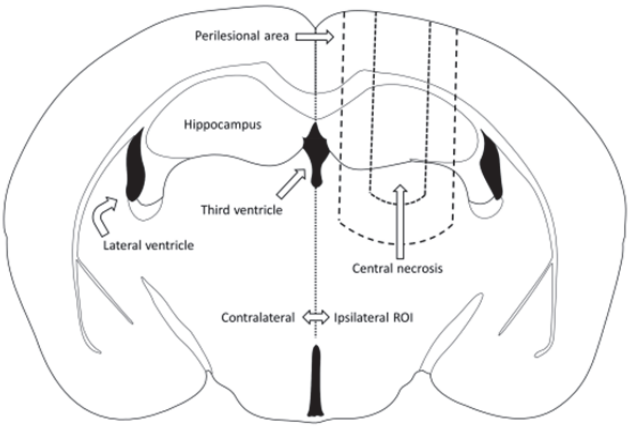


Figure 10 - Illustration of coronal rat brain sections with the central necrosis and the perilesional area outlined. The region of interest (ROI) was defined medially by the interhemispheric fissure and the midline, basally by the perilesional area and laterally by the lateral border of the right hemisphere.

### 3.7.2 In Situ hybridization

Quantification of ISH was done by batch processing in ImageJ (Schneider et al. 2012) by the following script: run("8-bit"); run("Invert"); setAutoThreshold ("Yen/default"); //run("Threshold..."); run("Measure"). The integrated intensity was calculated (∑ pixel intensity (corrected for background) x area), reflecting the level of staining. Quantification of COX-2 ISH was done manually by a blinded assessor due to the inability of the software to satisfactory measure the response for this probe. The pictures were ranked according to the following system: 0 = no staining, 1= weak staining, 2 = clearly defined staining, 3 = heavy staining. 16-80 pictures per animal were analyzed depending on resolution.

### 3.7.3 Western blot

Densiometric quantification and normalization to αTubulin was done in Image Studio v.2.1 (Li-Cor). In addition, all membranes contained an identical sample from rat macrophage cell line NR8383, stimulated with 500 ng/ml LPS from E-coli 0128:B12 (Sigma-Aldrich) and 100 ng/ml recombinant Rat IFN-ɣ (Millipore) for 24h and correspondingly treated for western blot. The sample expressed all proteins/protein-adducts examined and thus allowed all membranes to be

normalized to this sample, removing natural differences in western blot processing and staining, allowing for comparisons between different membranes.

### 3.7.4 In cell western

The images were analyzed in Odyssey Image Studio software. The integrated intensity for each well was calculated (∑ pixel intensity (corrected for background) x area), reflecting the primary antibody answer. The integrated intensity in each well was normalized to the actual cell number by DRAQ5 and Sapphire700 normalizing cell staining agents (Li-Cor), reducing any difference in protein expression as a result of varied cell amounts. A total of 20 wells were analyzed for each experiment.

## 3.8 STATISTICAL ANALYSES

Statistical analyses were done by GraphPad Prism versions 5.04 - 6.05 for Windows. All error bars represent the standard error of the mean. $P<.05$ was considered significant. Significance levels: * $p<.05$, ** $p<.01$, *** $p<.005$, **** $p<.001$.

In article I, the non-parametrical Kruskal-Wallis test was used.

In article II, all results were related to the baseline for that particular assay, probe and strain, and presented as percent of baseline. CellROX, western blots and LDH assays were analyzed by two-way ANOVAs with Šídák´s multiple comparisons test. Baselines were analyzed by the non-parametric Mann-Whitney test. Fetal size was analyzed by Fischer´s exact test. Fetus count, cell count at seeding and average viable cell count at seeding were analyzed by two tailed, unpaired t-test.

In article III, all systems (detection methods) were tested for normal distribution by the Kolmogorov-Smirnov test with Dallal-Wilkinson-Liliefor. The systems that passed the normality test were tested for statistical inference by one-way ANOVA followed by Tukey´s post-test. The systems that failed the normality test were tested for statistical inference by the non-parametric Kruskal Wallis ANOVA followed by Dunn´s post-hoc test.

In article IV, Affymetrix Transcriptome Analysis Console was used in addition. T-tests were used for gene array analysis, ICW, nitrite assay, western blot, and two-way repeated measures ANOVA with Šídák´s multiple comparisons test for Cell-IQ. $p<0.05$ was considered significant. All error bars represent the standard deviation.

In article V, all systems (detection methods) were tested for normal distribution by the Kolmogorov-Smirnov test with Dallal-Wilkinson-Liliefor. The systems that passed the normality

test were tested for statistical inference by one-way ANOVA followed by Fishers Least Significant difference test. The systems that failed the normality test were tested for statistical inference by Mann-Whitney nonparametric test. Outliers were by Grubbs' method and subsequently removed.

