Dissertation

DIE ROLLE DES STICKSTOFFMONOXID BEI CEREBRALEN SCHÄDEN VERURSACHT DURCH VERLETZUNGEN

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Dissertation

THE ROLE OF NITRIC OXIDE IN INJURY-INDUCED CEREBRAL DAMAGE

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STATUTORY DECLARATION

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this dissertation. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz".

Some of the results presented in this dissertation were already published as an original article in the Journal of Neurotrauma: *Ücal, M., Kraitsy, K., Weidinger, A., Paier-Pourani, J., Patz, S., Fink, B., Molcanyi, M., Schäfer, U. 2016.* **Comprehensive Profiling of Modulation of Nitric Oxide Levels and Mitochondrial Activity in the Injured Brain: An Experimental Study Based on the Fluid Percussion Injury Model in Rats**. *J Neurotrauma.*

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To my family

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ABBREVIATIONS and DEFINITIONS

3NT	3'-nitrotyrosine
ACh	Acetylcholine
ADM	Adrenomedullin
ADP	adenosine diphosphate
Akt	Akt serine threonine kinase
ANOVA	analysis of variance
ATP	adenosine triphosphate
Bax	BCL-2 associated protein
BCL2	B-Cell lymphoma leukemia 2, apoptosis regulator
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
BH3O3	boric acid
BH4	tetrahydrobiopterin
BSA	bovine serum albumin
Ca^{2+}	Ca2+ cation
CaMK	Ca2+/Calmodulin dependent protein kinase
cAMP	cyclic adenosine monophosphate
CBF	cerebral blood flow
CCI	controlled cortical impact, a brain trauma model
CcO	cyctochrome c oxidase, mitochondrial complex IV
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
CNS	central nervous system
CO2	carbon dioxide
COX1	cycloxygenase 1
COX2	cycloxygenase 2
CREB	cAMP repsonse element binding protein
Cu	Cupper
CVLM	caudal ventrolateral medulla
Cyt c	cytochrome c
DAF	diaminofluorescein
DAPI	4',6-diamidino-2-phenylindole
DETA-NONOate	(Z)-1-[N-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate
DETC	diethyl dithiocarbamate
DG	dentate gyrus
dlPAG	dorsolateral periaquidactal grey
DMNV	dorsal motor nucleus of vagus
DNA	deoxyribonucleic acid
Drp1	dynamin related protein 1
EDRF	endothelial derived relaxing factor
EDTA	Ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
eNOS	endothelial nitric oxide synthase

EPR	electron paramagnetic resonance
EPSP	excitatory post synaptic potential
Erk1/2	mitogen activated protein kinase
ETS	electron transport system
FAD	flavin adenin dinucleotide
FBS	fetal bovine serum
Fe (II)	iron (II)
$Fe(II)SO_4$ x $7H_2O$	iron (II) sulfate heptahydrate
FMN	flavin mononucleotide
FPI	fluid percussion injury
G6PDH	glucose 6-phosphate dehydrogenase
GABA	gamma aminobutyric acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDH	glutamate dehydrogenase
GLUT1	glucose transporter 1
GOT2	Glutamate oxaloacetate transaminase 2, mitochondrial isoform
GTP	guanosine triphosphate
H^+	proton
HCl	hydrochlorous acid
HD	Huntington's Disease
HDAC2	histone deacetylase 2
HIF1α	hypoxia inducible factor 1 alpha
HR	heart rate
ICP	intracranial pressure
IFNγ	interferon gamma
IgG	immunoglobulin G
IL-1, -1α, -1β	interleukin 1, -1 alpha, -1 beta
IL-2	interleukin 2
IL-6	interleukin 6
iNOS	inducible nitric oxide synthase
IPSP	inhibitory postsynaptic potential
JAK	Janus kinase
K^+	potassium cation
kDa	kilodalton
L-Arg	L-Arginine
LC	Locus Coeruleus
LFP	lateral fluid percussion
L-NAME	Nω-Nitro-L-arginine methyl ester
LPBN	lateral parabrachial nucleus
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase, same as Erk
MCAO	middle cerebral artery occlusion
Medullary LTF	medullary lateral tegmental field
MLKL	mixed lineage kinase domain-like protein
MNIC	mononitrosyl iron complex
MPT	mitochondrial permeability transition

mPTP	mitochondrial permeability transition pore
mRNA	messenger ribonucleic acid
NA	noradrenaline
Na^+	sodium cation
$Na_2B_4O_7$ x $10H_2O$	Borax
Na_2SO_4	sodium sulfate
NAD^+	nicotinamide adenin dinucleotide, oxidized
NADPH	nicotinamide adenin dinucleotide phosphate
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	nerve growth factor
NLPR3	NLR family, pyrin domain containing 3 (**NLR**: nucleotide-binding oligomerization domain-like receptors)
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NNFC	normoxic–normoglycemic flow cessation
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
-NO2	nitro group
NPC	neural precursor cell
NSC	neural stem cell
NT3	neurotrophin 3
NT4	neurotrophin 4
NT4/5	neurotrophin 4/5
NTS	nucleus tractus solitarii
O_2	molecular oxygen
O_2^-	superoxide anion
OB	olfactory bulb
OGDHC	oxoglutarate dehydrogenase complex
$ONOO^-$	peroxynitrate
p75NTR	nerve growth factor receptor
PAG	periaquiductal grey
PARP	Poly-(ADP-ribose) polymerase
PBBI	penetrating ballistic-like brain injury
PBS	phosphate buffered saline
PGAM5	phosphoglycerate mutase family member 5
PGD_2	prostaglandin D2
PGE_2	prostaglandin E2
PGF_2	prostaglandin F2
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKG	protein kinase G
PSD-95	post-synaptic density protein 95
PTEN	phosphatase and tensin homolog
PVN	paraventricular nucleus
RIPK1	receptor interacting serine/threonine kinase 1
RIPK3	receptor interacting serine/threonine kinase 3

RMS	rostral migratory system
RNA	ribonucleic acid
RNS	reactive nitrogen species
RONS	reactive oxygen and nitrogen species
ROS	reactive oxygen species
RSNA	renal sympathetic nerve activity
-RSNO	S-nitrosothiol
RVLM	rostral ventrolateral medulla
RVMM	rostral ventromedial medulla
SDH	succinate dehydrogenase
sGC	soluble guanylyl cyclase
SGZ	subgranular zone
SNP	sodium nitroprusside
SOD	superoxide dismutase
SOD1	superoxide dismutase, soluble; Cupper Zinc superoxide dismutase, soluble
SOD1G93A	SOD1 mutated at codon 93 coding for alanine instead of glycine
SPNs	sympathetic premotor neurons
STAT	signal transducer and activator of transcription
SUV39H1	suppressor of variegation 3-9 homolog 1
SVZ	subventricular zone
TBI	traumatic brain injury
TCA	tricarboxylic acid
Th1	Type 1 T helper cells
TRIS	Tris(hydroxymethyl)aminomethane
TrkA	neurotrophic tyrosine kinase, receptor, type 1
TrkB	neurotrophic tyrosine kinase, receptor, type 2
TrkC	neurotrophic tyrosine kinase, receptor, type 3
Tukey's HSD	Tukey's Honest Significant Difference Test
VEGF-A	Vascular endothelial growth factor A

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ZUSAMMENFASSUNG

Stickstoffmonoxid (NO) ist ein ungewöhnlicher interzellulärer Botenstoff und freies Radikal. Es ist bereits bekannt, dass NO eine Vielzahl von unterschiedlichen zellulären Prozesse reguliert wie z.B. die Lebensfähigkeit der Zelle, Proliferation, mitochondrialen Energiehaushalt und Zelltod. Des Weiteren, wirkt NO auf physiologische Prozesse ein, wie z.B. auf die kardiovaskulären Antworten, sympathische und parasympathische Nervaktivitäten, Schlaf und Hungergefühl, die alle durch das Zentrale Nervensystem (ZNS) reguliert werden. Im geschädigten Gehirn wurde NO stets mit Folgeschäden, sogenannte sekundäre Schäden, in Verbindung gebracht. Die durchschnittliche NO-Konzentration in verschiedenen Gehirnregionen vor und nach einem Schädelhirntraumata (SHT) und die Rolle von NO in der mitochondrialen Dysfunktion nach einem SHT sind jedoch noch nicht geklärt. Auch ist die Veränderung des NO Metabolismus aufgrund von SHT in entfernteren Organen wie Herz und Leber ist noch nicht erforscht.

In dieser Studie wurden NO Veränderungen im Gehirn, Leber und Herz nach einem Schädelhirntraumata gemessen. Des Weiteren wurde untersucht, ob es einen möglichen Zusammenhang zwischen schädlichen und nützlichen Effekten von NO nach SHT gibt.

Hier zeigen wir zum ersten Mal, dass sich die Menge vom basalen NO zwischen gesunden Kortex (0.44 ±0.04 μM), Hippocampus (0.26 ±0.03 μM) und Cerebellum (1.24 ±0.08 μM) signifikant unterscheiden. Innerhalb der ersten 4 Stunden eines schweren Lateral Fluid Percussion Verletzung kommt es in diesen Regionen nahezu zu einer Verdopplung der NO Konzentration, wobei die regionalen NO Konzentrationsunterschiede erhalten bleiben. SHT induzierte NO Produktion wurde mit erhöhter relativer Expression der induzierbaren NO Synthase (iNOS) in ipsilateralen aber nicht in contralateralen Regionen in Verbindung gebracht. Aufgrund des kurzzeitigen Anstiegs der NO Konzentration kommt es zu einer andauernden Nitrierung von Tyrosinresten rund um die Verletzungsstelle. Der nitrosative Stress-assoziierte Zellverlust durch Apoptose und Rezeptor-interagierender Serin/Threonin-Proteinkinase 3 (RIPK3)-vermittelte Nekrose wurden auch nur im ipsilateralen Kortex beobachtet, trotz gleich hoher NO Levels im contralateralen Kortex. Die NO-vermittelte Verschlechterung des mitochondrialen Zustands 3 der Atmungskontrolle war vorübergehend und auf den ipsilateralen Kortex begrenzt. Außerdem, wurde eine solche Verschlechterung nur mit mitochondrialen

Komplex I abhängigen Substraten beobachtet. Dabei war die Erniedrigung des gemessenen Zustands 3 über das Substrat Glutamat stärker als bei der Messung mit dem Substrat Pyruvat. Dies lässt vermuten, dass speziell die Katalyse von Glutamat im Tricarbonsäurezyklus durch SHT-induziertes NO im verletzten Gehirn verschlechtert wird.

Unsere Resultate zeigen, dass die NO Dynamik und dessen assoziierte Effekte (schädlich oder nützlich) sich in unterschiedlichen Regionen des verletzten Gehirns unterscheiden. Die potentielle Assoziation zwischen beobachtetem mitochondrialen Elektronenfluss durch Komplex I, aber nicht Komplex II und die Modulation von SHT-induzierten NO Produktion in verschiedenen Gehirnregionen muss in Zukunft noch weiter analysiert werden. Es konnte ein vorübergehender, jedoch signifikanter Anstieg der NO Produktion in der Leber und im Herz, gleichzeitig mit den Veränderungen in den Gehirnregionen, detektiert werden. Die klinische Relevanz von SHT-induzierten NO Veränderungen in entfernten Organen muss in zukünftigen Experimenten weiter analysiert werden.

ABSTRACT

Nitric oxide (NO) is a non-canonical intercellular messenger and a free radical. It is known to regulate a wide variety of cellular processes that include cell viability, proliferation, mitochondrial energy metabolism, cell death, and shown to mediate a broad spectrum of physiological processes regulated by the CNS, like regulation of cardiovascular responses, sympathetic and parasympathetic nerve activity, sleep and appetite. In traumatized brain, NO has frequently been associated with secondary damage after brain injury. However, average NO levels in different brain regions before and after traumatic brain injury (TBI) and its role in post-TBI mitochondrial dysfunction remain unclear. Furthermore, TBI-induced changes in NO metabolism in distant organs, such as heart and liver, have not been studied. In this study, NO changes in brain, liver and heart after TBI and possible association to its detrimental and beneficial effects were investigated. Here, we demonstrate for the first time that basal NO levels vary significantly in the healthy cortex (0.44 ±0.04 μM), hippocampus (0.26 ±0.03 μM), and cerebellum (1.24 ±0.08 μM). Within 4 h of severe lateral fluid percussion injury, NO levels almost doubled in these regions, thereby preserving regional differences in NO levels. TBI-induced NO generation was associated with inducible NO synthase (iNOS) increase in ipsilateral but not in contralateral regions. The transient NO increase resulted in a persistent tyrosine nitration adjacent to the injury site. Nitrosative stress-associated cell loss via apoptosis and receptor-interacting serine/threonine kinase 3 (RIPK3)-mediated necrosis were also observed in the ipsilateral cortex, despite high levels of NO in the contralateral cortex. NO-mediated impairment of mitochondrial state 3 respiration dependent on complex I substrates was transient and confined to the ipsilateral cortex. Decrease in glutamate-dependent state 3 respiration was more prominent as compared to pyruvate-dependent state 3 respiration, suggestive of particular vulnerability of glutamate node in the mitochondrial respiration to TBI-induced NO changes in injured brain. Our results demonstrate that NO dynamics and associated effects (detrimental or beneficial) differ in various regions of the injured brain. A potential association between the observed mitochondrial electron flow through complex I, but not complex II, and the modulation of TBI induced NO levels in different brain regions has to be prospectively analyzed in more detail. In liver and heart, a transient and but significant increase in NO levels was detected isochronous to the changes observed in brain regions. Clinical relevance of TBI-induced NO changes in distant organs has to be analysed in further research.

1. INTRODUCTION

1.1. Traumatic brain injury

Trauma of the central nervous system is well accepted as one of the leading causes of death or long-term disabilities all around the world. In Europe more than 1.7 million head injured patients are estimated to be admitted to hospitals every year, around 70 thousand of which eventuate by death [Estimated over population of Europe by year 2007, 731 million (United_Nations, 2007)] (Tagliaferri et al., 2006). Traumatic brain injury (TBI) creates a heavy burden on both the individuals and the public health system since it is a major cause for young to middle age casualties, long-term disabilities, loss of productive years as well as patient care costs (Berg et al., 2005). The most recent estimates indicated that more than 3 million civilian residents only in the United States are living with disability following hospitalization with TBI (Corrigan et al., 2010).

Although the prevalence change by age groups or societies, the major causes of TBI could be given as vehicle accidents, falls, violence, injuries of sports and military (CDC, 2014).

1.1.1. General Pathophysiology of TBI

TBI occurs when the brain is subjected to damage by an external force. It can be classified with respect to situation of the skull bone (closed, penetrating), location of injury (focal, diffuse) or strength (mild, moderate, and severe).

The physical damage at the moment of impact on head is defined as primary damage. A break in the skull, ruptures and laceration in the brain tissue, a concussion of the head or impact of blast waves could be counted among them. Needless to mention, primary damage could only be subjected to preventive, but not therapeutic measures. Therefore, therapeutic interventions mainly target pathophysiological processes relating to secondary damage.

1.1.2. Secondary Damage after TBI

After the primary mechanical damage, a cascade of events starts in minutes to hours and days (**Figure 1**). Major pathophysiological events occurring after TBI could be listed as follows: dysregulated cerebral blood flow (Golding, 2002), impaired cerebral oxygenation and hence ischemic insult (Maloney-Wilensky et al., 2009) neurotransmitter

(glutamate, in particular) excitotoxicity (Palmer et al., 1993, Yi and Hazell, 2006), blood brain barrier (BBB) breakdown (Shlosberg et al., 2010), cerebral oedema (Unterberg et al., 2004, Donkin and Vink, 2010), oxidative and nitrosative stress (Lewen et al., 2000, Abdul-Muneer et al., 2013) and by no means less important, cerebral inflammation (Schmidt et al., 2005, Morganti-Kossmann et al., 2007). One could extend the list to include several other physiological or molecular pathologies and sub-categories related to these major mechanisms, like hypo- and hyper perfusion (Thomale et al., 2002, Dore-Duffy et al., 2011), mitochondrial dysfunction (Cheng et al., 2012), haemorrhage (Kurland et al., 2012) or hyperaemia (Kelly et al., 1996, Chieregato et al., 2009). All of these events are somehow interrelated and influence each other regardless of the fact that some of them start earlier than others. The presence or prominence of such factors could vary with the type of injury (closed or penetrating, focal or diffuse, mild or severe). Even so, one feature common to all is that they exacerbate secondary damage to brain tissue and contribute to delayed loss of neuronal cells, directly or indirectly. As a matter of fact, this sooth formed the basis of virtually all clinical and experimental research in the field of TBI: to stop the progression of tissue damage and recover or repair what has been lost until then.

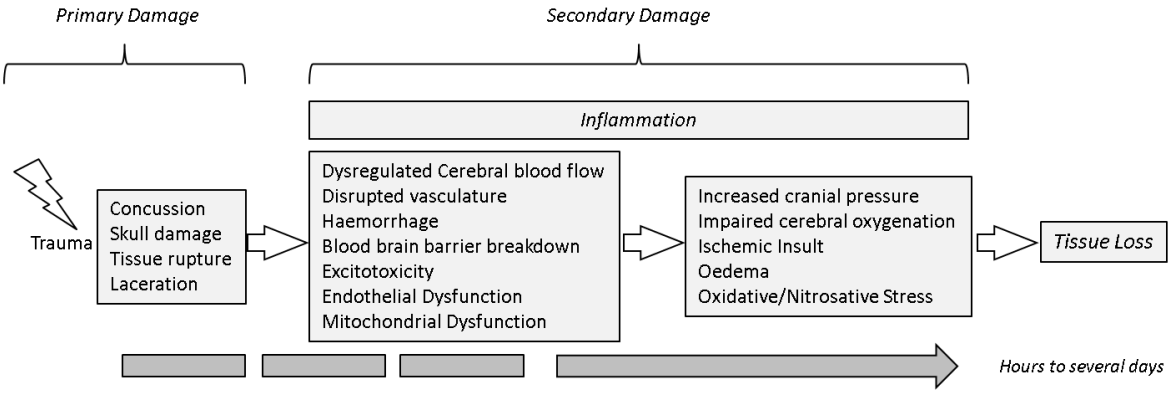

Figure 1: Major pathophysiological events after traumatic brain injury.
Traumatic brain injury leads to tissue loss through a series of multiple events from primary physical damage to secondary damage. Many of these pathophysiological processes are interconnected.

Several years of research has extensively improved our knowledge on the pathophysiology of TBI. Thanks to the clinical and experimental data accumulated over years, a considerable improvement has been achieved on the clinical management of TBI, which undoubtedly came with a remarkable and gradual reduction in the mortality due to

head injury (Gennarelli et al., 1989, Lu et al., 2005). Nevertheless, our understanding is far from being complete, a plethora of questions waiting to be answered and we are still far from a substantial therapy for TBI. Neurological deficits, cognitive and motor disabilities, psychiatric disorders or other morbidities following a brain trauma still remain as major sequelae of a TBI (McAllister, 2008). While limited capability of repair of brain tissue could account as one reason for this, progressive loss of neural cells due to uncurbed secondary injury account as another.

Research focused on individual physiological or molecular pathological mechanisms following trauma has helped a lot for our understanding. Indeed, attempts to inhibit or prevent individual pathological processes have shown improved outcome in experimental models. On the other hand, there have been also holistic approaches which suggest combination of multiple pharmacological interventions targeting diverse pathologies after TBI (Margulies et al., 2009, Margulies et al., 2015). Studies to date has revealed that complicated cascade of post traumatic events are not independent of each other, yet are equally far from being orchestrated or triggered by a single molecular mechanism. Rather, they are interrelated and mechanisms connecting them could be targeted as candidates to control or attenuate multiple secondary damage effectors. This study itself is a product of such approach as it focuses on nitric oxide (NO) on the grounds of its connective nature which might potentially link many of the major (patho)physiological events from cerebral blood flow (CBF) to mitochondrial function, regulation of inflammation, oxidative-nitrosative stress and neuroprotection, regeneration and repair. There is a huge body of literature, pointing towards both detrimental and beneficial effects of NO. These contradictory effects of NO is thought to be dependent on several factors such as concentration, source, localization and distribution of NO, all of which remained unclear to date in a traumatized brain. Thereof, this work is dedicated to contribute understanding of the role of NO in the early phase of a traumatic brain injury in various sub regions of the brain.