# 4 RESULTS AND COMMENTS

## 4.1 GENETIC POLYMORPHISM AFTER TBI

### 4.1.1 In whole rat brain (I)

We demonstrated that genetic polymorphism affected the expression of the NO producing enzyme iNOS and the antioxidative enzyme MnSOD in the posttraumatic inflammatory response following TBI. The inbred strain DA is susceptible to CNS inflammation, while PVG is resistant, which affected redox related enzymes differently after trauma. The expression of iNOS was significantly higher in PVG compared to DA ($p<0.05$). The total amount of w3/13 positive infiltrating inflammatory cells did not differ, hence the iNOS variance was attributed to the amount of iNOS expression in the inflammatory cells. The level of iNOS positive neurons did not differ between strains. The regulation was regarded as genotype dependent. The mechanism remained unclear. It could be coupled to kinetics or the total amount of iNOS. eNOS and nNOS

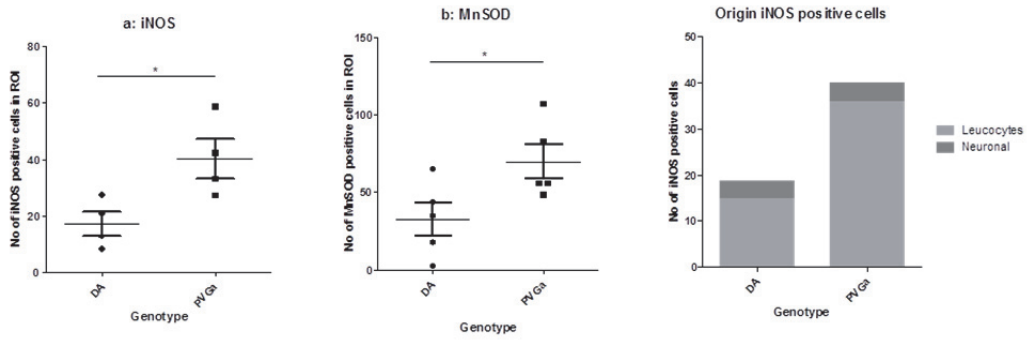


Figure 11 – Immunohistochemical quantification at 24h of (left) iNOS, (middle) MnSOD and (right) the morphological origin if iNOS positive cells. iNOS and MnSOD was increased in PVG rat brains compared to DA. The higher levels of iNOS were found in macrophages/microglia. No difference in neuronal iNOS formation was seen.

increased as a result of the trauma, but did not differ between strains. iNOS positive cells were spatially correlated to the nitrosative stress marker 3-NT and only detected in perilesional areas, agreeing with experimental ischemic brain injury (Ste-Marie et al. 2001). Unexpectedly, no differences were detected in the levels of 3-NT or Fluoro Jade positive neuronal degeneration. It is probable that higher levels of MnSOD in PVG rats ($p<0.05$), expressed in the perilesional area in cells morphologically identified as neurons, prevented an excessive formation of peroxynitrite to occur in spite of higher iNOS levels, thus protecting the PVG rat from oxidative stress. The

findings suggested a balance of the two potentially damaging and protective mechanisms. We could not assess whether underlying genetic differences determined the higher iNOS and MnSOD expression in one strain, or whether a rapid adaptive regulation led to secondary MnSOD synthesis in animals with higher levels of NO.

### 4.1.2 In isolated neuronal cultures (II)

The regulative difference in redox related enzymes in whole brain suggested that genetic susceptibility to CNS inflammation influenced the redox environment in the brain after TBI. However, it was unclear whether this heterogeneity directly affected intrinsic neuronal oxidative defenses. We therefore compared the intrinsic oxidative properties in primary neuronal cultures from the inbred rat strains DA and PVG after exposure to three oxidants with pivotal roles in the post traumatic inflammatory process: nitric oxide, superoxide and peroxynitrite as well as the lipid peroxidation product 4-HNE. Cell cultures allow for specific oxidative compound assessment by direct analysis without interference of supporting tissue or inflammatory cells. The cultures provide possibilities to manipulate the redox status in the cells and to analyze cell survival in absence of intrinsic buffer systems of the brain.

We found a clear genetic influence on neuronal susceptibility to oxidative stress. PVG was connected to a lower antioxidative response, elevated oxidative stress markers 3-Nitrotyrosine (3-NT) and 4-HNE and neuronal death measured by LDH release. The immediate stress response was higher in DA neurons than PVG after 2h for all oxidants and antioxidative enzymes were increased to higher levels after 24h. The induction of antioxidative enzymes must have been a result of de-novo synthesis, since increased levels were absent after 2h.

The MnSOD response was higher in DA than PVG after a peroxynitrite challenge, which differed from the results of a higher neuronal MnSOD response in PVG after TBI in article I. Probably, the inflammatory milieu in the brain shifted the antioxidative response related to the oxidative load, and hence the intrinsic MnSOD regulation of neurons could not be seen in vivo. Increased MnSOD in neurons and activated microglia after TBI resulting from increased superoxide formation lowers oxidative stress (Noack et al. 1998) and subsequently reduces lipid peroxidation, protein nitration and neuronal death (Keller et al. 1998).