1.2. Nitric Oxide

Nitric oxide (NO) was discovered by Joseph Priestly in 18th century and since then biological effects of this diatomic gaseous molecule has been a point of interest. However, the major step in biological actions of NO was taken after 1980s when “endothelial-derived relaxing factor” (EDRF), a substance responsible for the dilatation of blood vessels

by inducing relaxation of smooth muscle cells in response to activation of *N*-methyl *D*-aspartate (NMDA) receptors, was turned out to be NO (**Figure 2**). First Furchgott and Zawadzki identified the critical role of endothelial cells in acetylcholine (ACh)-induced relaxation of capillaries and proposed this capillary relaxation to be mediated by a previously unrecognized substance, which they have called endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980). Later in 1986, laboratories of Paul Vanhoutte and Salvador Moncada independently showed that EDRF was scavenged by superoxide anions (Rubanyi and Vanhoutte, 1986, Gryglewski et al., 1986). In the same year, Louis Ignarro and colleagues showed that EDRF was able to activate soluble guanylyl cyclase (sGC) in vascular smooth muscle cells (Ignarro et al., 1986).

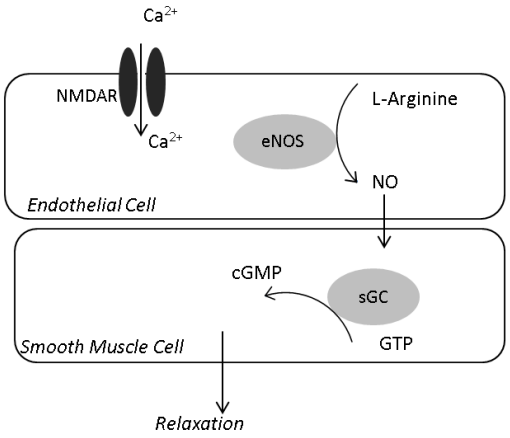

Figure 2: Vascular smooth muscle cell relaxation by NO.
Nitric oxide, previously known as endothelial derived relaxation factor (EDRF) mediates vascular smooth muscle cell relaxation and thus regulates blood flow. *N*-methyl *D*-aspartate receptor (NMDAR)-mediated Ca^{2+} influx leads to enzymatic activation of endothelial nitric oxide synthase (eNOS) to produce NO from L-Arginine. Endothelial released NO binds to the soluble guanylyl cyclase (sGC) in vascular smooth muscle cells, which catalyses guanosine triphosphate to cyclic guanosine monophosphate (GTP-cGMP) conversion. cGMP activates protein kinase G (PKG), which in turn mediates relaxation through modulation of intracellular Ca^{2+} levels.

These observations were in exact consistency with earlier findings of Ferid Murad and colleagues which showed NO was activating sGC to produce guanosine 3′,5′-cyclic monophosphate (cGMP) (Arnold et al., 1977) and it was scavenged by haemoglobin and myoglobin (Murad et al., 1978). Indeed, based on their further findings both Furchgott and Ignarro proposed in 1986 at the 4th International Symposium on Mechanism of

Vasodilatation that EDRF was in fact NO (Vanhoutte, 2009, Furchgott, 1986, Ignarro LJ, 1986). This was followed by publication of conclusive evidence showing EDRF was NO (Ignarro et al., 1987, Palmer et al., 1987). This as well meant that the mammalian cells should be able to produce NO, something not known until then. In a year, vascular endothelial cells were proven to produce NO (Palmer et al., 1988) and this was followed by the cloning of the first NO synthase from the rat cerebellum (Bredt and Snyder, 1990). Later, there has been an immense interest in NO in biology and medicine which made it the Molecule of The Year in 1992 (Koshland, 1992).

5-year record of published articles from nitric oxide research is around a thousand from 1986 to 1991. From 1992 to 1997 this number reaches to 17,000 showing the tremendous increase of interest both in number and extent of fields (Extracted from PubMed Search). In 1998, the seminal works of Robert Furchgott, Ferid Murad and Louis Ignarro leading to the "re-discovery" of NO and its effects on the cardiovascular system were recognized with the Nobel Prize in Physiology and Medicine.

Although earlier research was mainly focused on the cardiovascular system, NO was subsequently revealed to be involved in several other physiological functions. Today we know that NO plays key roles in numerous bodily functions including regulation of vascular tone, heart beat and rhythm, cellular bioenergetics, cell proliferation as well as apoptosis, inflammatory processes, synaptic transmission and synaptogenesis, hormone release, sleep-wake cycle and appetite.

1.2.1. Nitric Oxide in Biology

1.2.1.1. Synthesis of NO

There are three isoforms of nitric oxide synthases (NOSs) identified: neuronal NOS (nNOS, NOS I), inducible NOS (iNOS (NOS II) and endothelial NOS (eNOS, NOS III), numbered in the order of their cloning. Among these, nNOS and eNOS are constitutively expressed in neuronal and endothelial tissues, respectively and are activated by calcium (Ca^{2+})/calmodulin complex. In nervous system, nNOS is considered to be the main producer of NO under physiological conditions, in a Ca^{2+}-dependent manner, upon NMDA receptor stimulation (Garthwaite and Garthwaite, 1987, Garthwaite et al., 1988) . The discovery of physical association of nNOSα, the most abundant of the three nNOS isoforms in nervous tissue, and the NMDA receptor subunit NR2B with post synaptic

density protein-95 (PSD-95) through their PDZ domains (Brenman et al., 1996) further helped to explain the link between NMDA activity and NO production. Moreover, this close relationship, functional and physical, also forms a major basis for the involvement of NO in synaptic activities. The expression of iNOS is induced in response to damage, stress or inflammation and it is Ca^{2+}-independent. iNOS expression could be induced in a variety of cell types including immune (Vig et al., 2004, Tsukahara et al., 2001, Jacobs and Ignarro, 2001), glial (Saha and Pahan, 2006), neuronal (Heneka and Feinstein, 2001), epithelial (Kolios et al., 1998), endothelial (Cortese-Krott et al., 2014) and muscle cells (Balligand et al., 1994, Hecker et al., 1999). All three isoforms utilize L-Arginine, oxygen (O_2) and nicotinamide adenine dinucleotide phosphate (NAPDH) along with a number of cofactors to produce NO and L-Citrulline (**Figure 3**). Apart from enzymatic synthesis, NO could be produced from inorganic sources, which are taken up with nutrients (such as nitrate) (Lundberg and Govoni, 2004).

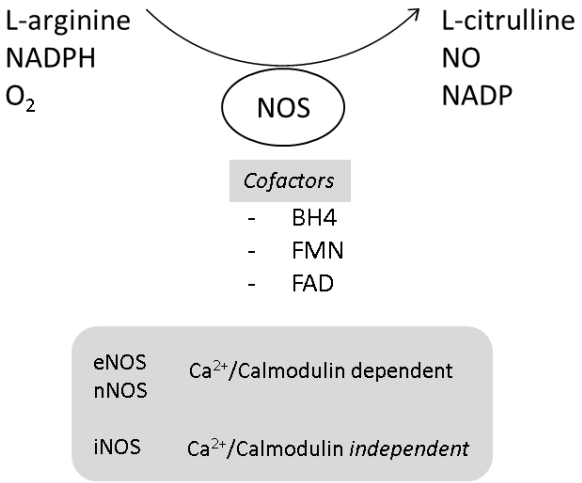

Figure 3: Enzymatic synthesis of NO by NOSs.
NADP: nicotinamide adenine dinucleotide phosphate, NADPH: reduced NADP, O_2: diatomic oxygen molecule, BH4: tetrahydrobiopterin, FMN: Flavin mononucleotide, FAD: Flavin adenine dinucleotide. All nitric oxide synthases (NOSs) produce NO through the same reactions, but eNOS and nNOS activities are regulated by Ca^{2+}/Calmodulin binding while iNOS operates Ca^{2+} independently.

1.2.1.2. Major Mechanisms of Action

Diverse actions of NO are based on its interaction with metal centres, amino acid residues and reactive oxygen species (ROS).

The best described interaction of NO with a metal centre is the activation of soluble guanylyl cyclase (sGC) by NO binding to the haem prosthetic group of the β1 subunit (**Figure 4**) (Bellamy et al., 2002). Here, the conformational change activates sGC to produce guanosine 3′,5′-cyclic monophosphate (cGMP), which in turn activates cGMP-dependent protein kinase (PKG) to induce smooth muscle relaxation through modulation of intracellular Ca^{2+} levels (Carvajal et al., 2000). NO-cGMP signalling is also utilized in synaptic activities of NO (Garthwaite, 2008).

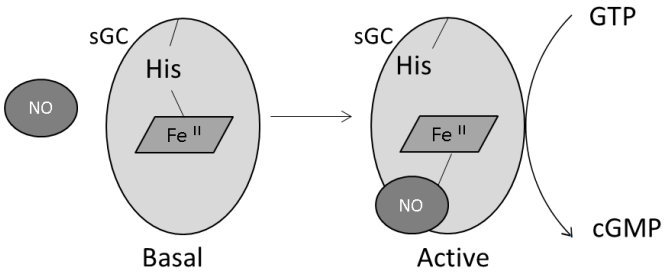

Figure 4: sGC activation by NO.
Activation of soluble guanylyl cyclase (sGC) is achieved by a conformational change upon NO binding to the haem group of beta1 subunit. Resulting active enzyme catalyses the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP).

Other examples are the binding of NO to the iron centres of haemoglobin (Gow and Stamler, 1998) or cytochrome P450 (Khatsenko et al., 1993, Ost et al., 2011). NO also binds to the iron-copper centre of cytochrome c oxidase (CcO, Complex IV) (Sarti et al., 2000, Antunes et al., 2004). In the case of CcO, NO competes with O_2 resulting in inhibition and hence decreased mitochondrial respiration (Brown and Cooper, 1994) (**Figure 5**).

The most studied interactions of NO/derivatives and amino acids include nitrosylation of cysteine and nitration of tyrosine residues. S-nitrosylation (also called S-nitrosation) is the covalent addition of NO to the thiol groups of cysteine residues to form R–S=NO (**Figure 6**) and has been documented in several studies [reviewed in (Martinez-Ruiz and Lamas, 2004) and (Martinez-Ruiz et al., 2011)]. The reversible nature of such modification suggested that it could serve as a signalling mechanism (Gould et al., 2013). In tyrosine nitration, a nitro group (−NO2) is incorporated to the phenolic ring of tyrosine residue at the 3rd position resulting in a 3-nitrotyrosine residue (**Figure 6**). It is an

irreversible modification and is considered as a marker of oxidative damage by peroxynitrite (ONOO−) (Radi, 2004, Radi, 2013).

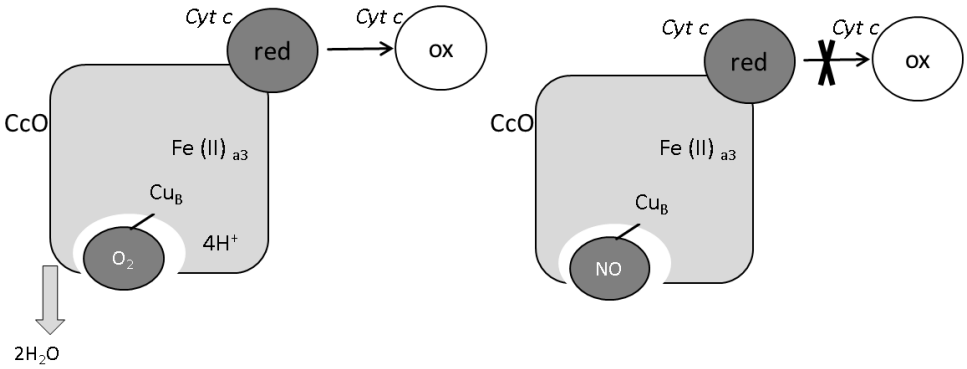

Figure 5: Mechanism of NO-mediated inhibition of cytochrome c oxidase.
NO binds to the iron-copper centre of cytochrome c oxidase (CcO) competing with oxygen (O_2). When O_2 is bound, electrons are transferred through iron-copper centres till the water synthesis and cytochrome c (Cyt c) is oxidized (ox) to recycle as an electron carrier. NO binding blocks the electron flow and cytochrome c remains reduced (red), preventing its recycling as an electron acceptor. This results in decreased ATP production.

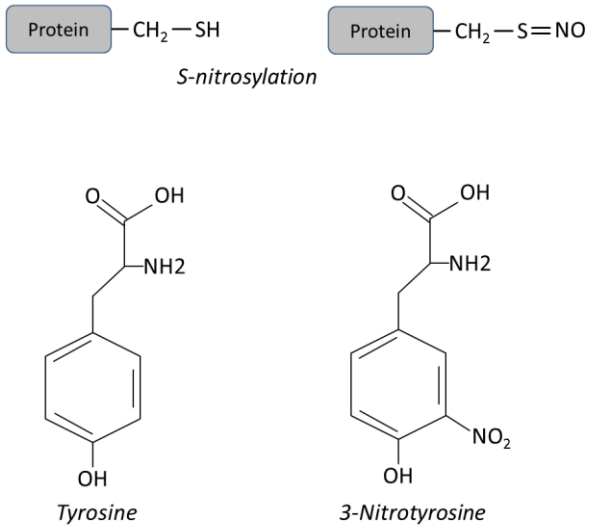

Figure 6: Structures showing S-nitrosylation and tyrosine nitration.
S-nitrosylation occurs in thiol containing residues (upper) where a nitrosyl (-N=O) reacts with Sulphur to result in S-nitrosyl (-S-N=O). In tyrosine nitration at nitro group ($-NO_2$) is incorporated to the phenolic ring of tyrosine residue at the third position (lower). S-nitrosylation is reversible, while tyrosine nitration is not. Both modifications affect protein functionality.

It should be noted that $ONOO^-$ is not the only, although the most prominent, nitrating agent on tyrosine residues, additional mechanisms, which are also based on NO or reactive nitrite, have been reported (Wu et al., 1999, van Dalen et al., 2000, Heinecke, 2002, Souza et al., 2008). Unlike classical post-translational signalling modifications (e.g. phosphorylation), neither nitration nor nitrosylation (or denitrosylation) take place via an enzymatic activity. In other words, although some proteins might be more prone to such reactions, these modifications are not considered as signalling modifications per se since both reactions lack specificity. However, both nitrosylation and nitration are of functional importance on the grounds of their ability to modulate activity of target proteins. One interesting example is the neural growth factor (NGF)-induced differentiation of PC12 cells, where protein nitration significantly increased upon incubation with NGF and the subcellular distribution displayed a shift from the cytosolic to the cytoskeletal fraction, alpha-tubulin being the major target (Cappelletti et al., 2003).

The reaction of NO with superoxide (O_2^-) is almost at diffusion-limited rate constant (Huie and Padmaja, 1993) allowing them to react spontaneously to form $ONOO^-$, a much stronger reactive species. Such reaction would have multiple consequences. On one hand, $ONOO^-$, when produced at high levels leads to nitrosative stress via tyrosine nitration; yet on the other hand, in the same reaction NO acts as a scavenger of O_2^- thus in another point of view considered beneficial. Third, inversely O_2^- could be considered to scavenge NO and it might reduce the bioavailability of NO itself, hindering signalling mechanisms mediated by NO in its normal diffusion distance. Although there is not enough evidence to assert such interactions as strictly regulated mechanisms, it is obvious that they might provide functionally distinct subcellular micro compartments with unequal distribution of NO.

1.2.2. Quantification of NO in Biological Samples

The main problem for NO quantification in biological samples comes from its short half-life (<2 milliseconds in whole blood) (Liu et al., 1998) although this may change with concentration and sample type (Kelm, 1999). Therefore, a number of techniques have been developed in order to detect and quantify NO in biological samples.

One of the common techniques is the measurement of end products of NO metabolism (Tsikas, 2007). Nitrite/nitrate levels give a quite good estimation of total NO levels in a specimen and this could be measured with the help of a colorimetric reaction in

a Griess assay. Several commercial kits have been made available and this method is the easiest to handle. However, in this method it is not possible to distinguish NO produced in living subject (cells, animals or patients) from nitrite/nitrate that is taken through nutrition. Further, the end product does not tell much about the dynamics, which might be critical for immediate biological effects in the cellular or tissue microenvironment. In principle a transient but strong peak in NO production may result in similar levels of end product after a moderate increase that spans a longer period.

For the cell culture experiments, diaminofluroscein (DAF) compunds have been developed, which are better selective for NO since they do not react with nitrite or nitrate. They provide concentration dependent fluorescence intensity and a detection limit of about 5 nM. Disadvantages of this method are i) the pH sensitivity of DAFs, which can cause complications in the presence of other agents that affect the pH value (e.g. bradykinin, a common NO stimulator); ii) it is not possible to use it for in vivo or ex vivo measurements [reviewed in (Hong et al., 2009)].

Electrodes that detect NO-evoked currents are another possibility for direct measurement of NO changes in in vivo research [reviewed in (Serpe and Zhang, 2007)]. The method utilizes the oxidation of NO at a working electrode and thereby records NO-evoked currents. While it allows for a high temporal resolution, the spatial resolution is lost since the recordings reflect changes at immediate vicinity at the electrode tip. Another disadvantage is that the changes are recorded as deviations from the baseline, without representing the actual basal NO levels.

Usage of spin traps provides another way of direct NO quantification in biological samples [reviewed in (Hogg, 2010)]. Diethyl dithiocarbamate-iron (DETC-Fe) is the most frequently used agent in this technique. Briefly, NO forms a mononitrosyl-iron complex (MNIC) with the DETC-Fe, in which the unpaired electron of NO (which also gives it the free radical property) is preserved, hence the name spin trap comes from (**Figure 7**). In an atom, each orbit contains a pair of electrons that spin in different planar directions and magnetic field created by one of them cancels that of other's. In MNIC, unpaired electron of NO gives its paramagnetic properties, which can be detected in electron paramagnetic resonance (EPR) spectrometry with a characteristic spectrum, unique to NO. Thereby it allows highly selective quantification of NO content in a given volume of a specimen. It also overcomes the problem of short half-life of NO, since MNIC is rather a stable product

with a half-life of more than six months when stored at -80°C. Disadvantages of this technique are that i) it is not suitable for in vivo detection and ii) the temporal resolution is limited. Advantages are i) selectivity, ii) sensitivity, iii) spatial resolution that it offers, and iv) possibility to measure basal levels. In preclinical research, once an animal subject is administered with spin trap then it is possible to quantify NO levels virtually in all tissues, ex vivo.

Each measurement technique has its advantages and limitations, and one can select the best suited one in order to meet the special requirements of a certain research question.

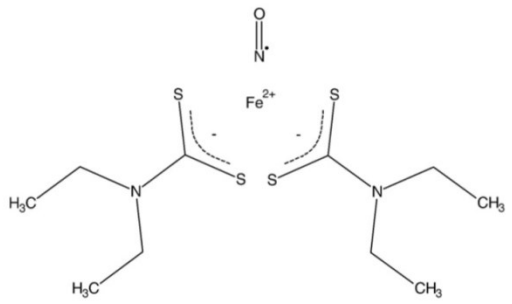

Figure 7: Molecular structure of a mononitrosyl-iron complex (MNIC).
Nitric oxide (NO) levels can be quantified by spectral analysis of MNICs in electron paramagnetic resonance (EPR) spectrometry, allowing for exact quantification of direct NO levels in a certain specimen. Figure adapted from (Hogg, 2010), copyright (2016), with permission from Elsevier (see Appendix C for agreement).

1.2.3. Cellular Effects of NO

Based on the aforementioned interactions (**Section 1.2.1.2**), NO has several cellular effects. Whether NO plays a detrimental or a beneficial role after TBI is tightly connected to its wide variety of cellular effects. Among these, three of them are foci of interest with respect to secondary tissue damage or neuroprotection, as well as repair and regeneration: regulation of cellular energetics, induction of cell death or support of cell viability.

1.2.3.1. NO and cellular energetics

NO can influence mitochondrial respiration through multiple mechanisms. One of these mechanisms is the reversible inhibition of cytochrome c oxidase (Complex IV, CcO), terminal electron acceptor and oxygen consuming enzyme in the mitochondrial electron transport system (ETS) (Brown and Cooper, 1994). Inhibition occurs via binding to the ferrous haem at the active site of the enzyme, therefore competitive with oxygen binding

(**Figure 5**) (Antunes et al., 2004). Nevertheless, physiological or pathological relevance of this inhibition remains under discussion, despite in vitro evidence for CcO inhibition by NO [reviewed in (Cooper and Giulivi, 2007)]. Although there has been studies showing increase in oxygen consumption in cardiac and skeletal muscles (Shen et al., 1995, Xie et al., 1996, King et al., 1994), kidney (Laycock et al., 1998) or whole body (Shen et al., 1994) in the presence of NOS inhibitors, controversial results were also reported in other studies (Grassi et al., 2005, Kirkeboen et al., 1994, Sadoff et al., 1996, Sherman et al., 1997).