It is possible that the lower MnSOD levels in DA neurons in vivo in article I were related to an increased need for reduction due to increased inflammation. Depletion of SOD after TBI may reflect an increased reductive demand (Hicdonmez et al. 2006). PVG neurons displayed a higher baseline of MnSOD, which may present an increased intrinsic protection against superoxide, or a response to higher native levels of superoxide. Superoxide caused twice the amount of MnSOD

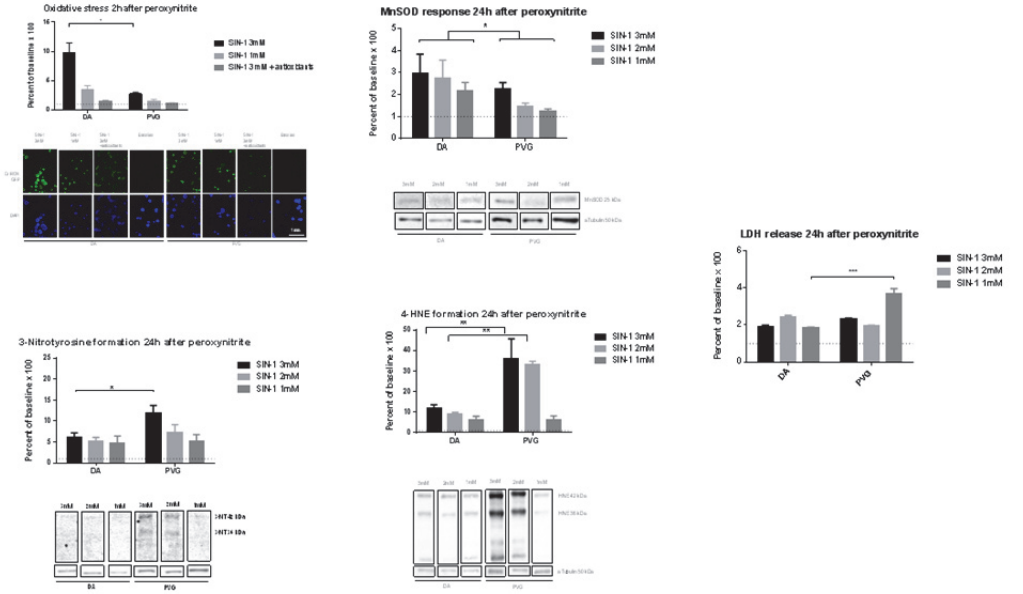


**Figure 12 -** Genetic susceptibility to CNS inflammation was connected to redox active enzymes. Oxidative stress by peroxynitrite (SIN-1) in neuronal primary hippocampal cultures. DA neurons had an initial increased oxidative stress response at 2h compared to PVG and increased antioxidative MnSOD at 24h. The lower antioxidative response in PVG led to higher levels of protein nitration (3-NT), lipid peroxidation (4-HNE) and neuronal death in PVG compared to DA.

induction compared to NO and peroxynitrite, confirming the specificity of superoxide compared to NO or peroxynitrite as the main substrate for MnSOD. In contrast, this specificity was not found in PRDX5, which decreased by half after peroxynitrite only, confirming the specificity of PRDX5 as a peroxynitrite reductase (Dubuisson et al. 2004). In addition, DA had higher levels of PRDX5 after NO and superoxide, although statistical significance was not reached. After TBI, PRDX5 lowers nitro-oxidative stress and cell death (Szabo et al. 2007), while PRDX5 gene silencing makes cells more vulnerable (Knoops et al. 2011). Neither NO, superoxide, peroxynitrite or 4-HNE affected iNOS regulation. The absence of differential strain related regulation further indicated that iNOS induction by these oxidants was not under genetic control in neurons.

The lower antioxidative capacity in PVG led to higher levels of protein nitration (3-NT) and lipid peroxidation (4-HNE) ultimately causing higher levels of cell death in PVG. However, superoxide did not lead to higher protein nitration in PVG, possibly due to the different MnSOD regulation. Protein nitration and lipid peroxidation occurred rapidly, within 2h of peroxynitrite exposure. The powerful oxidant effect of peroxynitrite in neurons was demonstrated by substantial lipid peroxidation, 35 times over baseline, compared to 5-10 times by NO and

superoxide. Nitrosylation was seen at 10-15 times over baseline in all oxidants, suggesting that peroxynitrite caused more lipid peroxidation than nitrosylation in neurons, and that nitrosylation occurred indiscriminate of oxidant.

| | Nitric Oxide | | | | | | Superoxide | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Oxidative stress | MnSOD | PRDX5 | 4-HNE | 3-NT | LDH | Oxidative stress | MnSOD | PRDX5 | 4-HNE | 3-NT | LDH |
| **DA** | up | same | same | down | down | down | up | same | same | down | same | down |
| **PVG** | down | same | same | up | up | up | down | same | same | up | same | up |

| | Peroxynitrite | | | | | | 4-HNE | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Oxidative stress | MnSOD | PRDX5 | 4-HNE | 3-NT | LDH | Oxidative stress | MnSOD | PRDX5 | 4-HNE | 3-NT | LDH |
| **DA** | up | up | same | down | down | down | same | same | same | same | same | same |
| **PVG** | down | down | same | up | up | up | same | same | same | same | same | same |

Figure 13 – Main differences in oxidative properties of DA and PVG neurons. The lower antioxidative response in PVG led to higher levels of protein nitration (3-NT), lipid peroxidation (4-HNE) and neuronal death in PVG compared to DA. Genetic susceptibility to CNS inflammation was connected to redox active enzymes after in vivo experimental TBI. A higher response than the other strain is termed "up" while a lower response is termed "down".