Reflecting the competitive nature of the mechanism, one of the earliest studies demonstrated that the inhibition is enhanced at low oxygen levels (Brown and Cooper, 1994). Such a regulation would presumably suggest a detrimental effect of NO in post-traumatic secondary damage since brain tissue undergoes regional or global hypoxia after injury, but whether it is pathophysiologically relevant remained to be elucidated. On the other hand, experiments using non-selective NOS inhibitor L-NAME, indirectly showed that NO might not have any effect on the redox state of CcO after brief anoxia in rat brain (De Visscher et al., 2002). Accordingly, the excessive capacity of CcO relative to the maximum flux of the ETS or abundance of CcO over other complexes in the ETS were suggested to allow for severe inhibition before detectable mitochondrial respiration decreases (Gnaiger and Kuznetsov, 2002). Therefore, it would be interesting to know whether such inhibition is functionally involved in traumatic brain injury where prolonged hypoxic insult is associated with increased NO levels.

NO-mediated inhibition of mitochondrial respiration also includes nitrosative damage to mitochondrial proteins by peroxynitrite (ONOO-) or S-nitrosothiols (RSNO). S-nitrosylation of Complex I and creatine kinase have been shown to inhibit mitochondrial activity; however, ONOO- mediated damage has been implicated in inhibition of multiple respiratory complexes (Complex I-II-III- and IV), the ATP synthetase, creatine kinase and aconitase [extensively reviewed in (Brown, 2007)] (**Figure 8**). Among these, aconitase inhibition might serve as a potential negative feedback system, since inhibition of the electron feed to the ETS would also limit ROS production. It should be noted that these mechanisms were studied mostly in vitro with exposure of tissue lysates, cells or isolated mitochondria to NO, ONOO- or NO donors. Therefore, although these findings point towards potential mitochondrial targets of NO, clinical or therapeutic value of such inhibitions is obviously dependent on validation of these observations in vivo under

pathological conditions. Furthermore, whether NO/ONOO- levels used/obtained in these in vitro studies mimic in vivo NO levels in patho-/physiological processes needs to be validated.

NO can induce mitochondrial permeability transition pore (mPTP) opening (Hortelano et al., 1997, Brookes et al., 2000), a process known to increase permeability of mitochondrial inner membrane to molecules with a size less than 1.5 kDa. This process is frequently associated with cell death, either necrotic or apoptotic (Kim et al., 2003), and NO/RNS mediated inhibition of respiration and thereby loss of mitochondrial membrane potential are thought to play facilitating role in the process (Borutaite and Brown, 2003, Brown, 2010). On the other hand, others also showed that NO might inhibit mPTP opening, probably through cGMP-PKG signalling and reduced Ca^{2+} accumulation in mitochondria (Takuma et al., 2001).

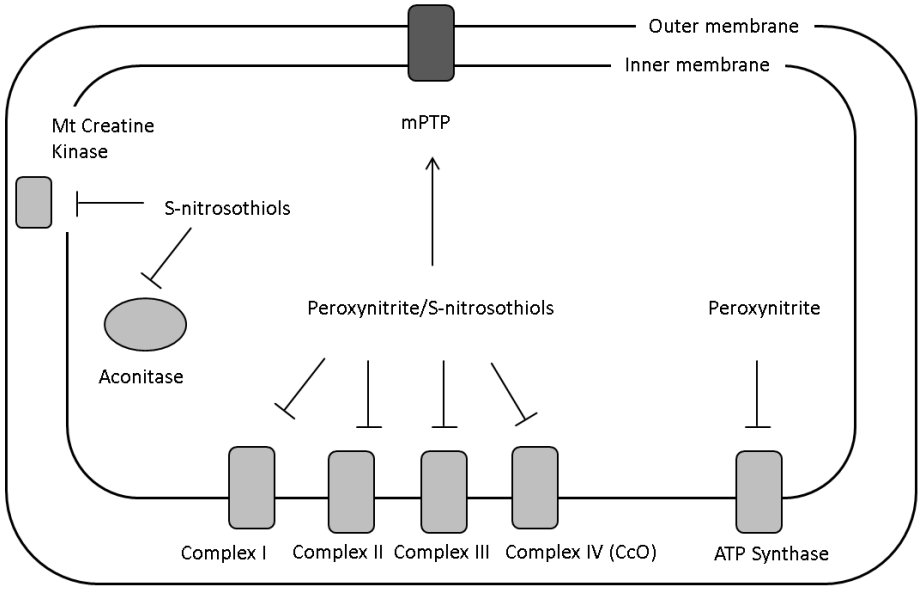

Figure 8: NO-mediated mitochondrial inhibition.
NO or derivatives have been shown to target several mitochondrial proteins, which ultimately contribute to impaired mitochondrial respiration, cellular energetics and even cell viability. Potential mechanisms include inhibition of a variety of mitochondrial proteins or induction of mitochondrial permeability transition pore opening. CcO: cytochrome c oxidase, Mt Creatine Kinase: mitochondrial isoform of Creatine Kinase, mPTP: Mitochondrial permeability transitions pore.

Finally, NO is thought to be involved in regulation of mitochondrial biogenesis, fission and fusion. Elongated, large mitochondria are generated via fusion of many

mitochondria and considered as healthy and efficient in ATP production, while fission results in smaller mitochondria under low energy demand (Westermann, 2012). Under physiological conditions mitochondrial dynamics is regulated to respond to changing energy demands, to repair or remove damaged mitochondria. Instead, elevated mitochondrial fission due to stress factors results in fractionated, small mitochondria which are less efficient in ATP biosynthesis and thereby contribute to ROS production, and they are vulnerable to oxidative stress (Wu et al., 2011). There are controversial findings regarding the exact role of NO in these processes. Low levels of endogenous NO were shown to stimulate mitochondrial biogenesis via cGMP signalling pathway (Nisoli et al., 2003, Nisoli et al., 2004) and there is evidence for NO-mediated mitochondrial elongation in myogenic differentiation (De Palma et al., 2010). On the other hand, another study showed dynamin-related protein-1 (Drp1)-dependent mitochondrial fission was enhanced with S-nitrosylation of Drp1, which promotes β-amyloid neurotoxicity in Alzheimer's disease (Cho et al., 2009a). A later study indicated S-nitrosylation did not have any effect on Drp1 activation; however, NO-induced Drp1 activation rather takes place via phosphorylation (Bossy et al., 2010). For a better understanding of the role of NO in mitochondrial biogenesis further research is needed. Nonetheless, these studies confirm the involvement of NO in the positive or negative regulation of mitochondrial biogenesis and would have an impact on pathological conditions of dysregulated NO signalling.

1.2.3.2. NO and cell death

Induction of cell death by NO is not independent of its effects on mitochondrial respiration. Based on the energy status of the cell, NO-induced cell death could be divided into two major classes: 1) Necrosis mediated by energy depletion and 2) apoptosis mediated by oxidative/nitrosative stress.

1.2.3.2.1. *Necrosis*

NO can contribute to impaired energetics in multiple ways. Induction of mPTP opening is one of them as sustained opening generally leads to rapid hydrolysis of mitochondrial and cytosolic ATP. In addition, multiple components of the respiratory chain are prone to inhibition via S-nitrosylation or nitration leading to reduced ATP production. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been shown to undergo S-nitrosylation or oxidation in its active site by ONOO- and other RONS, which in turn uncouples glycolytic flux from ATP synthesis (Souza and Radi, 1998, Brune et al.,

1996, Albina et al., 1999, Mohr et al., 1999). Additionally, ONOO- has been shown to induce DNA double strand breaks and hence poly ADP-ribose polymerase (PARP) activation (Pacher and Szabo, 2008). Accordingly, PARP activation was shown to be decreased in nNOS-knockout mice after cerebral ischemia and the effect was mediated by ONOO- rather than NO itself (Endres et al., 1998). In another study, a cold injury model of cerebral cortex provided similar results when pre-treated with nNOS inhibitor (Hortobagyi et al., 2003). Both studies indicated that peroxynitrite-induced DNA damage and PARP activation was a functionally important contributor of secondary damage in brain injury. PARP1, the predominant form of the enzyme, utilizes NAD+ as substrate, thereby reduces bioavailability of NAD+, unique electron carrier of glycolysis. All of these processes can potentially contribute to ATP depletion in the cell, which in turn results in failure of ATP-driven ionic pumps, loss of electrolyte equilibrium and osmotic rupture of plasma membrane to eventuate in necrotic cell death. Sustained availability of ATP is of crucial importance in injured brain, which already goes under metabolic stress through glutamate excitotoxicity and impaired cerebral blood flow.

1.2.3.2.2. *Apoptosis*

Apoptotic cell death has also been shown to be induced via NO-mediated oxidative/nitrosative stress through diverse mechanisms. When transiently induced, mPTP causes cytochrome c release from mitochondria to trigger intrinsic pathway of apoptosis. Indeed, there is evidence for the functional presence of this pathway in NO-mediated apoptotic cell death (Borutaite et al., 2000). In addition, p53 has been shown to be induced by high levels of NO (Thomas et al., 2004) via ROS/RNS induced DNA damage. p53 phosphorylation and Bax translocation to mitochondria was documented in NO-mediated apoptotic death of astrocytes, albeit without caspase activation (Yung et al., 2004), potentially due to caspase inactivation by S-nitrosylation (Tenneti et al., 1997). However, another study indicated that nitrosation of p53 may prevent its translocation to mitochondria (Hernlund et al., 2009). Apoptotic cell death via p38 MAP kinase pathway was also shown to be induced by peroxynitrite or high levels of NO (Guner et al., 2009). Further, functional presence of this pathway was documented in a model of ischemic brain damage in the same year by another group (Chen et al., 2009). Nevertheless, whether this pathway is downstream of mPTP or functions independently is unclear (Bossy-Wetzel et al., 2004).

1.2.3.2.3. *Necroptosis*

Regulated necrosis is an expanding research focus since its discovery, as a form of necrotic cell death that is regulated by a molecular mechanism (Vandenabeele et al., 2010). Later the term 'necroptosis' was coined, referring to its molecular controlled character, which was previously thought to be exclusive to 'apoptosis'. Two protein kinases, receptor-interacting serin/threonine kinase -1 and -3 (RIPK1 and RIPK3) have been shown to be activated by inflammatory signalling and mediate necroptotic cell death via a phosphorylation driven assembly of a 'necrosome complex', which comprises of RIPK1, RIPK3 and mixed lineage kinase domain-like (MLKL) proteins (Pasparakis and Vandenabeele, 2015, Cho et al., 2009b, Sun et al., 2012). Later, phosphoglycerate mutase family member 5 (PGAM5), a mitochondrial phosphatase, was shown to be included in various necrosomal complexes and mediate necrosis via mitochondrial fragmentation through Drp1 activation (Wang et al., 2012), suggesting mitochondria as a main target in necroptotic processes. Mitochondria seem to be important targets in NO-mediated cell death and potentially in necroptosis. Accordingly, inhibition of iNOS resulted in a decrease of hemin-induced necroptosis in mouse astrocytes (Laird et al., 2008). Later, high levels of NO were demonstrated to mediate necroptosis in endothelial cells in vitro via nitration of mitochondrial Complex I (Davis et al., 2010). Pathological conditions with dysregulated NO production and inflammation therefore could also induce necroptotic cell death. To that matter, treatment with necrostatin-1, a known inhibitor of necroptosis, resulted in improved outcome after brain trauma (You et al., 2008) and neonatal hypoxic-ischemic brain injury (Northington et al., 2011) in mice. It appears that NO can contribute to all three types of cell death mechanisms in pathological conditions and outcome to be determined by energy status and availability of caspase activation in damaged cells, which are also partly influenced by NO.

1.2.3.3. NO and cell viability

Although there are several lines of evidence on NO-mediated cell death, NO has also been shown to be cytoprotective and support cell viability. Physiologically, due to its vasodilatative activity NO seems to be neuroprotective under ischaemic conditions, as shown in a model of transient focal ischemia (Hashiguchi et al., 2004). Authors showed that eNOS was upregulated in endothelial cells secondary to phosphatidylinositol 3-kinase (PI3K)/Akt activity and eNOS-derived NO was critical for ischemic tolerance.

Alternatively, NO-induced PI3K/Akt survival pathway was reported to support survival of retinal neuronal cultures (Mejia-Garcia and Paes-de-Carvalho, 2007). The effect was dependent on sGC, PKG, PI3K, protein kinase A (PKA) and calmodulin-dependent kinase (CaMK) activities. NO-mediated cell survival through cGMP-dependent Akt activity has also been shown in several other studies (Sata et al., 2000, Dash et al., 2003, Ha et al., 2003, Ciani et al., 2002, Tejedo et al., 2004, Fujita et al., 2014). NO also seems to partially inhibit mPTP opening through cGMP-PKG signalling when released at low levels. Apoptosis inhibition via this pathway has been documented in astrocytes (Takuma et al., 2001), although there is no evidence supporting or disproving this notion in neurons. Convergence of these effects on PKG's regulatory role in Ca^{2+} homeostasis is noteworthy. cGMP-PKG signalling is considered to mediate NO actions mostly at low concentrations. Hence, the dilemma whether NO acts as cytotoxic or cytoprotective might be a matter of NO tissue levels. To that matter, elucidation of NO levels in injury-distant brain regions as well as in injury vicinity is crucial to understand whether post-TBI NO changes have beneficial or detrimental effects. Nonetheless, there are exceptions to such generalization. Bonthius and colleagues used substantial levels of NO (1mM DETA-NONOate) and reported its anti-apoptotic activity against alcohol-induced neurotoxicity, which they also confirmed that the effect is mediated through cGMP-PKG pathway and NF-κB activation (Bonthius et al., 2009). Others suggested that high levels of NO could be protective via S-nitrosylation of NMDA receptors (NMDARs) (Lipton et al., 1993, Choi et al., 2000) and caspases (Tenneti et al., 1997). On the other hand, whether inactivation of NMDAR could functionally help prevention of glutamate excitotoxicity or whether S-nitrosylation of caspases could stop progression of apoptotic or non-apoptotic cell death is unclear.

1.2.3.4. NO and Neurogenesis

There have been numerous studies investigating the influence of NO on neurogenesis. Effect of NO on neurogenesis is of critical importance for post-traumatic neuroprotection, regeneration and repair as they would serve beneficial to the injured brain. Data available so far points towards a dual role for NO in neurogenesis; inhibition by nNOS derived NO under physiological conditions and induction by eNOS or iNOS derived NO under pathological conditions. One of the early contributions to the quest came with the morphological observation that the subventricular zone (SVZ) is surrounded by differentiated neurons expressing nNOS and also that some neuroblasts express nNOS once they reach the olfactory bulb (OB) periglomerular area (Moreno-Lopez et al., 2000).

This was an important observation since nNOS expressing cells were excluded from the neurogenic regions and migratory neuroblasts started expressing nNOS on the way of differentiation. Conclusive evidence came when pharmacological inhibition or genetic ablation of nNOS resulted in increased number of mitotic cells in SVZ, rostral migratory system (RMS), OB, and dentate gyrus (DG) of hippocampus (Packer et al., 2003, Moreno-Lopez et al., 2004, Park et al., 2002). A later study showed that proliferation in DG was inhibited by nNOS derived NO and suggested that this effect might be mediated through NMDA receptors and by a reduction in cAMP response element binding protein (CREB) phosphorylation (Zhu et al., 2006). It appears under physiological conditions NO helps preservation of neurogenic reserves by suppressing proliferation. However, the mechanism was poorly understood. First, sGC expression in proliferating cell regions were not assessed in these studies and neural stem or progenitor cells (NSCs) might still be subjected to NO mediated mitotic inhibition in a paracrine manner. In that case, nNOS expressing cells around them would indeed provide an inhibitory control over cell proliferation, rather than being 'excluded' from these regions for disinhibition. Second, they might provide differentiation signals to the new-born cells. Indeed, there was evidence for such kind of regulation and a potential link between brain-derived neurotrophic factor (BDNF) and NO signalling was provided in that study (Cheng et al., 2003). Findings of a later study supported this hypothesis by showing that migratory neuroblasts in the RMS express catalytic (β1) subunit of NO receptor sGC, but astrocytes, immature precursors and ependymal cells do not (Gutierrez-Mecinas et al., 2007). A more recent in vitro study suggested a bidirectional regulation of neural stem cell (NSC) proliferation by autocrine-paracrine NO signalling (Luo et al., 2010). Utilizing co-culture of neurons with NSCs, authors showed that NO produced in NSCs is necessary for proliferation of NSCs and suggested a role for telomerase activation in this process. On the other hand, NO released from nNOS-expressing neurons inhibited NSC proliferation via reduced CREB phosphorylation. These findings provided useful insights to the mechanism, although NO production in NSCs seems to contradict earlier findings that have not detected nNOS expression in these cells in vivo.

Pathological conditions with dysregulated NO production would potentially have impact on proliferative and neurogenic regulation by NO. Nevertheless, studies have shown that it might not be simple to have a projection of pathological conditions based on the situation under physiological conditions. Indeed, NO has been shown to be a positive

regulator of injury induced neurogenesis and pharmacological or genetic approaches revealed that this was mediated by eNOS or iNOS after focal cerebral ischemia or depression (Zhu et al., 2003, Chen et al., 2005, Reif et al., 2004). Carreira et al used pharmacological high concentrations of NO, to resemble pathological NO levels, and induced proliferation of NSCs and they suggested this effect takes place by activation of the mitogen-activated protein kinase (MAPK) pathway by NO bypassing the EGFR (Carreira et al., 2010). This finding is particularly interesting on the grounds that an earlier study had showed physiological levels of NO inhibited NSC proliferation via inhibition of EGFR as well as PI3K/Akt pathway (Torroglosa et al., 2007). These findings suggest EGFR along with NO levels to be in a critical switch for inhibition or induction of NSC proliferation. Although, studies regarding NO and neurogenesis are much less than those showing its toxic or detrimental effects, they provided useful insights. Today we know that post-injury NO elevation might not be simply detrimental, but could be the part of a protective response to support cell viability via PI3K/Akt pathway (see previous section) or even to replace lost cells after the insult. In addition, NO could be a positive regulator of post-traumatic remyelination since in mice lacking iNOS fewer regenerating myelinated fibers, smaller caliber regenerating fibers, and slowed reinnervation of muscle endplates distal to the injury zone were observed after chronic constriction, nerve crush, and nerve transection injury models (Levy et al., 2001). Nevertheless, a careful interpretation of these results is necessary on the grounds that survival and successful integration of new-born cells are pre-requisites of a proper replacement of lost cells. Accordingly, there have been studies reporting that NSC cultures fail to differentiate into neuronal type, but rather the fate is diverted to astrogliogenesis after prolonged exposure (24h) to high levels of NO (Covacu et al., 2006).

1.2.3.5. NO and Neurotrophins

The earliest studies to suggest an interaction between neurotrophins and NO showed a robust, specific increase in NOS expression in basal forebrain cholinergic neurons in response to nerve growth factor (NGF) infusion (Holtzman et al., 1994). Later BDNF, neurotrophic factor-3 and -4 (NT3 and NT4) treatments were shown to induce an increase in NOS expressing neurons in rat spinal cord cultures (Huber et al., 1995). These studies were followed by an intensive research, which provided several lines of evidence indicative of a close, complex and reciprocal interplay between neurotrophins and NO, in terms of both neuroprotection and neurotoxicity.

BDNF, NT4, and NT3 (but not NGF) were shown to induce NO signals in the soma and in proximal dendrites of hippocampal neurons that were sensitive to NOS activity, neurotrophic tyrosine kinase receptor type 2 (TrkB) signaling, and intracellular calcium elevation (Kolarow et al., 2014), suggesting a role for NO in regulation of neurotrophin-induced neurite modulation. Accordingly, NO has been shown to enhance NGF-induced neurite outgrowth (Hindley et al., 1997) and mediate NT4-induced growth cone repulsion (Tojima et al., 2009). Both effects took place in a cGMP-dependent manner and probably through modulation of Ca^{2+}-induced Ca^{2+} levels. At low or endogenous levels it also supports BDNF-induced neurite outgrowth or axonal elongation (Ernst et al., 2000, Klocker et al., 2001), while, at higher levels or by exogenous application it mediates growth cone stabilization by counteracting the growth cone attraction induced by BDNF (Ernst et al., 2000).

1.2.3.5.1. Neurotrophin-induced suppression of NO in microglia

Microglial NO production might be suppressed by neurotrophins. NGF, BDNF, NT3 treatment suppressed the release of basal and lipopolysaccharide (LPS)-induced NO from microglial cells (Nakajima et al., 1998), reduced iNOS and tumor necrosis factor alpha (TNFα) expressions (Tzeng and Huang, 2003). Among these NT3 pre-treatment exerts its effect possibly through a MAPK- and PI3K-dependent pathway (Tzeng et al., 2005), while modulation of intracellular Ca^{2+} levels were suggested for BDNF effect (Mizoguchi et al., 2011). Similarly, NGF pre-treatment of cortical cultures attenuated delayed excitotoxicity induced by NO donors in another study (Kume et al., 2000). These findings suggest that the timing of post-TBI neurotrophin and NO releases could be critical for the outcome. These data together suggest that neurotrophin preconditioning counteracts the LPS effect, perhaps through alleviating the oxidative environment, which in turn would attenuate iNOS activation upon LPS treatment. Accordingly, a BDNF-induced decrease in ROS production was shown to take place in a model of gentamicin-induced vestibular sensory cell damage (Takumida and Anniko, 2002a, Takumida and Anniko, 2002b). NO suppressing effects of NT3, BDNF and NT4 were lost when treated together with or after LPS treatment of microglia (Tzeng and Huang, 2003) or after the injury of vestibular sensory cells (Barouch et al., 2001), which could be explained by the fact that iNOS activity is not regulated once activated. In macrophages, NT3 increased NO release when applied after LPS (Tzeng and Huang, 2003), perhaps as a secondary effect by saving NO

from being scavenged, although such increase was not observed with LPS-induced microglia (Barouch et al., 2001).

1.2.3.5.2. NO as a mediator of neurotrophin-induced neuroprotection

NO was also implied in mediating neuroprotective effects of neurotrophins. NT3 was supportive in recovery from erectile dysfunction through a mechanism that perhaps involves nNOS activation in major pelvic ganglia (Bennett et al., 2005). A similar role for NO as a downstream effector of neurotrophin stimulation was shown in other peripheral nerve injury models (Hsieh et al., 2003, Lin et al., 2003). In cultured striatal GABAergic interneurons, NT4/5 increased the number of nNOS+ neurons and a robust increase in nNOS protein levels, significant larger soma size and number of primary neurites (Ducray et al., 2006). Interestingly, striatal GABAergic interneurons expressing nNOS have been described to be relatively spared from the progressive cell loss in Huntington's disease (HD) (Behrens et al., 1996); however, whether NT4/5 is involved in the mechanism is not known. In a model of hippocampal neuronal cultures, NO was shown to be involved in neurotrophic tyrosine kinase receptor type 1 (TrkA), Akt and MAPK (Erk1/2) phosphorylation and prevented apoptosis in a sGC-cGMP-PKG dependent manner, and the authors suggested that NO might act as downstream effector in NGF-induced neuroprotection through TrkA receptor (Culmsee et al., 2005). Similarly, in another study, brief exposure to peroxynitrite enhanced BDNF-mediated phosphorylation of Akt (but not Trk receptors) and led to phosphatase and tensin homolog (PTEN) inhibition, thereby supported survival in primary neurons (Delgado-Esteban et al., 2007). On the contrary, in a rat model of spinal root avulsion injury, BDNF and glial-derived neurotrophic factor (GDNF) treatments supported the survival of injured motoneurons by up-regulating c-Jun and NGF receptor (p75NTR), but the effect was associated with a diminished nNOS expression (Wu et al., 2003).

1.2.3.5.3. NO-mediated modulation of neurotrophin expression or release

NO was also shown to modulate neurotrophin expression or release. Some studies suggested that the brain BDNF signalling was dependent on cerebrovascular endothelium-derived NO (Banoujaafar et al., 2016, Monnier et al., 2016). In isolated hippocampal neurons, NO donor (sodium nitroprusside (SNP); 500µM for 3 days) increased BDNF immunoreactivity (Chen and Russo-Neustadt, 2007). On the contrary, others reported that at lower levels (SNP; 10µM for 20 min) NO had inhibitory effect on BDNF release, in

cultured hippocampal neurons, in a cGMP-PKG-dependent manner (Canossa et al., 2002). In addition to NO levels, the underlying mechanisms might have also been different in these studies. In a later study, SNP (100μM, 5-10min) did not induce any change in BDNF and NT3 release; but induced an inhibition in depolarization-dependent release of these neurotrophins (Kolarow et al., 2014). Interestingly, endogenous NO either generated in response to depolarization or neurotrophin stimulation, did not result in a negative feedback on neurotrophin secretion, unlike exogenous NO (Kolarow et al., 2014). These findings might suggest that post-traumatic NO elevations caused by non-neuronal cells (microglia, astrocytes or macrophages, possibly through iNOS) might have a suppressing effect on neurotrophin expression and release in neuronal cells, hampering the neuroprotective response in injured brain.

1.2.3.5.4. Cross-talk between NO and neurotrophins in neural differentiation

There is also evidence of neurotrophin-NO cross-talk in neural differentiation. During the NGF-induced differentiation of PC12 cells, a significant increase in protein nitration and a shift in the subcellular distribution of nitrated proteins from cytosolic to cytoskeletal fraction were observed. The effect was due to endogenous NO production (Cappelletti et al., 2003). Similarly, in cultured hippocampal neural progenitor cells (NPCs), the production of NO and expression of nNOS, NT3, NT 4/5, and synapsin I increased markedly during neuronal differentiation (Park et al., 2016). All of them were abolished by nNOS inhibition (Park et al., 2016). In addition, endogenous NO was required for BDNF-induced NPC differentiation (Cheng et al., 2003). On the other hand, there is also data suggesting an effect in the opposite direction, i.e. NSC maintenance. NT3 produced and secreted by endothelial cells of brain and choroid plexus capillaries was shown to be required for the quiescence and long-term maintenance of NSCs in the mouse sub ependymal niche. Uptake of NT3 from irrigating vasculature and CSF induced the rapid phosphorylation of eNOS present in the NSCs, leading to the production of NO, which subsequently acts as a cytostatic factor (Delgado et al., 2014).

1.2.3.5.5. NO as a mediator of neurotrophin-induced gene expression

NO was also shown to mediate neurotrophin-induced gene expression by modulating epigenetic mechanisms. Pharmacological manipulation of neurons in vitro and analysis of mice lacking nNOS suggested that NO mediates BDNF- and activity-dependent expression of CREB target genes with a mechanism involving S-nitrosylation of nuclear

proteins that associate with CREB target genes (Riccio et al., 2006). Later studies have identified that histone deacetylase 2 (HDAC2) was S-nitrosylated, which did not directly change its deacetylase activity but led to its release from chromatin and made CREB target promoters available for acetylation and thereby activation (Nott et al., 2008). A similar phenomenon was observed to take place via suppression of SUV39H1, a histone methyltransferase, by BDNF and NGF through a NO-dependent manner; however in this mechanism S-nitrosylation of GAPDH seemed to be critical as nitrosylated GAPDH/Siah complex translocated to nucleus and mediated degradation of SUV39H1, suppressing histone methylation and thereby blocking deactivation (Sen and Snyder, 2011).

1.2.3.5.6. NO-neurotrophin interaction in cytotoxicity

NO was also shown to mediate cytotoxic effects of prolonged neurotrophin exposure. In rat and mouse primary neuronal cultures, NGF-induced NMDA neurotoxicity was shown to be dependent on NO as a downstream effector (Samdani et al., 1997). Similar phenomenon was observed with prolonged exposure to BDNF and NT4/5, where nNOS inhibition was shown to attenuate neurotrophin cytotoxicity (Hwang et al., 2002, Choi et al., 2004). In another study, astrocytes stimulated with the BDNF released NO, and conditioned medium from activated astrocytes had detrimental effects on the morphology and survival of neurons, which was amplified by NO produced by neurons (Colombo et al., 2012). As later studies suggested, NO released from these stimulated cells in turn react with and oxidize the neurotrophins available in the culture, which makes them to be detrimental. Accordingly, Pehar et al showed that exogenous NGF stimulated motor neuron apoptosis only in the presence of a low steady state concentration of NO (Pehar et al., 2004). Further, when mitochondrial superoxide dismutase (SOD1) is mutated (SOD1G93A), motor neurons got vulnerable to NGF-induced apoptosis even in the absence of an external source of NO (Pehar et al., 2007), suggesting a critical role for peroxynitrite formation. Indeed, they also showed that peroxynitrite triggered the formation of stable high-molecular-weight oligomers of NGF, which exerted much higher apoptotic activity even in the absence of exogenous NO, and the effect was abolished via prevention of tyrosine nitration by urate (Pehar et al., 2006). Others also have shown that motor neuron apoptosis resulting from both trophic factor deprivation and exposure to neurotrophins required the induction of nNOS and peroxynitrite formation (Ricart et al., 2006).

In TBI pathophysiology, nitrosative stress is considered as an important contributor to the secondary damage. Whether post-TBI peroxynitrite formation is involved in neurotrophin-mediated toxicity in injured brain is not known. Nevertheless, it has been shown that when cultured astrocytes were challenged with LPS or peroxynitrite, they became reactive and accumulated NGF in the medium (Pehar et al., 2004, Vargas et al., 2004). Further, in both NGF-induced cytotoxicity and cytokine-induced cell death, NO has been shown to be the downstream mediator, and both mechanisms required p75NTR signalling (Pehar et al., 2004, Pehar et al., 2006, Chiarini et al., 2006). Further, tonicity-mediated cell swelling rapidly activated transcription of the endogenous p75NTR gene, and the effect was NO-dependent (Peterson and Bogenmann, 2003). These findings suggest that NO elevations in injured brain might also exert detrimental effects by switching a neuroprotective response into a death signal. Therefore, a comparison of neurotrophin expression and NO production profiles after TBI is desirable for a better understanding of protective and detrimental responses that take place after TBI.

1.3. TBI Pathophysiology Revisited: Role of Nitric Oxide

Nitric oxide is a physiological regulator of a wide variety of processes, including cerebral blood flow or inflammation, which are dysregulated or disrupted after traumatic brain injury. Whether NO changes in traumatized brain have detrimental or beneficial effect is a matter of discussion: should NO changes be held responsible for dysregulation of these physiological processes, or are NO changes, in fact, part of a protective response serving for the recovery of them once disrupted? Inhibition or supplementation experiments, undoubtedly, contributed a lot to our understanding on that quest. However, resolution of the dilemma requires the assessment of NO levels not only in injury core but also in injury-distant brain sub regions, the latter of which has usually been overlooked, perhaps, due to the fact that contralateral regions are not always considered to be ‘really affected’. Therefore this untold assumption that has been intrinsic to many studies might not hold true.

1.3.1. Hypoxic Signalling

Traumatic brain injury may cause vascular damage leading to the intracerebral haemorrhage, disruption of blood circulation and thereby regional hypoxic insult. Tissue hypoxia may induce stabilization of hypoxia inducible factor 1 alpha (HIF1α) protein against prolyl hydroxylase-mediated degradation and can upregulate HIF1α mRNA

expression as well. HIF1α then dimerize with HIF1β to form hypoxic signalling complex HIF1, which in turn acts as a transcriptional activator of gene expression for hypoxic tolerance mechanisms.

TBI-induced robust increases of HIF1α in mRNA or protein levels has been reported in focal injury models (Anderson et al., 2009, Huang et al., 2010, Li et al., 2013, Schaible et al., 2014), although these were not always concurrent with active hypoxic signaling (Schaible et al., 2014). However, contradictory results were also published, possibly due to the differences between experimental models. In diffuse injury models a slight increase was reported, albeit without an increase in glucose transporter 1 (GLUT1; a HIF1α target) expression (Umschweif et al., 2013, Hamlin et al., 2001), and in penetrating ballistic-like brain injury (PBBI) significant decreases at 1, 3 and 7 days in protein levels of HIF1α were observed (Cartagena et al., 2014). In addition, another FPI study could detect neither HIF1α nor VEGF-A (HIF1α target) in the cortex at 24 hours post-TBI, although HIF1α stabilization and VEGF-A expression were increased in hippocampus (Park et al., 2009).

There are several lines of evidence indicating the role of NO in regulation of expression or stabilization of HIF1α [reviewed in (Brune and Zhou, 2007)]. NO has been shown to inhibit hypoxia-induced stabilization of HIF1α (Agani et al., 2002), possibly via a proteasome-independent, but a calpain-dependent pathway (Zhou et al., 2006). On the other hand, under normoxic conditions NO showed a stabilizing effect on HIF1α (Zhou et al., 2006), particularly with exposure to steady state concentration of NO in a time-dependent manner (Thomas et al., 2004). The difference appears to be due to the availability of ROS, because it was already known that HIF1α stabilization and transcriptional activation of its target genes were dependent on mitochondrial ROS (Chandel et al., 1998). Accordingly, Kohl et al showed that under normoxic conditions induction of a moderate ROS production promoted HIF1α stability and the effect was attenuated by addition of NO, while NO-mediated attenuation was not observed with a strong ROS induction (Kohl et al., 2006). On the other hand, another study showed that NO-mediated stabilisation of HIF1α was associated with an extensive S-nitrosation of the protein under normoxic conditions (Sumbayev et al., 2003). Among all S-nitrosated thiol groups in HIF1α, S-nitrosation of Cys-800 was shown to be critical for its interaction with p300, which is necessary for transcriptional activity of HIF1 complex (Yasinska and

Sumbayev, 2003). Whether post-TBI NO changes are involved in the regulation of hypoxic signalling remained largely unknown.

1.3.2. Mitochondrial Dysfunction

Mitochondrial dysfunction after brain trauma has been well documented after TBI by numerous clinical or experimental studies. TBI can induce mitochondrial damage via reductions in activity or expression of mitochondrial enzymes (Jiang et al., 1999, Jiang et al., 2000, Singh et al., 2006), disruptions in mitochondrial ultrastructure (Balan et al., 2013, Lifshitz et al., 2006), and fluctuations in respiration metabolites (Signoretti et al., 2001, Mazzeo et al., 2008). All of these disturbances lead to functional impairment in mitochondria which also has been well documented with reductions in respiratory measurement studies (Singh et al., 2006). On the other hand, several mitochondrial proteins were shown to be targeted for modification mediated by NO or its derivatives in numerous in vitro studies (Poderoso et al., 1996, Costa et al., 1997, Poderoso et al., 1998, Clementi et al., 1998, Beltran et al., 2000, Orsi et al., 2000, Riobo et al., 2001, Chinta and Andersen, 2006, Carreras and Poderoso, 2007, Davis et al., 2010). Nevertheless, whether these findings have pathophysiological correlates in TBI remained as an open question, since it is not known whether NO concentrations used in these studies are comparable to the tissue levels of NO in brain after trauma. Further, the effects observed in cell cultures or isolated mitochondria that are exposed to a given concentration of NO, may not reflect the situation in the inflamed or intact tissue, where other biological "sinks" of NO are also present, like blood. However, there is evidence for mitochondrial damage to be, at least partially, mediated by NO or its derivatives after TBI. 3-nitrotyrosine (3NT) has been frequently used as an oxidative/nitrosative damage marker in isolated mitochondria after TBI (Singh et al., 2006, Opii et al., 2007, Mbye et al., 2008, Gilmer et al., 2010, Mustafa et al., 2010, Miller et al., 2015), although the functional location of damage remained unassessed in these studies.

1.3.3. Other pathophysiological events

Several reports indicated increased NO levels after TBI, which was evaluated by NO metabolites (Hlatky et al., 2003, Yuksel et al., 2013, Ansari et al., 2014), iNOS activity (Wada et al., 1998b, Jafarian-Tehrani et al., 2005, Gahm et al., 2006) or expression (Gahm et al., 2000, Abdul-Muneer et al., 2013, Jung et al., 2014). Post-TBI perturbations in cerebral blood flow (CBF) at least partially were attributed to increased levels of NO based

on the correlation of higher levels of NO metabolites to CBF dysregulation (Hlatky et al., 2003) or vasoconstrictive effect of NO-derived radicals (Elliott et al., 1998). On the other hand, in other studies supplementation with NO-donor (Zhang et al., 2002a) and particularly with L-Arg (Liu et al., 2002, Mendez et al., 2004, Cherian et al., 2004) was shown to be helpful in recovery of CBF after TBI. Additionally, trauma-induced cerebral haemorrhage is thought to reduce NO bioavailability via fast scavenging of NO by oxy-haemoglobin, which in turn leads to cerebral vasospasm (Siuta et al., 2013) and contributes to dysregulated CBF.

Similar discrepancies were observed with respect to post-traumatic oedema. Using a cold injury model, Nag et al showed a close association of blood brain barrier (BBB) permeability with increased endothelial iNOS and eNOS immunoreactivities in the perilesional vessels (Nag et al., 2000). In another study, iNOS activity was inhibited using three different selective iNOS inhibitors, but only one of them resulted in a reduction of brain water content (Louin et al., 2006). These findings may suggest that NO production by iNOS might not necessarily be involved in oedema progression. However, these observations also raise questions on the reliability of NOS inhibitors, since an observed effect might not necessarily take place due to NOS inhibition, but rather due to the inhibitor itself. On the other hand, in a CCI model of mice, an improvement in cerebral oedema, BBB permeability and intracranial pressure (ICP) was observed with inhaled NO (Terpolilli et al., 2013). Therefore, a clear molecular and mechanistic explanation of how NO contributes to or protects against oedema, whether it is a secondary or a direct effect, remain unclear.

The exact reasons of controversial findings regarding the role of NO in post-TBI CBF dysregulation or oedema progression is currently unknown. Presumably, consequences of NO changes in injury core and injury distant regions differ from each other. Accordingly, studies in isolated, perfused lung (Mundy and Dorrington, 2000) and in isolated skeletal muscle (Persson et al., 2003) showed protective effect of endogenous NO against oedema by supporting endothelial impermeability in the absence of an injury. The findings of Terpolilli et al (Terpolilli et al., 2013) seem to be based on a similar mechanism, as inhaled NO systemically reach to intact micro vessels as well and lead to a reduction in overall permeability, perhaps by substituting reduced NO in the perilesional or lesion-distant micro vessels, caused by the loss of shear stress. Therefore, observed endogenous NO elevations following trauma, in fact, might have been part of a protective

response for a recovery of CBF in traumatized brain. However, it perhaps comes with a side effect due to peroxynitrite formation via superoxide increase in traumatized brain tissue. Accordingly, a beneficial effect of L-Arg supplementation (Liu et al., 2002, Mendez et al., 2004, Cherian et al., 2004) is particularly reasonable when one considers the capability of iNOS and eNOS to produce superoxide instead of NO in the absence of L-Arg (Xia et al., 1998a, Xia et al., 1998b), which reduces NO bioavailability and increases peroxynitrite. In addition to CBF and oedema, there is a huge body of literature on the pro-inflammatory and anti-inflammatory effects of NO [reviewed in (Wallace, 2005, Cirino et al., 2006, Dedon and Tannenbaum, 2004)]. Therefore, comprehensive characterization studies for NO changes in injury-distant brain regions as well as injury site are necessary to help resolution of these discrepancies.

1.3.4. Sytemic Effects of TBI: Possible Role for NO

Influence of brain injury on internal organs and bodily functions has been studied mainly in clinical settings since monitoring of vitals of TBI patients comprises an important part of intensive care. To that matter, considerable amount of research has been focused on understanding of so-called “sympathetic storm” after TBI, which by definition refers to a series of events rising from the disturbances of sympathetic neural transmission after brain injury. Several TBI-induced systemic complications have been reported [reviewed in (Lim and Smith, 2007)]. Injury of the brain can induce critical complications in the functioning of internal organs, particularly pulmonary and cardiovascular system, in the acute phase and later (Lee and Rincon, 2012, Krishnamoorthy et al., 2014, Prathep et al., 2014). Ischaemic stroke of right insular cortex was shown to increase patient mortality during hospitalization due to heart rate variabilities and autonomic imbalance (Tokgozoglu et al., 1999). On the other hand, there is a great body of literature on NO regulation in liver and particularly in heart, and influence of NO on physiological functioning of these organs. However, whether and how a brain injury influences NO metabolism in these organs remain largely overlooked.