Surprisingly, 4-HNE did not cause acute oxidative stress; MnSOD, PRDX5 and 3-NT were not markedly increased, and cell death was only half of the levels induced by NO, superoxide and peroxynitrite. 4-HNE is an α,β-unsaturated aldehyde generated by peroxidation of ω-6 polyunsaturated fatty acids. At low and physiological levels 4-HNE acts as an endogenous signaling molecule, but high concentrations result in injury to mitochondria and neuronal cell death through both apoptosis and necrosis (Kruman and Mattson 1999). 4-HNE and 3-NT increase in cortical tissue within 30 min after experimental TBI (Deng et al. 2007). DA also showed increased neurodegeneration compared to PVG in vivo by intracerebral injections of 4-HNE, which was connected to higher levels of Gsta4 in PVG after TBI. Gsta4 is known to effectively reduce 4-HNE (Al Nimer et al. 2013b). Probably, the oxidative effect of 4-HNE is multifactorial, and depends on cell type, strain and location, but may also be related to detection methods.

Our findings suggest that 4-HNE had noticeably lower oxidative capabilities than NO, superoxide and peroxynitrite and that the toxic effects of 4-HNE did not include excessive oxidative stress. The absence of strain differences also suggested that the previously described favorable trait of PVG after TBI related to factors other than the intrinsic 4-HNE elimination in neurons.

### 4.1.3 Gender influence on inflammation (III)

In this study the inflammatory response after TBI in male and female rats was investigated with the hypothesis that CNS inflammation is affected by gender related genes. The study was designed to measure the expression of major inflammatory enzymes after TBI. We found that male rats had an increased COX-2 response in mRNA and protein levels at both 24h and 72h ($p<.05$). COX-2 was increased in the perilesional area and the protein expression pattern matched spatially with COX-2 mRNA expression, corroborating earlier findings in diffuse TBI (Cernak et al. 2002). The expression was located in the cortex of the entire hemisphere rather than the perilesional area, similar to the pattern of nestin expression in KCl induced spreading depression (Holmin et al. 2001) (von Baumgarten et al. 2008).

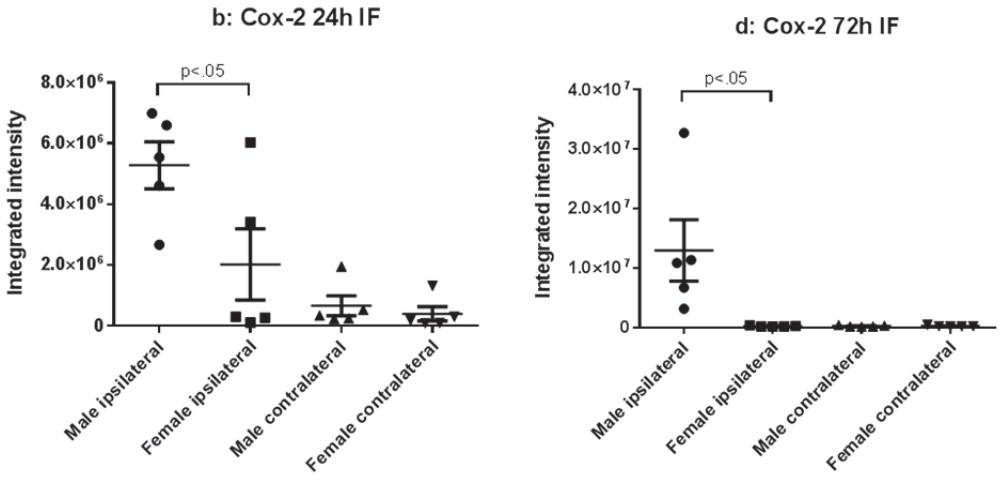


Figure 14 - Quantifications of COX-2 immunofluorescence showing higher expression of the inflammatory enzyme in male rats compared to females at both 24h and 72h, suggesting gender specific regulation.

iNOS mRNA increased in the perilesional area at 24h following TBI, corroborating earlier studies (Gahm et al. 2000). Higher expression was found in female rats ($p<.05$), although not paralleled in protein levels, contrasting a study of ischemic brain injury (Park et al. 2006). No difference was seen in 3-NT why we suggested that the gender specific inflammatory regulation did not primarily involve ROS. COX-2 and iNOS were not spatially co-expressed in the brain. iNOS and COX-2 share an inflammatory regulation (Mémet 2006), co-expression in microglia after TBI, multiple sclerosis and amyotrophic lateral sclerosis (Loane and Byrnes 2010) (Rose et al. 2004) (Minghetti 2004) and NO modulates Cox activity (Marnett et al. 2000). However, our

results did not support broad co-regulation of iNOS and COX-2 as a consequence of the inflammatory response.

TUNEL staining, indicative of apoptosis, was increased in the hippocampus on the ipsilateral side at 24h with higher expression in male rats ($p<.05$). Fluoro Jade stain showed that neuronal degeneration was increased in the perilesional area at 24h and 72h with no difference between genders. The general inflammatory response was assessed by osteopontin and GFAP. They were equally affected in both genders. Osteopontin is an extracellular glycosylated phosphoprotein synthesized by macrophages and activated microglia, which is increased following TBI as an indication of microgliosis (Plantman 2012). GFAP is an intermediate filament protein which is increased in reactive astrocytes following CNS damage (Eng et al. 2000). The inflammatory response hence included an equal macrophage-, microglial- and astrocytic activation, further emphasizing the specificity of the differential COX-2 regulation observed in the study.