In accordance with its actions as neuromodulator and neurotransmitter, NO has been implied in central control of physiological processes. In that respect, sympathetic and parasympathetic control of cardiovascular system has been of particular focus.

Autonomous control of cardiovascular system is achieved by different nuclei/regions located in cerebral cortex, midbrain and brain stem (**summarized in Table**

Appendix 1). Notably, NO activity has been implicated in many of these regions including paraventricular nucleus (PVN) of the hypothalamus, insular cortex, rostral- and caudal ventrolateral medulla (RVLM and CVLM), solitary tract of nucleus (NTS), nucleus ambiguus, and dorsal motor nucleus of the Vagus (DMNV). Both inhibitory and excitatory actions of NO on either sympathetic or parasympathetic nerve activity within these autonomic control areas have been reported. Although a detailed review of the role of NO in these brain nuclei is beyond the scope of this current work, NO appears to play a generally depressor role in sympathetic outflow (RVLM, CVLM, PVN, Locus Coeruleus, midbrain PAG, NTS) and facilitates parasympathetic control of cardiovascular events (Vagus, DMNV, Nucleus Ambiguus). Thereof, post-traumatic changes in NO levels would certainly have impact on central regulation of cardiovascular system.

Much less is known about the control of liver function via specific brain regions when compared to the state of knowledge on the control of cardiovascular system. One reason could be that most of the cardiovascular readouts upon stimulation of a brain region comprise of momentary changes in blood pressure, heart rate or cardiac output. However, physiological changes in liver, like changes of blood glucose levels, gluconeogenesis, glycogen metabolism, are much slower to observe than cardiovascular reflexes, and they are under strong control of endocrine system, which could be the second reason, as it is often difficult to differentiate whether the observed effect is through innervation or secondary to an endocrine effect. Nevertheless, control of hepatic function by sympathetic and parasympathetic innervation is well-established (Puschel, 2004) and NO-mediated changes in sympathetic or parasympathetic outflow would also have an impact on the liver function as well as cardiovascular system. To that matter, consequences of injury-induced changes of NO levels in brain would certainly not be limited to the brain tissue, but also would have an impact on the systemic responses of the body to a brain trauma.

In cardiac tissue, NO effects include reduction of noradrenalin (NA) release and facilitation of ACh release; the end effect is inhibition of cardiac responses to sympathetic nerve stimulation and facilitation of negative chronotropic action of vagal stimulation [reviewed in (Sears et al., 2004)]. In liver tissue, NO has been shown to play various roles in hepatic metabolism, either by its direct effects in hepatocellular function or indirectly through its vasodilator properties. NO (from iNOS) supports liver regeneration (Rai et al., 1998) and have apoptotic or anti-apoptotic effects in liver (Li and Billiar, 1999). Impact of brain trauma in NO changes in heart or liver remains unknown.

2. HYPOTHESIS and AIMS

Nitric oxide is involved in a plethora of physiological processes including neurotransmission, synaptic plasticity, modulation of sympathetic/parasympathetic signalling and immune responses, regulation of cardiovascular changes as well as cellular processes such as neurogenesis, cell viability and proliferation, cellular bioenergetics, mitochondrial damage and cell death. In view of its pleiotropic effects, disturbances of NO metabolism upon brain injury would have impact on a broad spectrum of physiological processes. In another point of view, a change in the dynamics of NO could as well be implemented in order to cope with the consequences of brain injury. Equally inclusive, these facts give NO a central role in the TBI pathophysiology, as it connects several physiological and pathophysiological processes to each other. In addition, as a free radical messenger, fate of NO depends tightly on the oxidative status of the microenvironment in which it is produced. Based on this approach, this project was specifically designed to elucidate the changes in NO levels in different regions of injured brain, and detrimental or beneficial roles of these changes in injury induced cerebral damage.

We hypothesized that NO dynamics and associated effects (detrimental or beneficial) differ in various regions of the injured brain.

To address this question, ***the first aim*** of the project was to quantify direct NO levels in rat brain, rather than its secondary derivatives, in the first 3 days following a traumatic brain injury and compare them to the basal NO levels. On this quest, lateral fluid percussion (LFP) injury was chosen since it provides not only a focal injury site, but also models the concussive damage to the brain regions that are, to some extent, distant to the injury core, which makes LFP superior to other focal injury models such as controlled cortical impact (CCI) or focal penetration injuries. On the grounds of the small-size of the craniotomy applied before TBI induction, it also provides partially increased intracranial pressure, although to a lesser extent than that is induced by closed head trauma models.

In line with its physiological relevance and current knowledge, a homogenously distributed NO over the brain is not presumable. The extent and impact of NO changes should be variable with 1) the basal levels of NO at a given brain sub region 2) the distance of the region with respect to the injury site. Therefore, elucidation of NO levels in distinct brain sub regions before and after TBI comprises ***the second aim*** of this project.

The third aim was to clarify whether any change in NO levels imposed by a brain trauma is influenced by the severity of the damage.

As ***the fourth aim***, expression of iNOS and nNOS were assessed in different brain regions after moderate or severe brain trauma in order to understand the source of possible changes in NO levels imposed by TBI.

Once the NO changes are determined, it is crucial to understand the biological significance of them. Therefore, ***the fifth aim*** of the study was to elucidate whether any NO changes observed after TBI is detrimental or beneficial. For this purpose, a complete profile of mitochondrial function was assessed in ipsilateral cortex and hippocampus by respirometric analyses using different substrates (pyruvate, glutamate and succinate) in order to determine the presence of a possible mitochondrial dysfunction and components of mitochondria that are potentially vulnerable to traumatic brain injury. By comparing these results to NO profiles, determination of any possible association of changes in post-TBI NO and those in mitochondrial function was aimed. In vitro NO application of healthy tissue homogenates was utilized to validate whether observed changes in mitochondrial function after TBI are implemented by NO or not. In addition, availability of nitrosative stress in different sub regions of the brain following trauma was assessed by fluorescent 3-nitrotyrosine immunostaining and the results were compared to those observed in healthy controls. Apoptotic and necroptotic cell death after TBI was compared to NO and mitochondrial function profiles.

Sixth, cortical and hippocampal gene expression of the neuroprotective and neurotrophic factors (LIF, NT4, BDNF) and gene expression of hypoxic signalling pathway components (HIF1α and its target GLUT1) were analysed after moderate and severe brain injury at the same time points as NO measurements, in order to address any correlation between them.

Finally, NO changes in liver and heart tissues were measured after moderate and severe TBI in order to analyse whether NO metabolism in injury-distant internal organs was responsive to an injury of the brain and to understand whether NO plays a role in brain-heart or brain-liver axes.

3. MATERIALS AND METHODS

3.1. Ethics Statement and Animal Husbandry

Care and use of animals were conducted in accordance with the ethical guidelines and all animal experiments were approved by the Bundesministerium für Wissenschaft, Forschung und Wirtschaft (BMWF-66.010/0011-II/3b/2013). Adult male Sprague Dawley rats were kept in standard conditions with free access to food and drink in a controlled environment with 12h:12h light-dark cycle, in animal facility of Biomedical Research Institute in Medical University of Graz.

3.2. Experimental Trauma Model

Lateral Fluid Percussion (LFP) on Sprague Dawley rats (McIntosh et al., 1989) was used as the trauma model. A pressure sensor connected to both fluid percussion device (AmScien Instruments, Richmond, VA, USA) and a computer, was used for pressure recordings, which in turn was used for standardization of the primary physical damage. A severity scale was used for classification of the TBI based on pressure values as previously defined (Molcanyi et al., 2007, Kraitsy et al., 2014). Mortality rates were ~13% and ~19% for moderate and severe groups, respectively.

Group Statistics of Animal Experiments																
Groups	***Experiments***	*Nitric Oxide (CX-HPC-CB)*				*PCR (CX-HPC)*				*Mitochondrial activity (CX-HPC)*			*Immunostaining (3NT & Casp3)*			
Time *post-TBI/surgery*		*4h*	*1d*	*2d*	*3d*	*4h*	*1d*	*2d*	*3d*	*4h*	*1d*	*3d*	*4h*	*1d*	*2d*	*3d*
Sham		8*	9*	9	9	5	5	5	5	6	6	6	-	-	-	-
mTBI		8*	9*	9	9	5	5	5	5	-	-	-	-	-	-	-
sTBI		8	9#	8#	9#	5	5	5	5*	6	6	6	3	3	3	3

Table 1: Number of animals per group and testing parameter.
CX: cerebral cortex; HPC: hippocampus; CB: cerebellum; mTBI: moderate traumatic brain injury; sTBI: severe traumatic brain injury. Mitochondrial activity measurements were performed only in animals with severe brain injury. **Slightly different group sizes #: Applies only to cortex* (See **Table Appendix 2**, **Table Appendix 3** and **Table Appendix 4** for detailed group statistics).

Animals were numbered independent of injury and time after TBI/surgery and researchers were blinded to the groups during assessment of NO levels, mitochondrial

activity, gene expression and immunostainings. Number of animals used for different experimental analyses are given in **Table 1.**

3.3. Surgery and Trauma Procedure

Sprague Dawley rats were operated under anaesthesia. (Anaesthetics mixture (2.5 ml/kg rat): Fentanyl (Jannsen-Cilag Pharma, Vienna, Austria), Midazolam (Erwo Pharma, Brunn am Gebirge, Austria) and Domitor (Orion Pharma, Espoo, Finland) in volume ratio of 2:2:1, injected intraperitoneal). Animals were well fixed on a stereotactic frame (David Kopf Instruments, Tujunga, CA, USA), where temperature stability was controlled with the help of a rectal thermometer and a heating pad. Surgical procedure was performed as described previously (Kraitsy et al., 2014). Briefly, a longitudinal incision was applied to the head skin and the skull was exposed. Two parietotemporal cranial holes with diameter of ~4 mm and 0.5 mm were opened at the right side of the skull. A 0.5 mm diameter screw was screwed into the small hole by 2-3 full turns. A luer lock was placed onto the large hole and fixed with the help of dental cement (Heraeus Kulzer, Hanau, Germany). The stability of the setup was checked with sterile physiological saline filled into the luer lock to ensure that there was no leakage. Dura was intact throughout the whole procedure. After completion of the setup, animals were reanimated (Reanimation mixture (1.5 ml/kg rat): Anexate (Roche Austria, Vienna, Austria), Antisedan (Orion Pharma) and physiological saline in volume ratio 8:1:3, injected subcutaneously). Trauma was set with a rapid and brief increase in pressure with fluid percussion, which leads to cortical-subcortical tissue deformation, subdural haematoma, cerebral haemorrhage and immediate swelling of brain tissue. Following trauma luer lock, screw and dental cement were removed; wound was stitched and animals were taken to the cages and kept under observation for 1-2 hour(s) with red light in order to prevent hypothermia. Animals were injected antibiotics on the day of surgery (0.15 mg/kg body weight Cefotaxime Sandoz (Sandoz, Kundl, Austria) in physiological saline, s.c.). Antibiotics medication was continued together with painkiller medication (2% (v/v) Rimadyl (Zoetis Austria, Vienna, Austria) in physiological saline, s.c.) under temporary anesthesia with isofluorane (AbbVie, Vienna, Austria) (~2 min, mixed with air 2 l/min), for maximal 2 days, depending on the sacrifice time. Sham animals received all the procedure including medications except craniotomy and trauma. Animals were sacrificed at 4 hours, 1 day, 2 days and 3 days post-TBI.

3.4. Sample Preparation for EPR Spectrometry

NO was quantified by diethyldithiocarbamate-iron (DETC-Fe) spin trapping and electron paramagnetic resonance spectrometry. At 3 hours, 1 day, 2 days and 3 days post-TBI time points, animals were anaesthetized with i.p. injection of anaesthetics mixture. 500 mg/kg body-weight DETC (Sigma-Aldrich, Steinheim am Albuch, Germany) in EPR grade water (Noxygen, Elzach, Germany) was injected s.c. to the neck. 5 min later Fe-Citrate complex was injected s.c. to the thigh (40 mg/kg body weight $Fe(II)SO_4 \cdot 7H_2O$ (Sigma-Aldrich) and 160 mg/kg body weight Citrate pH7.4 (Sigma-Aldrich) in EPR grade water). 60 min later animals were sacrificed by decapitation under anaesthesia; brain, liver, heart, blood tissues were removed. Liver, heart, cerebral cortex, hippocampus and cerebellum regions of the brain were dissected on ice. Ipsilateral and contralateral regions were dissected separately for each brain region (Eight different samples per animal). Tissues were chopped with the help of a scalpel, mixed homogenously and were filled into a 100 µl portion of a 1 ml disposable pipette so that each sample to be measured has a standard shape and size appropriate for EPR spectrometer (cylindrical shape; 3 mm diameter and 15 mm height; for hippocampus ~7,5 mm height due to small size). Samples were snap-frozen in liquid nitrogen and stored at -80°C until measurement.

3.5. Quantification of NO levels by EPR spectroscopy

Quantification of NO levels using EPR spectroscopy was conducted in Ludwig Boltzmann Institute of Clinical and Experimental Neurotraumatology by Dr. Adelheid Weidinger. Mononitrosyl iron complex (MNIC) spectra were determined by EPR spectroscopy of frozen tissue samples. Spectra were recorded at liquid nitrogen temperature with the *MiniScope* MS200 EPR spectrometer (Magnettech, Berlin, Germany). The EPR settings were as follows: microwave frequency, 9.431 GHz; modulation frequency, 100 kHz; microwave power, 10 mW and modulation amplitude, 5 G. In order to quantify NO concentration, the ferrous diethyldithiocarbamate ($Fe(DETC)_2$)-nitrosyl signals observed in tissue samples were compared with those obtained from $Fe(DETC)_2$-NO standards. Calibration was carried out with an excess of $Fe(DETC)_2$ (5 mM, Sigma-Aldrich, Vienna, Austria) and a range of 0 to 50 µM NO concentrations.

3.6. Assessment of mitochondrial respiration in injured brain tissue

Assessment of mitochondrial respiration in injured brain tissues was conducted at the Medical University of Graz with the help of Dr. Jamile Paier-Pourani from Ludwig Boltzmann Institute of Clinical and Experimental Neurotraumatology. Cortical and hippocampal tissues were removed at 4 hours, 1 day and 3 days post-FPI or sham surgery. Tissue homogenate (1mg tissue/30 µl buffer) was prepared in a buffer containing 0.25 M sucrose (Sigma-Aldrich), 10 mM TRIS (Sigma-Aldrich), 0.5 mM EDTA (Sigma-Aldrich) and 0.05% BSA (Sigma-Aldrich), pH 7.2. State 3 respiration was induced by addition of 2 mM ADP (Sigma-Aldrich) in the presence of either pyruvate (10 mM) or glutamate (10 mM) as substrates for Complex I. Then electron flow through Complex I was inhibited by rotenone (2 µM, Sigma-Aldrich) and succinate (10 mM, Sigma-Aldrich) was added to supply electrons through Complex II. Respiration rate at State 3 was determined by means of high-resolution respirometry (Oroboros Oxygraph-2K, Innsbruck, Austria) by measurement of oxygen consumption in measurement chambers. Results were presented as oxygen uptake per second per ml homogenate.

3.7. Exposure of tissue lysates to exogenous NO

Aqueous NO solution was prepared by bubbling compressed NO gas through the oxygen free distilled water. Freshly prepared healthy rat brain homogenates were placed in the chamber (2 ml) of high-resolution respirometer, 20 µL of saturated aqueous NO solution (1.8 mM) was added and incubated for 10 min. After 10 min mitochondrial function was determined as described in **Section 3.6**. These experiments were conducted in Ludwig Boltzmann Institute of Clinical and Experimental Neurotraumatology.

3.8. Gene Expression Analyses with RT-PCR

Animals were euthanized by decapitation with guillotine under deep isofluorane anaesthesia. Tissues were removed and snap frozen in liquid nitrogen and stored at -80°C for further analysis. Total RNA was isolated from frozen tissue with mirVana PARIS Kit (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. Following RNA quality control on 1.2% formaldehyde agarose gel electrophoresis, cDNA was synthesized from 200 ng total RNA depleted from small RNA (RNA larger than 200

nucleotide size) with Fermentas First Aid cDNA First Strand Synthesis Kit (Life Technologies) according to the manufacturer's instructions.

Amplicon sizes, primer sequences, annealing temperatures and cycle numbers are given in **Table 2**.

PCR conditions were as follows: first denaturation was at 95°C for 5 min; denaturation in cycle was at 95°C for 30 sec, annealing time was 30 sec (for annealing temperatures See **Table 2**) and extension was at 72°C for 30 sec. Final extension was at 72°C for 10 min.

PCR products were run on 2% agarose gel (1.5% for RIPK3) and densitometric analysis was done with Quantity One software (BioRad, Hercules, CA, USA). G6PDH was used as reference gene. Results are presented as signal intensity of the target amplicon normalized against the signal intensity of G6PDH amplicon.

Gene Name	Primer pair sequence (3´ – 5´)	Annealing Temp (°C)	Cycle number	Amplicon Size (bp)
nNOS	*GCATGCGGTGGACACCCTCC*	60	40	487
	GTTGGCAGGCGGTGCATCCT			
iNOS	*CCTTGTTCAGCTACGCCTTC*	60	38	179
	GGTATGCCCGAGTTCTTTCA			
LIF	*TACACAGCTCAAGGGGAACC*	60	40	400
	GGACCACCGCACTAATGACT			
NT4	*CACTGGCTCTCAGAATGCAA*	60	40	512
	GCCCCAGCTCATACATTGAT			
BDNF	*GGACAAGGCAACTTGGCC*	57	40	235
	CAGAGGAGGCTCCAAAGG			
HIF1α	*TGCAGCACGATCTCGGCGAA*	60	40	476
	TCATCGTCCTCCCCCGGCTT			
GLUT1	*GTCCGGCGGGAGACGCATAG*	64	38	154
	AGGGATCCGAGCACTGCCCC			
RIPK3	*ACCACTGAGCGAGCATCCTTCCA*	62	39	603
	AGTCTGCTAACTTGGCGTGGAGC			
G6PDH	*TGCTACTTGACATTCCTCCTCA*	60	40	160
	CTAGCTGCTGCTCTTACCTTCC			

Table 2: List of primers used in gene expression analyses.
Details of DNA primers used in gene expression analyses in cortex and hippocampus after brain trauma, their sequences, annealing temperatures and amplicon sizes and number of cycles used in PCR reactions. nNOS and iNOS, neuronal- and inducible nitric oxide synthases; LIF, leukemia inhibitory factor; NT4, neurotrophin-4; BDNF, brain-derived neurotrophic factor; HIF1α, hypoxia-inducible factor 1 alpha; GLUT1, glucose transporter 1; RIPK3, receptor-interacting protein kinase 3; G6PDH, glucose-6-phosphate dehydrogenase.

3.9. Tissue preparation for immunostainings

Animals for immunostainings were sacrificed with transcardial perfusion of 4% formaldehyde prepared from 37% stock (Merck, Darmstadt, Germany) in phosphate buffered saline (PBS, pH 7.4) following i.p. injection of 50mg/ml Thiopental (Sandoz) and

brains were removed and stored in 4% formalin overnight at 4°C. Following fixation, brains were incubated in 15% sucrose (in sterile PBS) overnight and tissue slices were prepared as frozen coronal sections in 10 µm thickness using Microm HM 560 M (Microm GmbH, Walldorf, Germany).

3.10. Immunostainings

Tissue sections were incubated in 4% formaldehyde solution (prepared from 37% stock) for post-fixation, and then washed three times in PBS (Sigma-Aldrich) for 5 min. After that, sections were incubated in 2N HCl (Merck) for 30 min and in borate buffer pH 9.0 for 6 min (50 mM BH_3O_3 (Sigma-Aldrich), 5 mM $Na_2B_4O_7$ x$10H_2O$ (Sigma-Aldrich), 10 mM Na_2SO_4 (Sigma-Aldrich)). They were three times washed in PBS for 2 min. For permeation, sections were incubated in 0.3% Triton X-100 (Sigma-Aldrich) in PBS (Sigma-Aldrich) for 30 min. Blocking was performed for 1 hour at room temperature (7% fetal bovine serum (FBS) (Sigma-Aldrich), 0.5% bovine serum albumin (BSA) (Sigma-Aldrich) in PBS) to avoid non-specific antibody binding. For 3-nitrotyrosine (3NT) detection, mouse monoclonal antibody anti-3NT (ab61392, clone 39B6, Abcam, Cambridge, UK) was used in 1:1000 dilution in block with overnight incubation at +4°C. Cleaved caspase-3 (Casp-3) was detected by a rabbit polyclonal anti-active caspase 3 (ab4051, Abcam) with a 1:100 dilution in block and incubation overnight at +4°C. For co-immunostainings a mixture of two antibodies with the same dilution factors was used. Secondary antibodies were 1:2000 goat anti-mouse immunoglobulin G (IgG) conjugated to Alexa568 dye (A11019, Life Technologies) or 1:200 goat polyclonal anti-rabbit IgG conjugated to Cy5 (ab6564, Abcam). For co-immunostainings a mixture of both antibodies with the same dilution factors was used. For counterstaining and mounting, an aqueous mounting media containing 4',6-diamidino-2-phenylindole (DAPI) (Fluoroshield with DAPI, Sigma-Aldrich) was utilized.