COX-2 increases in the CNS following signaling by growth factors, tumor promoters, hormones, bacterial endotoxin and cytokines (Smith and Dewitt 1996). Its role is contradictory with both adverse and protective effects in CNS disease (Minghetti 2004). After TBI, COX-2 inhibition improves cognition and motor function (Cernak et al. 2002), and COX-2 derived prostanoids appear to be toxic in NMDA related neurotoxicity (Manabe et al. 2004) although prostaglandins also induce VEGF expression and angiogenesis after CNS trauma (Sköld et al. 2000). The higher COX-2 expression in male rats provided for a putatively higher production of prostaglandins and a more extensive inflammatory response, which would be expected to cause increased neuronal damage unless balanced by simultaneous up regulation of protective mechanisms. We aimed to describe major inflammatory markers and enzymes in male and female rats given the unknown mechanistic links in female neuroprotection, which may be unrelated to sex hormones. It would be experimentally difficult to control for the rapid four day estrous cycle in rats (Marcondes et al. 2002) (Schank 2001) (Westwood 2008). Even so, the difference in COX-2 regulation was robust at two different times. The estrous cycle was probably not a probable confounding factor. We suggest a detrimental influence of COX-2 mediated inflammation and a possible link to differences in outcome between genders after TBI. A mechanistic relation between progesterone and COX-2 agrees with our findings: progesterone treatment decreased COX-2 expression and the levels of $PGE_2$ and TNFα in male rats (Si et al. 2014).

## 4.2 INFLAMMATORY CELL ACTIVATION BY TRAUMATIC FORCES (IV)

Microglia and macrophages play a vital part in the posttraumatic inflammation and account for a major part of the NO produced. However, it was not known how trauma could induce activation and iNOS synthesis in these inflammatory cells. Activation routes for inflammatory cells, other than cytokine mediated, may cause additional production of oxidative stress by traumatic forces, not seen in pathological contexts without traumatic forces. To study this hypothesis, we used two cell lines with microglial or macrophage lineage, from two different rodent strains, rat (NR8383) and mouse (RAW264.7). The omission of supportive tissue and the circulatory system reduces the number of confounding mechanisms and allows for isolation of traumatic effects at a cellular level (Kumaria and Tolias 2008) (Morrison et al. 1998) (Chopra et al. 1987).

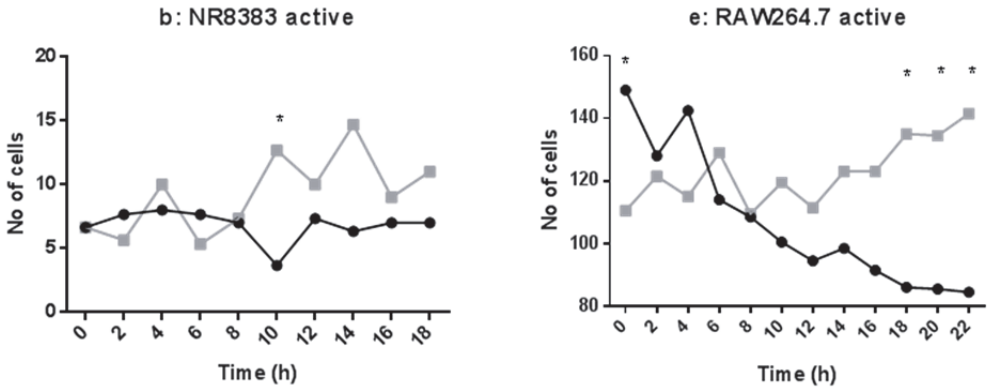


Figure 15 - Cell count as a function of time by Cell-IQ. RAW264.7 macrophages were significantly activated by shock wave trauma. NR8383 were activated by flyer plate, although not passing the significance threshold.

Morphological signs of activation were detected in both macrophage cell-lines after shock wave trauma. In RAW264.7 the difference was statistically significant ($p<0.05$), and in NR8383 the activation was higher than in controls but the difference passed the threshold of statistical significance alpha ($p<0.05$) only at one time point. Shock wave trauma did not cause formation of iNOS mRNA in gene arrays or iNOS protein in ICW. Nitrite was not detected in cell culture medium by nitrite assay in any cell line. Shock wave trauma to NR8383 macrophages caused an increase of 167 genes compared to controls ($p<0.05$, fold change >1.4). Functional enrichment analysis by DAVID resulted in high enrichment for detection of chemical stimuli and membrane bound G-protein signaling with enrichment scores 7.01 and 4.87. Shock wave trauma to RAW264.7 macrophages caused an increase of 494 genes compared to controls ($p<0.05$, fold

change >1.4). Functional enrichment analysis by DAVID showed high enrichment for inflammatory response (enrichment score 3.18), response to wounding and defense response and detection of chemical stimuli and membrane bound G-protein signaling. Hierarchical clustering of the 100 genes with highest differential regulation between Flyer Plate and control allowed identification of a number of gene group regulations specific to Flyer Plate, differing from regulation following Flyer Plate combined with LPS.

Macrophage activation was previously described to follow two different routes, both requiring humoral signaling. The classical activation route (M1) occurs during cell-mediated immune response with IFN-γ and TNF, inhibiting cell proliferation and cause tissue damage (Mosser and Edwards 2008), and the alternative route, mediated by T helper 2 cytokines IL-4 and IL-13. Activated macrophages promote cell proliferation and tissue repair (Gordon and Martinez 2010). The alternatively activated macrophages may produce little or no proinflammatory cytokines and may also express arginase, which can inhibit NO production (Lumeng et al. 2007).

We detected morphological and biochemical signs of activation in the absence of humoral stimuli, just by transfer of mechanical energy. The absence of NO induction suggests that traumatic activation may be associated with the alternative route of induction. It is also probable that macrophages in vivo are further activated by cytokines included in the inflammatory response (Feuerstein et al. 1998). These include IFN-α, IFN-β, IFN-ɣ, TNF, IL-1, IL-2, IL-4, IL-10, IL-13, IL-17 and TGF-β (Miljkovic and Trajkovic 2004) (MacMicking et al. 1997) (Bogdan 2001) and may work in concert with traumatic forces to trigger a more extensive activation of iNOS than would be expected from cytokine activation only. It is possible that the addition of energy, which is unique to traumatic neurodegeneration, provides an initial step for the subsequent initiation of inflammation.

We concluded that shock wave trauma caused an inflammatory response and morphological signs of activation of the macrophage cell lines. We proposed a novel activation mechanism of macrophages by shock wave trauma, independent of cytokine activation. However, iNOS and NO were produced only in response to humoral signaling.

## 4.3 NEUROPROTECTION BY AN ANTIOXIDATIVE SUBSTANCE (V)

Redox intervention may provide new pharmacological targets (Marklund et al. 2006). We examined the effects of NACA in the secondary inflammatory response following focal penetrating TBI in rats. NACA is a modified form of NAC, which exhibits higher membrane- and blood brain barrier permeability than NAC (Offen et al. 2004) (Grinberg et al. 2005). NAC has limited but well documented neuroprotective effects after experimental CNS ischemia and TBI (Pandya et al. 2014), but the bioavailability is very low (Gilgun-Sherki et al. 2002). NAC reduces extracellular cystine to cysteine, supplies sulphydryl (-SH) groups that stimulate glutathione biosynthesis and enhances glutathione-S-transferase (GST) activity (Issels et al. 1988) (Nakata et al. 1996) (De Vries and De Flora 1993). Glutathione is an essential antioxidant, and depletion occurs in neurodegenerative diseases and CNS injuries (Drake et al. 2002) (Kamencic et al. 2001). NAC is also a potent free radical scavenger and antioxidant as a result of its nucleophilic reactions with ROS (Aruoma et al. 1989).

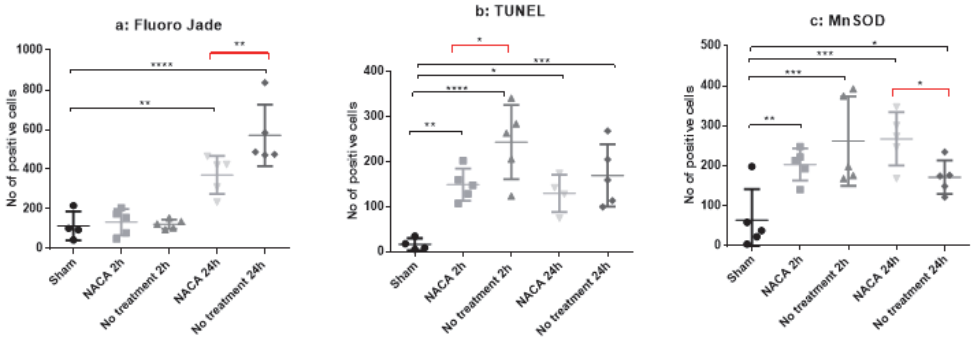


Figure 16 – NACA treatment decreased Fluoro Jade neuronal degeneration at 24h ($p<0.01$), TUNEL staining indicative of apoptosis at 2h ($p<0.05$), and increased MnSOD at 24h ($p<0.05$).

Combining the antioxidative properties of NAC with high CNS penetrance would create a promising substance for pharmacological intervention. We found that NACA treatment decreased neuronal degeneration, visualized by Fluoro Jade, at 24h with a mean decrease of 35.0% ($p<0.05$) and decreased TUNEL staining, indicative of apoptosis, at 2h. The mean change was 38.7% ($p<0.05$). The findings agreed with earlier studies of reduced apoptosis by NAC in mild TBI (Chen et al. 2008) and NACA-reduced apoptosis in renal epithelial cells induced with iohexol (Gong et al. 2010). It is probable that NACA treatment lowers both necrosis and apoptosis in the acute phase after TBI. MnSOD increased in the NACA treatment group at 24h with a mean increase of 35.9% ($p<0.05$), similar to increased MnSOD after NAC administration in experimental closed head trauma (Hicdonmez et al. 2006), which suggests that NAC and NACA

affects MnSOD expression similarly. The increased levels of MnSOD may reflect higher de-novo synthesis, or a decreased elimination due to lower oxidation levels. Levels of migrating macrophages and activated microglia (Ox-42) were not affected, similarly to a previous study (Chavko et al. 2009), hence the inflammation-regulative properties of NACA probably are not related to macrophage migration and microglia activation.