3.11. Microscopy

Microscopy was performed using Axio Imager 2.1 (Carl Zeiss AG, Oberkochen, Germany) installed with TissueFaxs Cell Analysis System (TissueGnostics GmbH, Vienna, Austria); or with Olympus BX51 (Olympus Corp., Tokyo, Japan) installed with an Olympus DP71 camera (Olympus). Images were taken either at 2.5X or 63X magnification.

3.12. Statistical Analysis

NO levels in brain sections of animals in the sham groups were comparable (**Figure 10**). Therefore all sham animals were pooled (n=32 for Cortex-ipsi; n=35 for each of the following groups: Cortex-contra, Cerebellum-ipsi, Cerebellum-contra) as previously described (Tomura et al., 2012, Oliva et al., 2012) and implemented in statistical analyses for the time course of the NO profile after TBI. During analyses of ipsi- and contralateral cortex, moderate and severe TBI groups were separately tested against the same sham group and presented in separate graphs for the sake of clarity and ease of interpretation. Statistical significance was assessed with one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD). Variance homogeneity was tested with Levene's Test for Equality of Variances. When variance heterogeneity was observed across the tested groups, Welch's variance-weighted ANOVA and Games-Howell post-hoc test were used. NO levels in liver and heart tissues were analysed the same way.

NO levels in hippocampal samples of sham animals had higher variance at different days, although not statistically significant. Therefore, for the time course analysis of moderate brain injury nitric oxide levels in this region, sham groups of different days were not pooled. Instead, NO levels in injured animals were compared to the sham controls at consistent time points using Mann-Whitney U Test.

For the analysis of severity-dependent NO levels at 4 h time point (ipsi- and contralateral hippocampus, ipsi- and contralateral cerebellum, liver and heart) only sham controls at 4 h time point was used. Statistical significance was assessed with one-way ANOVA followed by Tukey's HSD. Welch's variance-weighted ANOVA and Games-Howell post-hoc test were used, when significant variance heterogeneity was observed across the tested groups at Levene's Test.

Statistical significance of mitochondrial respiration results was assessed with one-way ANOVA followed by Fisher's least significant difference (LSD) test. Normality of variable distributions was confirmed by Kolmogorov-Smirnov test.

Mann-Whitney U Test was used for statistical assessment of mRNA expression levels in moderate or severe groups compared to the sham groups at consistent time points. SPSS (versions 19.0, 22.0 or 23.0) (IBM, NY, USA) was used for statistical analyses.

NO levels and mitochondrial activity were presented as box-whisker-plots, the interquartile range was represented as the box, the median value was represented by a bar in the box; whiskers represent minima and maxima. Gene expression results were presented as mean ± SEM (standard error of the mean) in bar charts.

4. RESULTS

Nitric oxide (NO) levels were measured by diethyl dithiocarbamate – iron (DETC-Fe) spin trapping and electron paramagnetic resonance (EPR) spectrometry in brain, liver and heart tissues after sham surgery or traumatic brain injury by lateral fluid percussion (LFP). NO forms a mononitrosyl iron complex (MNIC) with the DETC-Fe without losing its unpaired electron, thereby making it possible to quantify it in a magnetic field in EPR. DETC-Fe spin trapping was the best suited approach for the requirements of our study since it 1) allows the direct quantification of NO, rather than the secondary products (superior to Griess reaction based assays), 2) overcomes the time problem of short half-life of NO by trapping NO in a stable product, 3) offers high selectivity, as MNIC has a unique spectral signature in EPR spectroscopy 4) allows enhanced spatial resolution; it is possible to measure NO levels in various brain sub regions and different organs from the same animal, and finally 5) it allows quantification of basal NO levels (superior to microelectrode based methods).

Sources of NO production in injured brain tissues were assessed by gene expression analyses of nitric oxide synthases (NOSs). Possible detrimental and beneficial effects of NO in injured brain was analysed by associating measured NO levels with analyses of mitochondrial dysfunction, neuroprotective gene expression, nitrosative stress and apoptotic or necroptotic cell death. Finally, NO levels in liver and heart were measured to assess whether NO metabolism in other organs could play a role in systemic response after a unilateral injury of brain. A scheme for experimental design is given in **Figure 9**.

4.1. Dynamics of NO in Brain

NO levels were quantified at 4 hours, 1 day, 2 days and 3 days post-TBI using spin trapping and EPR spectrometry. (For group statistics see **Table Appendix 2**). NO levels were measured and quantified in cerebral cortex, hippocampus and cerebellum of sham controls and moderately and severely brain injured animals. Ipsilateral and contralateral portions were analysed separately for each region.

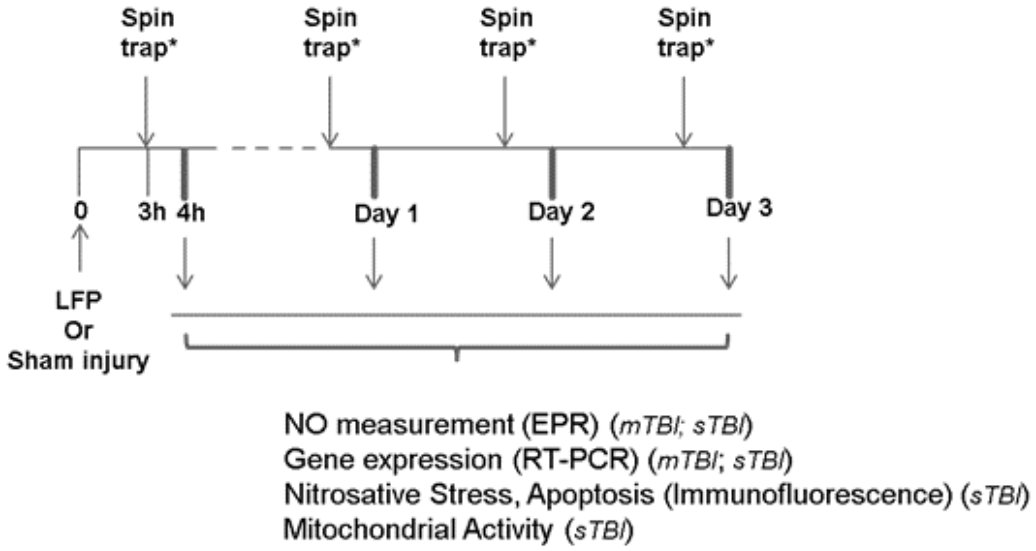

Figure 9: Schematic presentation of the experimental study schedule.
Following induction of trauma by lateral fluid percussion (LFP) injury, animals were euthanized at 4h, and 1, 2, or 3 days after traumatic brain injury (TBI) for analysis. Different animals were used for different experimental analyses. *Spin trap (diethyldithiocarbamate-iron [DETC-Fe]) administration was only applied to the animals used for nitric oxide (NO) quantification, under stable anesthesia, 1 h before euthanasia.

There was not any significant difference among the sham controls at any of the time points analysed (**Figure 10**). Therefore, they were pooled as one large sham group and statistical significance of the possible differences in injured animals were assessed in comparison to this large sham group (For adjustment of statistical methods, See **Section 3.12**).

4.1.1. Basal Levels of NO in Brain

Although NO has been subjected to extensive research for its various roles in nervous system, basal levels of NO in different brain regions remained undetermined. Our results indicated average NO levels to differ significantly in brain sub regions. Among the regions analysed, hippocampus showed the lowest NO concentration (0.26 ±0.03 μM), while cortex showed NO concentrations almost double as hippocampus (0.44 ±0.04 μM). Cerebellum had the highest basal levels of NO among the three regions (1.24 ±0.08 μM). These results have been published elsewhere as an original article (Ucal et al., 2016).

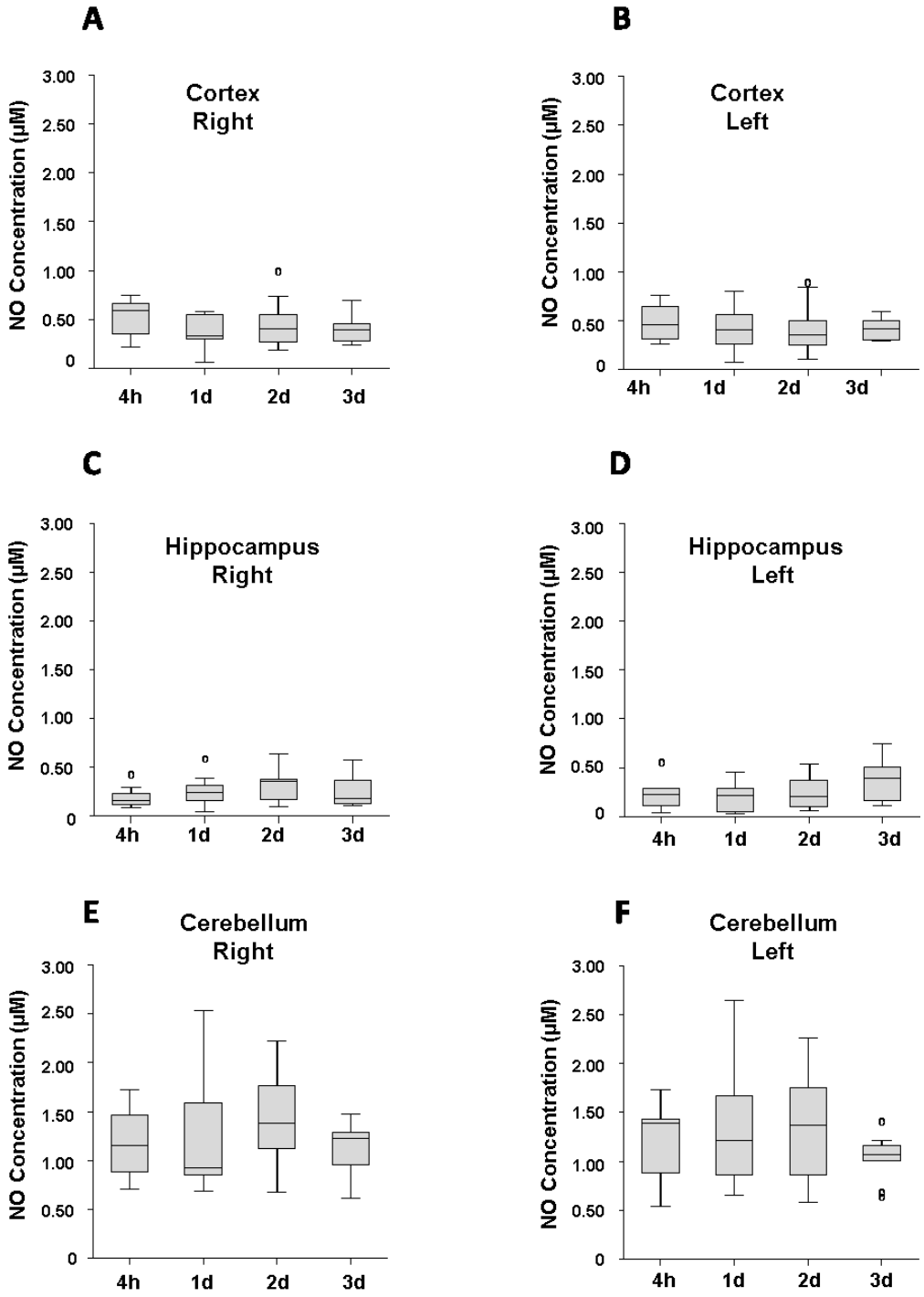

Figure 10: NO levels in brain sub regions of sham controls.
Nitric oxide (NO) levels determined by diethyldithiocarbamate-iron (DETC-Fe) spin trapping and electron paramagnetic resonance (EPR) spectrometry in cortical (**A, B**), hippocampal (**C, D**), and cerebellar (**E, F**) tissues of sham controls at 4 h and 1, 2, and 3 days post-surgery. There was no significant change in NO levels in either the ipsilateral or contralateral portions at different time points. However, the differences of NO levels among different brain regions were remarkable. Statistical significance was tested by one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test. Open circles show outliers.

4.1.2. Changes in Brain NO Levels Following Traumatic Brain Injury

4.1.2.1. Cerebral Cortex

In the ipsilateral cortex of moderately injured animals, remarkable increase in NO levels was observed (**Figure 11A**). However, the effect was statistically not significant (F[4,20]=2.516, $p=0.073$). The increase of NO levels at 4h following trauma was noteworthy, doubled in average as compared to sham controls (**moderate:** 0.87 ±0.12 µM; **sham** 0.44 ±0.04 µM). Within 24h following moderate TBI, NO levels were reduced to

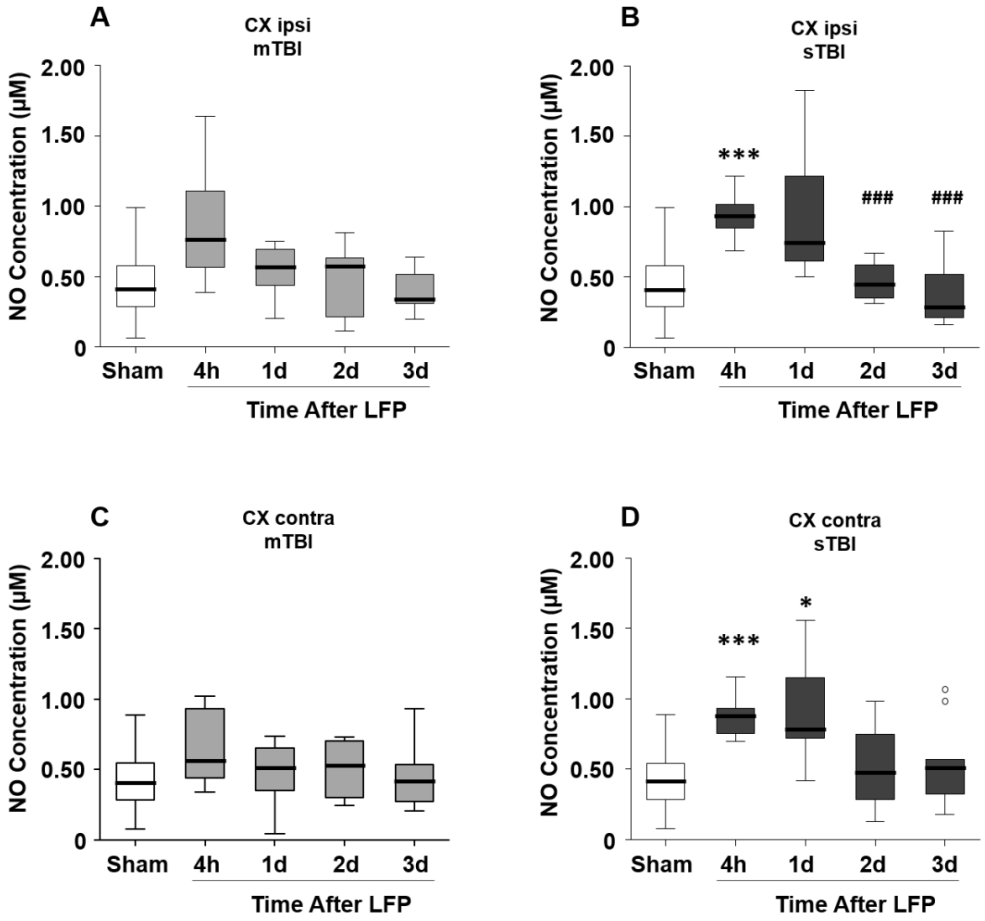

Figure 11: NO levels in cerebral cortex following TBI.
Changes in nitric oxide (NO) levels in the cerebral cortex at the ipsilateral (**A, B**) and contralateral sides (**C, D**) at 4 h, and 1, 2, and 3 days following moderate (**grey boxes**) and severe brain injuries (**black boxes**) determined by diethyldithiocarbamate-iron (DETC-Fe) spin trapping and electron paramagnetic resonance (EPR) spectrometry. A significant increase in NO levels was detected in the ipsilateral cortex at 4 h, but sustained up to 2 days in the severe group. Statistical significance was tested with one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test or Welch's variance-weighted ANOVA followed by Games–Howell test. *$p<0.05$, ***$p<0.001$ compared with sham group. ###$p<0.001$ compared to 4 h post traumatic brain injury (TBI) group. Open circles show outliers. For number of animals see Table 1. CX, cortex; mTBI, moderate traumatic brain injury; sTBI, severe traumatic brain injury; LFP, lateral fluid percussion injury.

basal levels and remained low up to 3d following injury. In severely injured animals, on the other hand, NO levels were significantly differed from the control group (F[4,20]=15.781, $p=6.2x10^{-6}$). Post-hoc analysis revealed that ipsilateral cortical NO levels were significantly higher at 4h (0.94 ±0.06 μM, $p=3.1x10^{-5}$) as compared to sham controls (0.44 ±0.04 μM). Average NO levels at 1 day post-TBI were comparable to those measured at 4h, however the inter-animal variance was higher and the effect was not statistically significant (0.96 ±0.17 μM, $p=0.089$) (**Figure 11B**). NO levels at day 2 (0.47 ±0.05 μM) and day 3 after severe TBI (0.39 ±0.08 μM) were comparable to sham controls and significantly lower than 4h ($p=1.61x10^{-4}$, $p=6.09x10^{-4}$ for 2d and 3d, respectively, in comparison to 4h).

In the contralateral cortex, similar profiles were observed. A non-significant change in contralateral cortical NO levels was observed in moderately injured animals (F[4,66]=1.738, $p=0.152$) with a noticeable increase at 4h (**moderate:** 0.65 ±0.08; **sham:** 0.44 ±0.03). Within 24h following moderate TBI, NO levels were reduced to basal levels and remained low up to 3d following injury (**Figure 11C**). In severely injured animals, however, significant changes in NO levels were observed (F[4,64]=8.968, $p=8.2x10^{-6}$). The profile of NO changes were similar to the ipsilateral side (**4h** 0.87 ±0.05 μM, $p=4.81x10^{-4}$; **1d** 0.90 ±0.13 μM, $p=0.92x10^{-4}$ as compared to **sham** 0.44 ±0.03 μM) (**Figure 11D**). At 2d (0.52 ±0.10 μM) and 3d (0.53 ±0.10 μM), NO levels were comparable to sham controls.

These results indicate that a unilateral damage of cerebral cortex induces elevated production of NO and both the timing of the elevation and the affected distance is increased proportionate to increase in severity of the damage. On the other hand, the magnitude of NO elevation after severe brain injury was comparable to those after moderate injury, ~2-fold compared to basal levels. These results have been published elsewhere as an original article (Ucal et al., 2016).

4.1.2.2. Hippocampus and Cerebellum

In order to analyse a possible association in post-TBI NO changes and the distance of cerebral regions with respect to the injury site, NO levels in hippocampus and cerebellum were quantified.

In the hippocampus, the profile of changes in NO levels in hippocampus after moderate TBI was similar to that of cortical tissues – a transient increase was observed at 4h after moderate TBI, albeit restricted to the ipsilateral side (**Figure Appendix 1**). Therefore, further analyses were focused on the 4h critical time point.

In the ipsilateral hippocampus, a significant increase in NO levels was induced by LFP injury within 4h, independent of injury severity (F[2,24]=8.507; $p=0.0016$) (**Figure 12A**, **moderate**: 0.38 ±0.05 µM, $p=0.0013$, **severe:** 0.33 ±0.09 µM, $p=0.034$ as compared to **sham:** 0.19 ±0.04 µM). In the contralateral hippocampus, however, significant change in NO levels was observed in response to a severe but not moderate injury (F[2,24]=3.386; $p=0.05$) (**Figure 12B**; **moderate**: 0.28 ±0.03 µM, *not significant*; **severe**: 0.38 ±0.04 µM, $p=0.045$ as compared to **sham:** 0.23 ±0.05 µM).

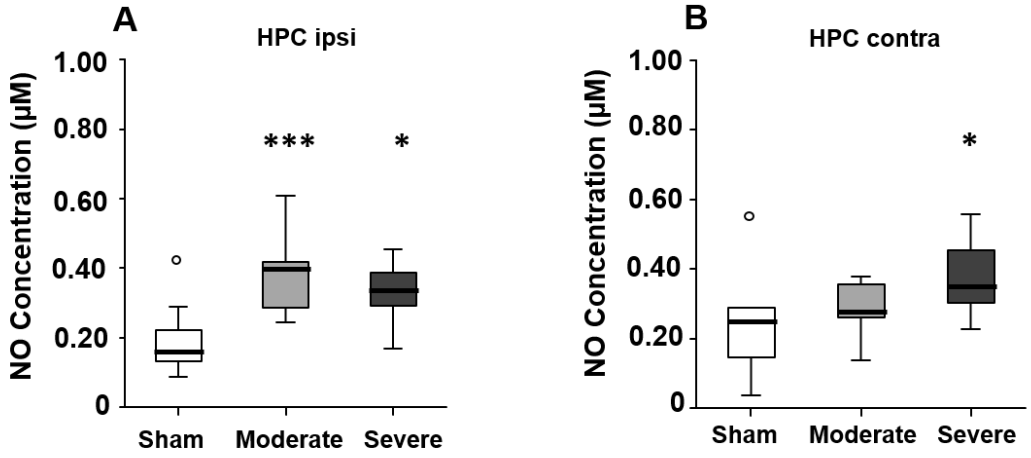

Figure 12: NO levels in hippocampus following moderate TBI.
Changes in nitric oxide (NO) levels in the hippocampus at the ipsilateral (**A**) and contralateral sides (**B**) at 4 h following moderate (**grey boxes**) and severe brain injuries (**black boxes**) determined by diethyldithiocarbamate-iron (DETC-Fe) spin trapping and electron paramagnetic resonance (EPR) spectrometry. A significant increase in NO levels was detected in the ipsilateral hippocampus at 4 h, independent of injury severity, while in the contralateral side a similar increase was only observed in severely injured animals. Statistical significance was tested with one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test. *$p<0.05$, ***$p<0.001$ compared with sham group. Open circles show outliers. For number of animals see Table 1. HPC, hippocampus.

In the cerebellum, the most distal sub region with respect to the cortical injury site, NO levels were comparable to the sham controls at 4h, 1 day, 2 days and 3 days after

moderate TBI (**ipsilateral** $F[4,18]=0.482$, $p=0.749$; **contralateral** $F[4,65]=0.736$, $p=0.571$) (**Figure Appendix 2**). However, a severity-dependent analysis of NO levels at 4h after TBI showed significant changes in both ipsi- and contralateral cerebellar hemispheres (**ipsilateral**: $F[2,24]=4.979$; $p=0.016$ and **contralateral**: $F[2,24]=6.680$; $p=0.0049$). Severe TBI induced significant increases in NO levels of both cerebellar hemispheres (**Figure 13A, B**; **ipsilateral**: 2.08 ±0.17 µM, $p=0.016$; **contralateral**: 1.96 ±0.13 µM, $p=0.007$) as compared to sham controls (**right:** 1.23 ±0.13 µM; **left:** 1.22 ±0.12 µM). A comparison of severely injured group to moderately injured group also revealed remarkable and significant differences in ipsi- ($p=0.058$) and contralateral ($p=0.014$) cerebellum, respectively, at 4h after TBI (**ipsilateral:** 1.41 ±0.24 µM; **contralateral:** 1.30 ±0.18 µM after moderate TBI).

These results indicate that severe injury was able to trigger NO elevations in all three regions of the brain bilaterally, regardless of their distance to the injury site, whereas the effect was limited to the injured cortex and the closest region, the ipsilateral hippocampus, in moderate injury.

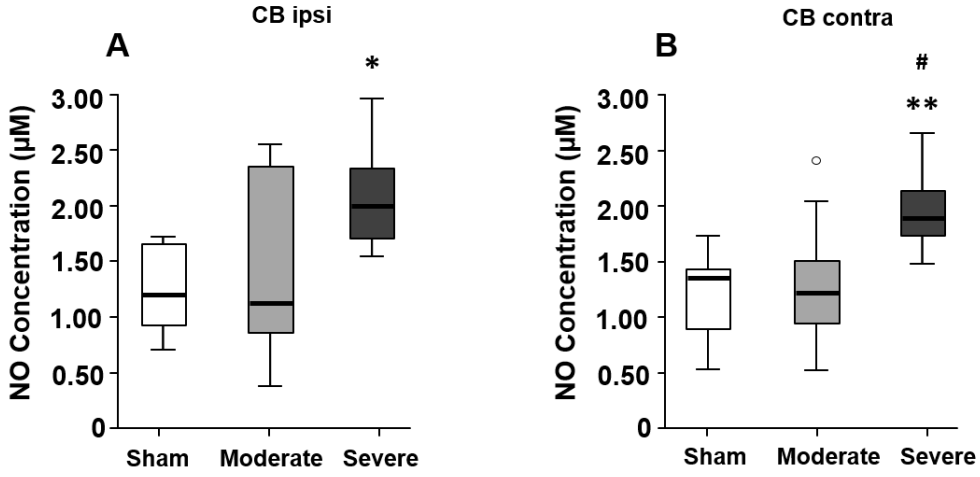

Figure 13: NO levels in cerebellum following severe TBI.
Changes in nitric oxide (NO) levels in the cerebellum at the ipsilateral (**A**) and contralateral sides (**B**) at 4 h following moderate (**grey boxes**) and severe brain injuries (**black boxes**) determined by diethyldithiocarbamate-iron (DETC-Fe) spin trapping and electron paramagnetic resonance (EPR) spectrometry. A significant increase in NO levels was detected in both ipsi- and contralateral cerebellum at 4 h following severe, but not moderate brain injury. Statistical significance was tested with one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test. *$p<0.05$, **$p<0.01$ compared with sham controls; #$p<0.05$ compared with moderately injured group. Open circles show outliers. For number of animals see Table 1. CB, cerebellum.

Fold Change in NO levels at 4h after severe TBI			
Region	**Cortex**	**Hippocampus**	**Cerebellum**
Ipsilateral	1.83	1.74	1.76
Contralateral	1.80	1.63	1.62

Table 3: Fold increase of NO levels in brain following severe TBI.
Fold changes of NO levels in the cortex, hippocampus and cerebellum at the ipsilateral and contralateral sides at 4 h following severe brain injury were comparable. Similar increase in NO levels preserved the regional differences observed at basal conditions.

There was a considerable difference among the NO levels in the cortex, hippocampus, and cerebellum (0.44 ±0.04 µM; 0.26 ±0.03 µM; 1.24 ±0.08 µM, respectively). However, within 4h of a unilateral severe injury of cortex, fold increase in NO levels in these regions were comparable, preserving the regional differences (**Table 3**).

These observations underline that trauma severity regulated the intensity of modulation independent of the distance of the respective regions from the injury site. The overall NO concentration (basal and trauma induced), on the other hand, seemed to be determined by the regional microenvironment (**Figure 14**). These results have been published elsewhere as an original article (Ucal et al., 2016).

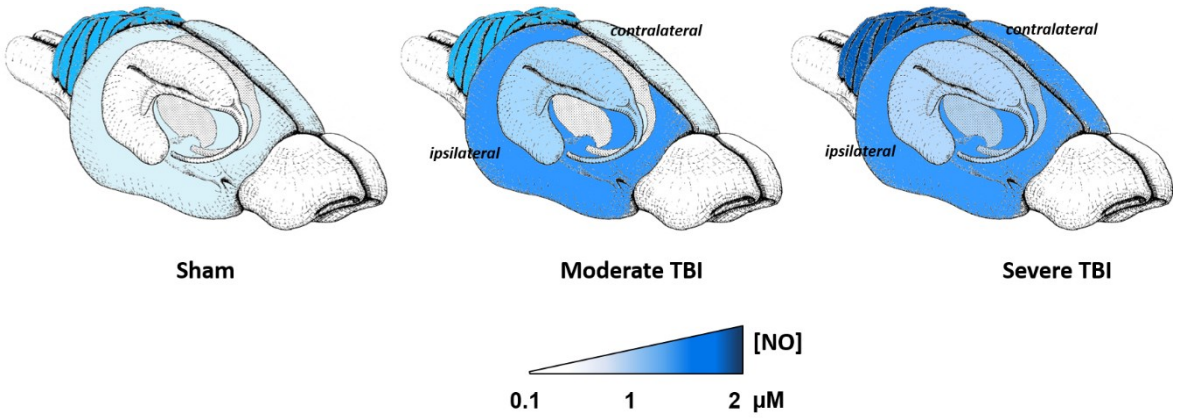

Figure 14: Schematic representation of changes in NO levels induced by TBI at 4 h.
A unilateral moderate injury of the cerebral cortex induced increases in NO levels of cortex and hippocampus ipsilateral to the injury while the contralateral regions and the cerebellum remained unaffected. A severe TBI, however, induced NO increases in all three regions regardless of their distance to the injury site. Brain pictures were adapted from (Cheung and Cardinal, 2005), copyright (2016), with permission from BioMed Central (for permission see Appendix C).

4.2. Expression of NOS isoforms after moderate and severe TBI

Quantitative analyses of NO levels in different brain sub regions provided insights into the changes of NO metabolism in response to a unilateral trauma of cerebral cortex. Among three different NOS isoforms, neuronal NOS is responsible for NO production in CNS under physiological conditions, while iNOS is synthesized in response to injury or inflammatory conditions in various tissues and known to be responsible for elevated NO production. For further understanding of the details of trauma induced NO changes in brain, expression of these two NOS isoforms, nNOS and iNOS, were analysed in respective tissues.

4.2.1. NOS expression in cortex

Induction of a TBI induced increases in mRNA expression of iNOS, inducible isoform of nitric oxide synthase, in the ipsilateral cortex at 4h post-TBI (**Figure 15A**), corresponding to the increase in NO levels. iNOS expression was comparable in moderate or severe groups, although severe TBI induced changes were below the significance cut-off level (**moderate** 0.54 ±0.20, *p=0.032*; **severe** 0.56 ±0.20, *p= 0.056* as compared to **sham** 0.08 ±0.02). Within 1 day following injury, iNOS mRNA expression returned to the basal levels. In contralateral cortex, on the other hand, there was no change in mRNA expression of iNOS at 4h regardless of the injury severity (**Figure 15B**), despite high levels of NO at the same time point in this region following particularly severe TBI. There was no change in expression of neuronal isoform, nNOS, in either of the cerebral hemispheres of cortex regardless of the injury severity (**Figure 15C, D**). These results suggest iNOS as the primary source of NO elevations in the ipsi-, but not in the contralateral cortex. These results have been published elsewhere as an original article (Ucal et al., 2016).

4.2.2. NOS expression in hippocampus

Similar to the observations in cortex, hippocampal expression of iNOS showed a transient increase at 4h following a moderate or severe injury in the ipsilateral side (**moderate** 0.30 ±0.10, *p=0.008*; **severe** 0.60 ±0.26, *p= 0.008* as compared to **sham** 0.04 ±0.01) (**Figure 16A**). Contralateral hippocampus, on the other hand, did not show any significant changes in iNOS mRNA expression (**Figure 16B**). nNOS mRNA expression remained unchanged at both ipsi- and contralateral hippocampal regions (**Figure 16C, D**).

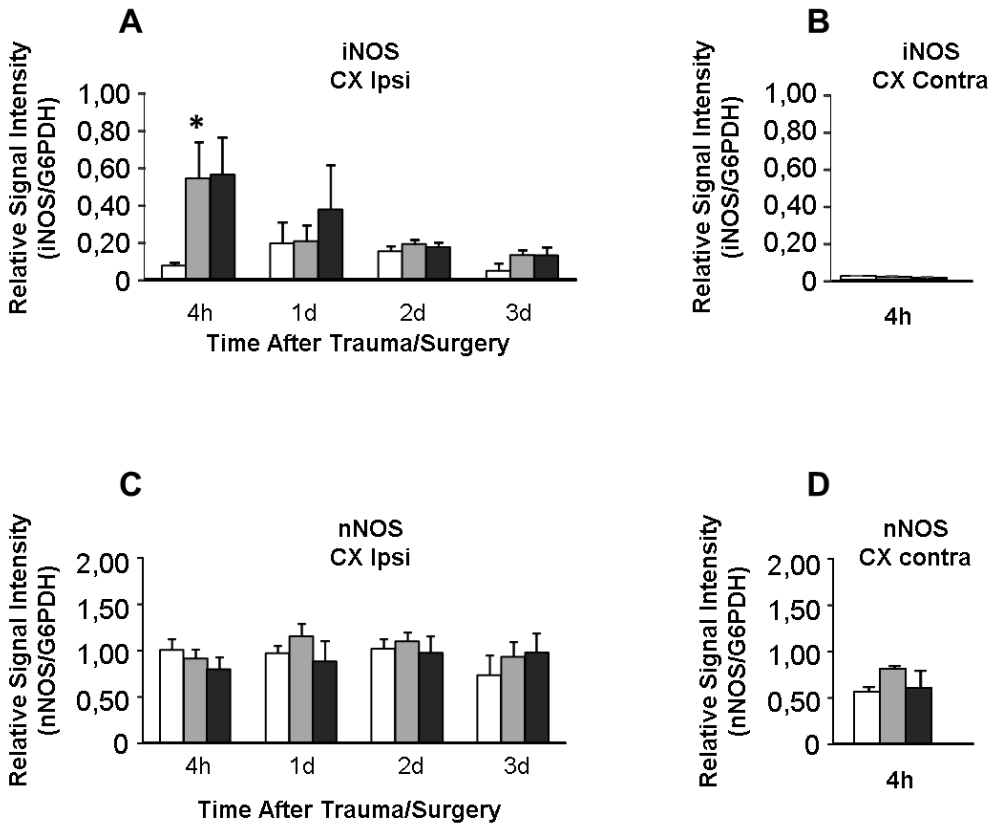

Figure 15: Expression of NOSs in cerebral cortex after TBI.
mRNA expression levels of inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS) in the ipsilateral cortex (CX) (**A, C**), and in the contralateral cortex (**B, D**) at 4 h and 1, 2, and 3 days after traumatic brain injury (TBI), determined by reverse transcription polymerase chain reaction (RT-PCR). There was a significant increase in iNOS, but not in nNOS expression at 4 h, consistent with nitric oxide (NO) elevation at the same time point. Glucose-6-phosphate dehydrogenase (G6PDH) was used as the reference gene. Data are presented as relative signal intensity of product bands on agarose gel. White columns show sham controls; grey columns show moderate TBI groups and black columns show severe TBI groups. Statistical significance of changes compared with the sham group was tested with the Mann–Whitney U test. *$p<0.05$ compared with the sham group at a consistent time point.

It should be noted though, absence of an increase in *de novo* synthesis of nNOS in either of the hemispheres in both cortex and hippocampus, does not necessarily exclude a possible increase in activity of resident enzymes in these tissues. Altogether these results indicate iNOS to be the primarily responsible source for the post traumatic increase in NO levels in the ipsi- but not contralateral regions. These results have been published elsewhere as an original article (Ucal et al., 2016).

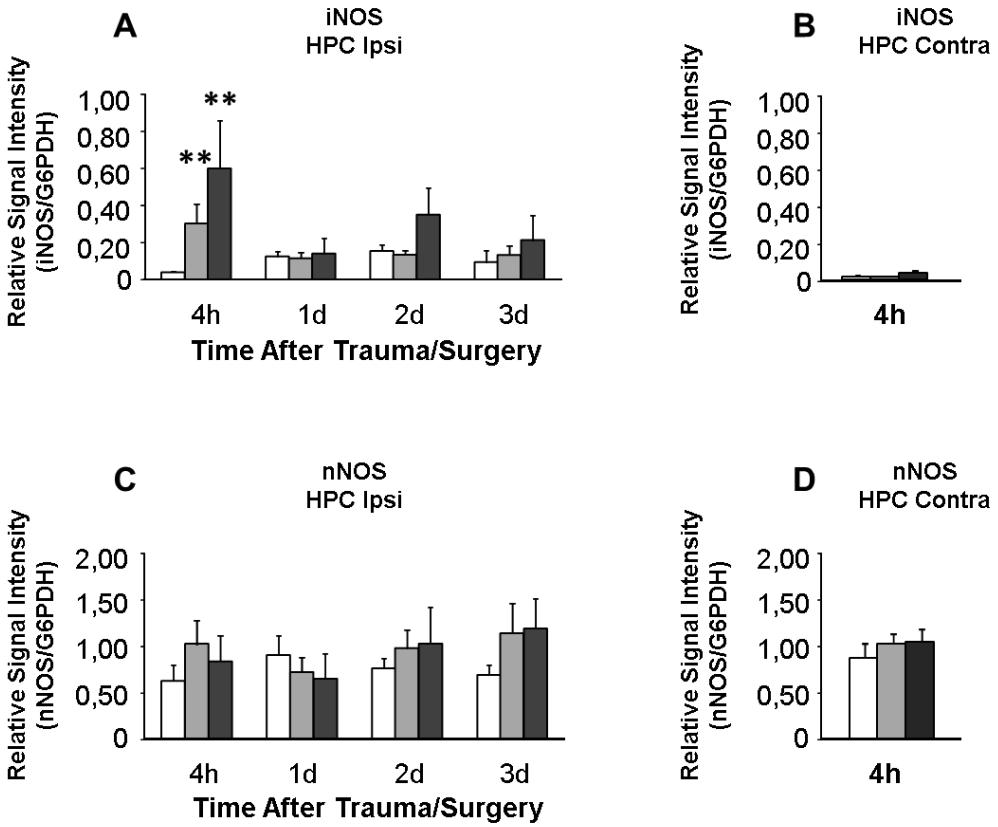

Figure 16: Expression of NOSs in hippocampus following TBI.
mRNA expression levels of inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS) in the ipsilateral hippocampus (HPC) (**A, C**), and in the contralateral hippocampus (**B, D**) at 4 h and 1, 2, and 3 days after traumatic brain injury (TBI), determined by reverse transcription polymerase chain reaction (RT-PCR). There was a significant increase in iNOS, but not in nNOS expression at 4 h, consistent with nitric oxide (NO) elevation at the same time point. Glucose-6-phosphate dehydrogenase (G6PDH) was used as the reference gene. Data are presented as relative signal intensity of product bands on agarose gel. White columns show sham controls; grey columns show moderate TBI groups and black columns show severe TBI groups. Statistical significance of changes compared with the sham group was tested with the Mann–Whitney U test. ***p<0.01* compared with the sham group at a consistent time point.

4.3. NO and secondary damage

NO has frequently been implicated in secondary damage following a CNS injury. In order to assess whether these NO levels were associated to the secondary damage in injured brain we investigated nitrosative stress, mitochondrial dysfunction and cell death in consistent time points after severe TBI.

4.3.1. Tyrosine nitration after TBI

Tyrosine nitration is one of the hallmarks of the peroxynitrite mediated oxidative damage (Radi, 2004). Peroxynitrite is produced by a diffusion limited reaction of NO and O_2^-, and it is a much more powerful oxidizing reagent than both NO and O_2^-. Peroxynitrite targets tyrosine residues to irreversibly modify them by transfer of a nitro group, resulting in 3-nitrotyrosine (3NT) formation. Therefore, 3NT positivity was investigated in injured brain by immunofluorescent staining in order to assess whether measured levels of NO elevations are associated to detrimental effects or not. 3NT staining was analysed following severe brain injury, since the most significant changes in NO levels were observed in severe TBI groups.

Although NO elevations after TBI were transient (4h to 1 day), there was a persistent 3NT formation at the ipsilateral cortex close to the lesion site following severe brain injury (**Figure 17**). 3NT was not detected in the healthy brain (**Figure 17a**). As early as 4h post-LFP, nitrotyrosine positivity was already detectable but the signal was confined to some capillary-like structures close to the lesion (**Figure 17b, c**). However, at day 1 and day 2 after injury, the entire region adjacent to the lesion site was 3NT positive while injury-distant regions did not show any 3NT staining (**Figure 17d-g**). The number of 3NT positive cells was notably reduced at day 3 and were comparable to injury-distant cortical regions (**Figure 17h, i**).