In agreement with earlier findings, iNOS increased at 24h following trauma (Gahm et al. 2000) (Miljkovic and Trajkovic 2004). NACA treatment did not affect iNOS, contrasting to a cell study (Gong et al. 2010). It is likely that the antioxidative properties of NACA did not primarily involve NO in the brain, but instead the antioxidative systems, as suggested in an early study of the compound (Bartov et al. 2006). NACA did not affect the levels of 3-NT at either time, corroborating results in moderate TBI (Pandya et al. 2014). This finding contrasted to a cell culture study (Bartov et al. 2006), probably a difference due to the absence of inflammatory cells, and glial cells. It is possible that higher MnSOD levels in the NACA treatment group were insufficient to eliminate the superoxide radical, or that increased superoxide resulted in effects other than protein nitration. NACA treatment did not alter NFkB, which translocates from the cytosol to the nucleus after TBI (Hang et al. 2006) (Chen et al. 2008). It is likely that NACA acts mainly antioxidatively in processes known to occur 2-24h after TBI (Bains and Hall 2011). NACA treatment did not affect caspase 3 activation, fundamental for apoptosis (Cheng et al. 2012), total levels of Cytochrome c, released from the mitochondria to the cytosol to activate Caspase 3 (Cheng et al. 2012) (Sullivan et al. 2002), or Bcl-2, preventing Cytochrome c efflux from the mitochondria in caspase mediated apoptosis (Cheng et al. 2012). Therefore, the effect of NACA on TUNEL labeled apoptosis is likely regulated by pathways other than Cytochrome c. We conclude that NACA prevented brain tissue damage after focal penetrating TBI, which we suggest was connected to the regulation of antioxidative enzymes rather than inflammatory cell migration.

# 5 GENERAL DISCUSSION

This thesis shows that genetic factors affect the secondary inflammation and redox systems after TBI. Genetically derived host factors in two male rat strains with different susceptibility to CNS inflammation and gender associated differences between male and female rats caused specific responses and affected outcomes. The findings increase knowledge about inflammation and oxidation mechanisms in experimental TBI and demonstrate the importance of host factors for the injury. It was shown that macrophages/microglia can be activated as a result of traumatic forces, an essential and unique component of TBI. Finally, a drug with antioxidative properties was shown to provide neuroprotection after TBI. Interestingly, iNOS, which is an important mediator of secondary reactions, was not part of traumatic induction in macrophages or differentially regulated in neurons.

Two different models of experimental TBI were used in the experimental settings. Weight drop injury produces a mild focal injury (Feeney et al. 1981). The penetrating injury model produces a moderate focal penetrating injury. Both models resemble brain contusions and produce a perilesional neuroinflammatory response, important for relevant hypothesis testing in CNS trauma research. TBI is inherently heterogeneous. Subsequently, several pre-clinical models are needed in order to mimic pathological mechanisms (Diaz-Arrastia et al. 2014). One principal finding of the thesis, that genetic polymorphism affects CNS inflammation and oxidative stress, was corroborated in two different TBI models and in a cell culture model. We demonstrated that the iNOS and MnSOD regulation differed. iNOS is induced during inflammation and accounts for a majority of NO production. It adds oxidative stress in the brain. MnSOD is an antioxidative enzyme well known to provide neuroprotection after TBI. By comparing homogenous rat strains with fundamental differences in CNS inflammation, we detected differences that may be important for the future direction of TBI research, but too subtle to be detected in a general heterogeneous human population.

The differences found in DA and PVG whole brains were corroborated in isolated neuronal cell cultures, a study designed to describe the intrinsic antioxidative mechanisms in neurons with special regard to MnSOD and iNOS. The inability of the post-mitotic neuron to divide to replace or dilute damaged components together with low antioxidant levels (Almeida et al. 2002), make neurons vulnerable to oxidative stress, emphasizing the need for an effective antioxidative protection. Neuroprotection is dependent on an array of supporting cells and inflammatory migratory cells, but the intrinsic neuronal protection mechanisms may ultimately decide whether the neurons live or die.

The higher inflammatory activation in DA brains would have provided a highly oxidative environment after TBI, and we initially believed that DA rats would have elevated susceptibility to oxidative stress and neuronal damage. Interestingly, when taking the findings from the whole brain to isolated neurons, we discovered that the PVG neurons with a low CNS inflammatory response instead had a decreased defense against oxidative stress. Possibly, neurons primed by an environment with high inflammatory activity had increased the compensatory antioxidative systems. Neurons may adapt the antioxidative mechanisms correspondingly in order to maintain survival. The mechanistic links remain to be elucidated, but it may be speculated that hormesis; oxidative stress adaptation by which cells respond to, and cope with environmental and physiological shifts in level of oxidative stress, may also affect genetic traits. Repeated stress by hydrogen peroxide led to significant extension of adaptive processes in cell cultures (Pickering et al. 2013).

Coping with oxidative stress may be secondary to the general inflammatory response, and not a main feature of neuroprotection. The oxidative stress in inflammation may also be situationally and spatially dependent. The oxidative environment is a consequence of inflammatory cells, why it may be suggested that targeting the cellular inflammatory response is a priority in oxidative stress related adverse effects. Also, oxidative stress may be beneficial in autoimmune diseases affecting organs other than the immune-privileged CNS. DA, with increased susceptibility to autoimmune associated disease arthritis, has lower oxidative burst than arthritis-resistant strains (Olofsson et al. 2002).

Our findings that genetic polymorphism affected the inflammatory response led to analyses of the hypothesis that gender could also affect posttraumatic CNS inflammation. Gender correlated with differential risks and vulnerability to inflammatory disease (Mirandola et al. 2015). Although results are conflicting regarding outcome after TBI in human cohorts, experimental studies point towards a protection by the female gender after TBI. We demonstrated that the inflammatory enzyme COX-2 was elevated in males after TBI. COX-2 is pivotal in inflammation, and the target enzyme for the widely used non-steroidal anti-inflammatory drugs (NSAID). A possible mechanism connected to different outcomes of TBI may provide suggestions for future treatment targets, although mechanisms need to be further elucidated. The female sex hormone progesterone did not show beneficial results in outcomes in patients with acute TBI in the recent ProTECT III study (Wright et al. 2014). Gender related protective mechanisms are probably more complex, and continued fundamental research is vital for future findings of exploitable mechanistic links.

Physical trauma to the nervous system is usually the result of many forces with different directions and kinetics. The fact that traumatic injuries entail energy transfer to the brain provides a fundamental difference from ischemic pathology. The latter is a consequence of deprivation of energy. Fundamental relations between traumatic forces and inflammation such as whether

traumatic energy transfer to macrophages can produce an inflammatory response are insufficiently studied. We used an experimental model that delivers shock waves similar to those registered inside of rat brains after blast injury (Chavko et al. 2011), and demonstrated that shock wave trauma caused an onset of intracellular sensory- and inflammatory reactions in two macrophage cell lines. The fact that energy transfer by itself can activate macrophages is highly intriguing, since much knowledge of neurodegenerative mechanisms in trauma reflects extrapolation of findings from models of ischemia, where the causal factor is energy deprivation. Whether shock wave induced macrophages augment the post-traumatic inflammation or act protectively remains to be elucidated. Surprisingly, the activated macrophages did not produce iNOS or NO. It is possible that the macrophages did not contribute to NO-derived oxidative stress or that NO production requires a crosstalk with additional cellular elements.

Finally, we demonstrated neuroprotection by NACA following penetrating focal brain injury by decreased neuronal degeneration, apoptosis and increased MnSOD. Depletion of SOD in the brain has been suggested to reflect an increased reductive demand after TBI (Hicdonmez et al. 2006). Our findings in neuronal cell cultures were that elevated MnSOD levels correlated with reduced oxidative markers and cell death. The findings agreed with this mechanistic model of explanation, and, together with other findings (Pandya et al. 2014) (Patel et al. 2014), suggest that NACA is a potential pharmacological candidate in the treatment of TBI.

Taken together, our data support the need to study oxidative mechanisms in TBI and show that genetic polymorphisms determine the secondary inflammation and oxidative stress following TBI. The findings are important to (a) corroborate findings of neuroprotection in animal models in humans, (b) achieve significant neuroprotection in human clinical trials and (c) suggest an explanatory model for differences in outcome in a human population, essential for updated outcome predictions.

# 6 CONCLUSIONS

This thesis verified the complexity of the secondary inflammatory process after TBI and confirmed the need for wider explanatory models for outcome predictions and identification of pharmaceutical targets. The importance of host factors for outcome was demonstrated and a unique effect of energy transfer by trauma was shown to activate inflammatory traits. We did not elucidate the regulation of iNOS, but made several other novel observations.

Genetic susceptibility to CNS inflammation was connected to redox active enzymes after in vivo experimental TBI. NO producing enzyme iNOS and antioxidative enzyme MnSOD were increased at 24h in the PVG rat strain, protected from CNS inflammation compared to DA, susceptible to CNS inflammation, indicating genetic regulation. The difference in iNOS regulation was seen in inflammatory cells while neuronal iNOS expression did not differ. The increased levels of iNOS did not lead to increased neuronal degeneration at 24h.

The genetic regulation of oxidative stress vulnerability was corroborated in primary neuronal cultures from DA and PVG rats. This was the first demonstration that inbred strains could have different oxidative responses. While PVG neurons had decreased oxidative stress levels at 2h, at 24h oxidative stress markers for nitrosylation and lipid peroxidation and neuronal death were elevated compared to DA neurons. Neurons primed in an environment of high susceptibility to inflammatory activity seem to have increased compensatory antioxidative systems.

iNOS was not increased by any of the oxidants, suggesting that humoral stimulation was necessary for neuronal iNOS induction and that iNOS induction by these oxidants were not under genetic control in isolated neurons.

COX-2 regulation differed between genders after experimental TBI. Male rats expressed higher levels of COX-2 mRNA and protein than female rats at 24h and 72h, correlating with increased apoptotic cell death at 24h, but not with neuronal degeneration. Females had higher iNOS mRNA expression although not in protein levels. No difference was seen between genders in astrogliosis, microgliosis or nitrosylation.

Macrophage cell lines from mouse and rat were activated by shock wave trauma, causing an inflammatory response which did not include iNOS or NO. iNOS induction appeared to require humoral signaling by LPS/IFN-γ. Direct energy transfer by trauma activated the macrophages directly without humoral mediators, comprising a novel activation mechanism in macrophages.

The final article corroborated the relevance of antioxidant therapy in experimental neurotrauma. Posttraumatic treatment with antioxidative compound NACA reduced neuronal degeneration and

increased levels of MnSOD at 24h. Apoptosis was reduced at 2h, which regulation did not include cytochrome C or Bcl-2. NACA did not affect levels of migrating macrophages/activated microglia, iNOS, nitrosylation or NFkB.

We have found that direct energy transfer, which does not occur in non-traumatic neurodegeneration, can cause activation on the cellular levels and showed several examples and analyses of how host factors influence inflammation and oxidation. The findings show a higher complexity in post-traumatic responses than usually acknowledged and considered.

Our findings can improve preclinical and clinical pharmacological trials, and provide new knowledge to reach the main objective of finding effective pharmacological interventions in the treatment of TBI.

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