In contralateral cortex, 3NT formation was not detected at any time point, apart from some capillaries (**Figure Appendix 3**), despite significant NO elevations in this region as high as ipsilateral cortex. Additionally, 3NT signals were not detected in hippocampus (**Figure Appendix 4**) or cerebellum (**data not shown**), although NO levels were doubled in these regions as well at 4h after severe TBI. Taken together, these findings indicate that lack of NO-related detrimental outcomes, like nitrosative stress, in distant regions after LFP injury was not due to the lack of NO bioavailability, but rather should be dependent on other factors as well.

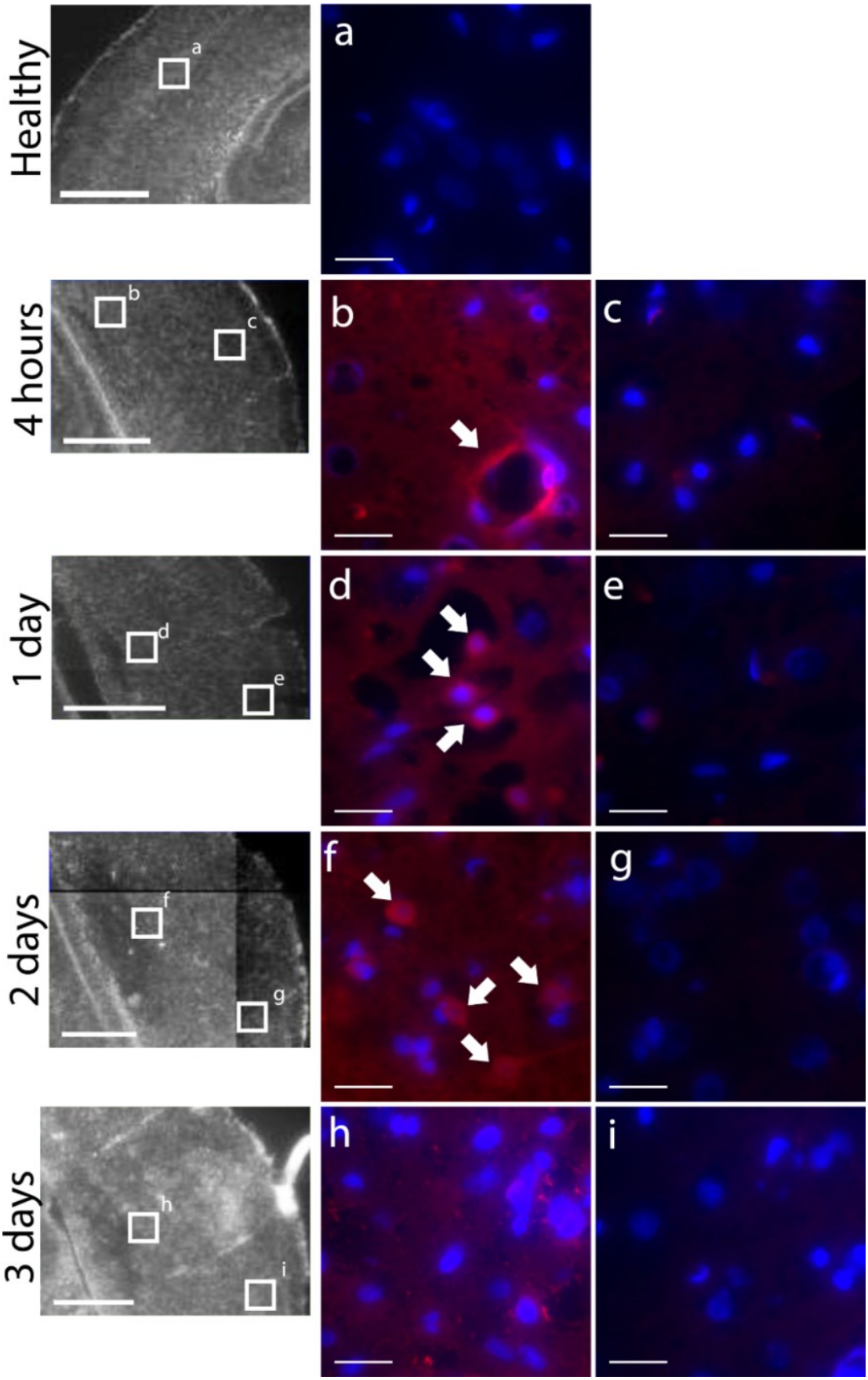
Healthy
4 hours
1 day
2 days
3 days
a
b
c
d
e
f
g
h
i

Figure 17: Tyrosine nitration in ipsilateral cortex following severe TBI.
3-nitrotyrosine (3NT) levels in cortical (ipsilateral to the injury) sections of healthy (**a**) and severely injured animals at 4 h, and 1, 2, and 3 days after traumatic brain injury (TBI), in regions close to (**b, d, f, h**) or away from the lesion site (**c, e, g, i**). Note that high 3NT levels in the injury vicinity were persistent from 1 to 2 days post-TBI. Arrows indicate 3NT-positive cells. Cell nuclei were stained with DAPI for counterstaining. Note that low magnification, overview pictures were taken with DAPI channel only to provide an anatomical location for the regions visualized with high resolution pictures. Scale bars: 10 μm in (**a–i**); 500 μm in overview pictures. These results have been published elsewhere as an original article (Ucal et al., 2016).

4.3.2. Mitochondrial Dysfunction after LFP and the Role of NO

NO and the associated generation of peroxynitrite have been implicated to impair mitochondrial respiration (Davis et al., 2010, Werner and Engelhard, 2007). Several in vitro studies pointed to a role for NO in respiratory inhibition through targeting several mitochondrial proteins (**See Section 1.2.3.1**). Therefore, in our settings post-TBI mitochondrial respiration was assessed in order to check whether a post-TBI mitochondrial dysfunction was associated to elevated NO levels and tyrosine nitration. Severe brain injury and the ipsilateral regions were used for the respiration analyses since the most significant changes in NO levels and tyrosine nitration were observed in this group.

For this purpose, Complex I and Complex II activities were analysed in the cortex and hippocampus after TBI. Substrate dependent state 3 rates of oxygen consumption was measured with high resolution respirometry in homogenates of ipsilateral cortex and hippocampus at 4h, 1 day and 3 days following severe injury by LFP. Pyruvate or glutamate was used as substrates feeding electrons to the Complex I and succinate was used to assess activity of Complex II.

At 4h post TBI a modest but significant reduction in pyruvate dependent state 3 respiration rate was observed in ipsilateral cortex of injured animals as compared to sham controls ($p<0.05$) (**Figure 18A**). In glutamate-dependent state 3 respiration rate, on the other hand, there was a more pronounced reduction at 4h (~50% of sham controls) ($p<0.001$) (**Figure 18B**). State 3 oxygen consumption rates with both substrates were comparable to sham levels within 1 day following injury, reflecting the transient nature of mitochondrial dysfunction after LFP (**pyruvate 1d:** $p<0.05$; **glutamate 1d and 2d**: $p<0.001$, compared with 4h post-TBI counterparts). Succinate-dependent respiration rates, on the other hand, remain unchanged through all time points analysed (**Figure 18C**), excluding a deficiency in mitochondrial function related with Complex II in our settings.

It should be noted that in the ipsilateral hippocampus there was no change in mitochondrial function at any time point with any of the substrates analysed (**data not shown**), consistent with the lack of a tyrosine nitration in hippocampus. These results have been published elsewhere as an original article (Ucal et al., 2016).

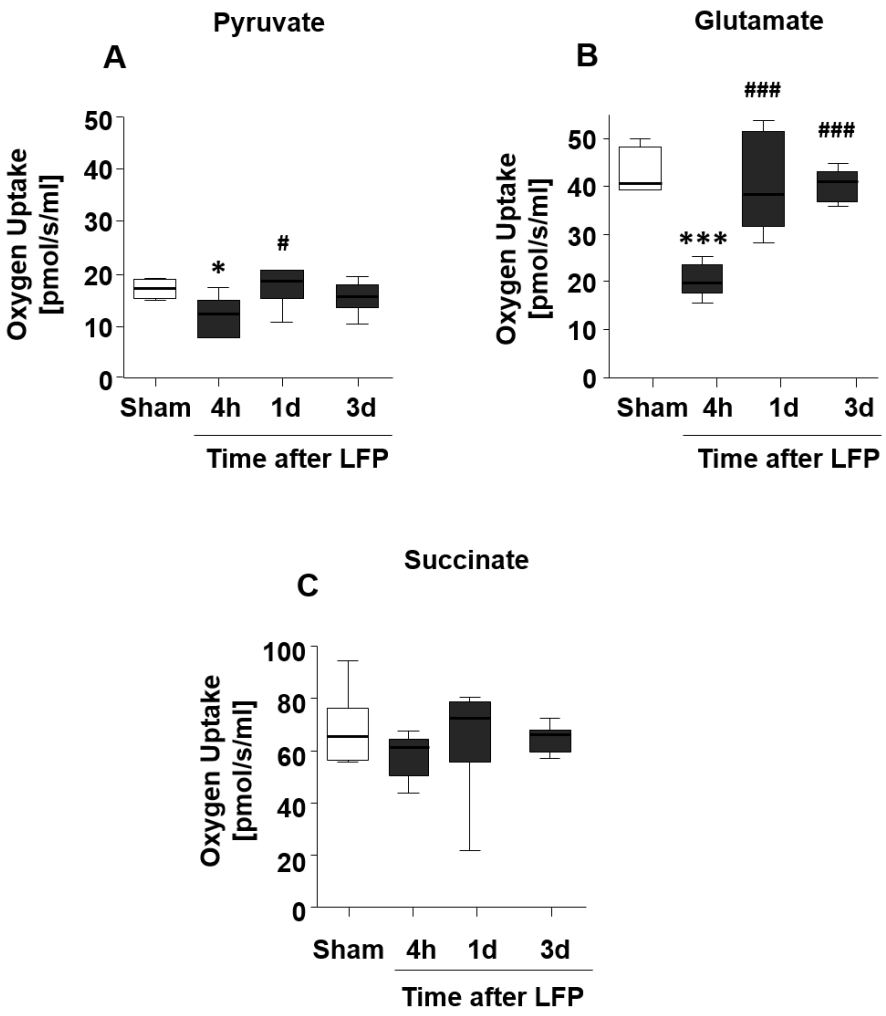

Figure 18: Mitochondrial dysfunction in ipsilateral cortex after TBI.
Substrate dependent State 3 respiration rates in the ipsilateral cortex after severe brain injury by fluid percussion. Pyruvate (**A**) and glutamate (**B**) were used as substrates for mitochondrial complex I, succinate (**C**) was used as substrate for complex II activity. Note that significant reductions in complex I activity at 4 h, when the highest NO levels were observed (**A, B**), together with complex II activity being stable over time (**C**). Statistical significance of the differences was tested with analysis of variance (ANOVA) followed by post-hoc least significant difference (LSD) test. *$p<0.05$, ***$p<0.001$ compared with sham; #$p<0.05$, ###$p<0.001$ compared with 4 h post-TBI.

Impaired utilization of pyruvate and glutamate in mitochondrial respiration after severe TBI in the ipsilateral cortex was isochronous to the NO elevations, suggesting the two mechanisms could be related. To assess whether there is such a relation, homogenates of cerebral cortex from healthy animals were exposed to aqueous NO solution (~20 μM; for 10 min). Surprisingly, a significant reduction in glutamate-dependent state 3 respiration rate was observed, similar to our observations in severe TBI animal cortical homogenates

(**Figure 19A**). Pyruvate-dependent state 3 respiration rates, however, remained unaffected when cortical homogenates of healthy animals were exposed to NO *in vitro* (**Figure 19B**). Together, these findings support the possibility that post-TBI impairment of mitochondrial glutamate utilization could be mediated by NO. Further, these results also suggest that glutamate node in mitochondrial respiration appears to be more vulnerable than the pyruvate utilization in response to TBI-induced changes in brain NO levels.

Succinate-dependent state 3 respiration rates of cortical homogenates exposed to aqueous NO were comparable to control levels (**Figure 19C**), in line with previous findings obtained after LFP (**Figure 18C**).

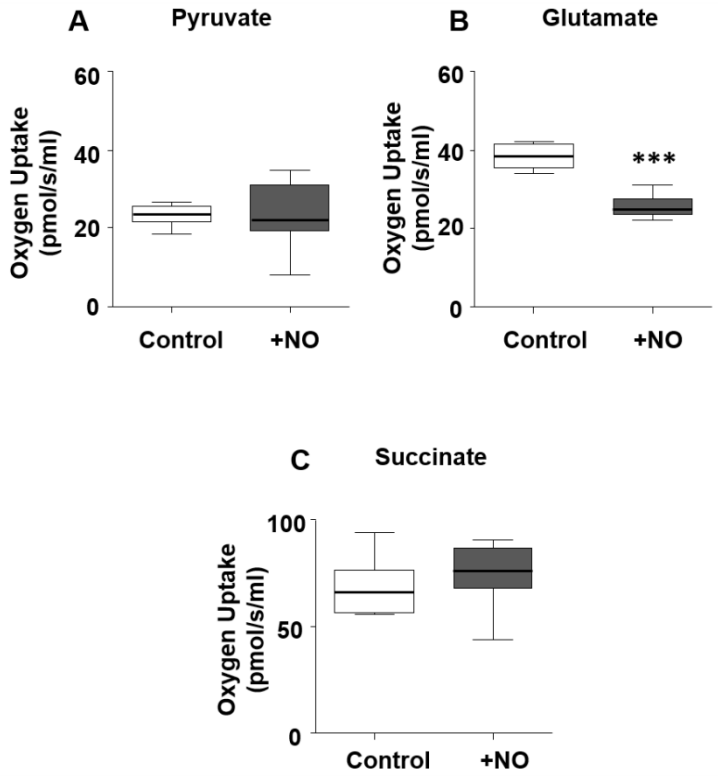

Figure 19: Mitochondrial dysfunction in healthy cortical tissue exposed to NO.
Substrate dependent State 3 respiration rates in the healthy cerebral cortical homogenates and upon exposure to 20 μM aqueous NO for 10 min. Pyruvate (**A**) and glutamate (**B**) were used as substrates for mitochondrial complex I, succinate (**C**) was used as substrate for complex II activity. Note that the utilization of glutamate in mitochondrial respiration was significantly reduced upon exposure to NO, while pyruvate and succinate dependent State 3 respiration rates were not affected. Statistical significance of the differences was tested with analysis of variance (ANOVA) followed by post-hoc least significant difference (LSD) test. ****p<0.001* compared with sham.

4.3.3. Post traumatic cell death and role of NO

Secondary damage contributes to the progression of brain injury by contributing to loss of neural cells in the post-acute phase. Both apoptosis and programmed necrosis have been shown to mediate delayed cell loss after brain injury (Werner and Engelhard, 2007, Rink et al., 1995). Various studies reported a role for NO or nitrosative stress in either type of cell death (Mitrovic et al., 1995, Uchiyama et al., 2002, Borutaite and Brown, 2003).

4.3.3.1. RIPK3 Dependent Necroptosis

High levels of NO/nitrosative stress and resulting mitochondrial dysfunction has been shown to lead to RIPK3-mediated necroptosis (Davis et al., 2010). Therefore, potential activation of programmed necrosis to contribute delayed cell death in injured brain was examined in our settings by RIPK3 expression analysis at consistent time points.

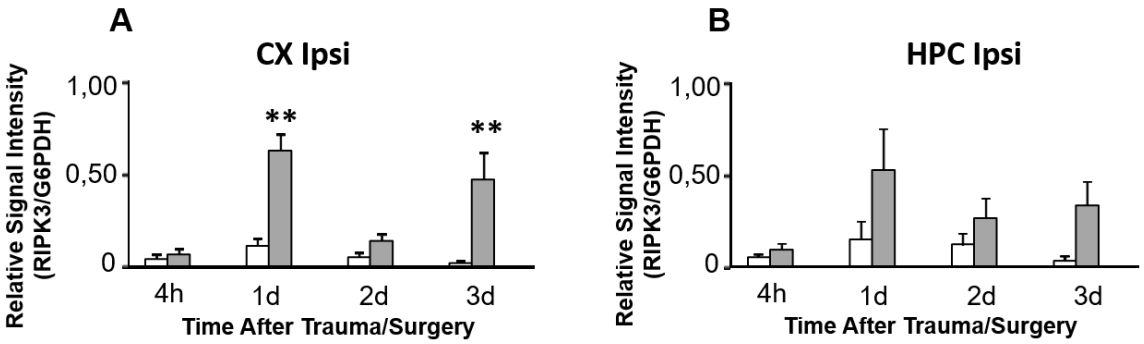

Figure 20: RIPK3 Expression following Moderate TBI.
mRNA expression levels of receptor interacting protein kinase 3 (RIPK3) in the ipsilateral cortex (CX) (**A**) and hippocampus (HPC) (**B**) at 4 h and 1, 2, and 3 days after moderate traumatic brain injury (TBI), determined by reverse transcription polymerase chain reaction (RT-PCR). A biphasic change was observed in CX with significant increases at 1 and 3 days, while the changes in hippocampus was not statistically significant although remarkable. Glucose-6-phosphate dehydrogenase (G6PDH) was used as the reference gene. Data are presented as relative signal intensity of product bands on agarose gel. White columns show sham controls; grey columns show moderate TBI groups. Statistical significance of changes compared with the sham group was tested with the Mann–Whitney U test. ***p<0.01* compared with the sham group at a consistent time point.

There was a drastic increase in expression of RIPK3 within 1 day after moderate TBI in the ipsilateral cortex (0.63 ±0.09, *p=0.008*) as compared to the sham group (0.11 ±0.04) (**Figure 20A**), followed by a reduction at day 2 to the levels comparable to sham (**moderate** 0.14 ±0.03, **sham** 0.05 ±0.02). At day 3, there was a second phase of increase

in RIPK3 expression (**moderate** 0.48 ±0.14, **sham** 0.02 ±0.01, *p=0.008*). Hippocampal expression of RIPK3 showed a biphasic pattern similar to cortex, but the inter-animal variances were higher for the RIPK3 changes in hippocampus, thus the differences were not statistically significant at any time point analysed (**Figure 20B**).

Severely injured animals, however, did not show a biphasic, but rather a steady increase in RIPK3 expression in both the ipsilateral cortex and hippocampus (**Figure 21**). With severe TBI, cortical RIPK3 expression increased significantly at day 2 (**severe** 0.37 ±0.06, **sham** 0.05 ±0.02, *p=0.008*) and sustained at day 3 post-TBI (**severe** 0.49 ±0.12, **sham** 0.02 ±0.01, *p=0.016*) (**Figure 21A**). These results have been published elsewhere as an original article (Ucal et al., 2016). Hippocampal expression showed a similar pattern as cortex, but the increase was statistically significant only at day 3 (**severe** 0.34 ±0.14, **sham** 0.03 ±0.02, *p=0.032*) (**Figure 21B**).

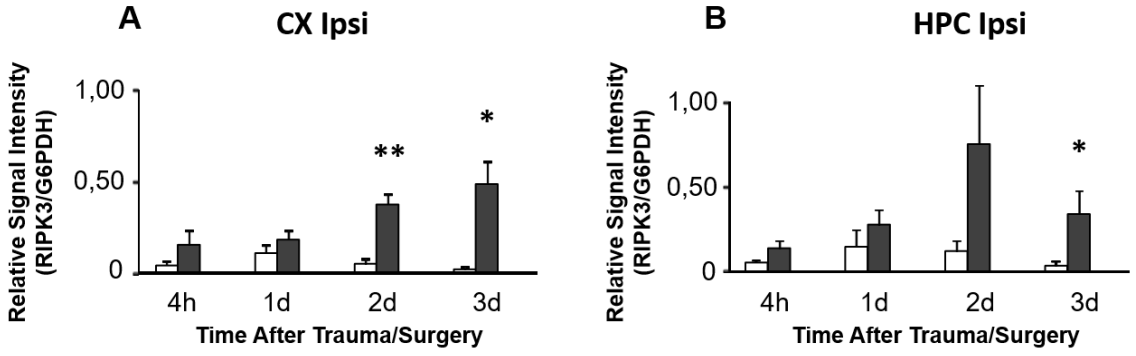

Figure 21: RIPK3 Expression following Severe TBI.
mRNA expression levels of receptor interacting protein kinase 3 (RIPK3) in the ipsilateral cortex (CX) (**A**) and hippocampus (HPC) (**B**) at 4 h and 1, 2, and 3 days after severe traumatic brain injury (TBI), determined by reverse transcription polymerase chain reaction (RT-PCR). Significant increases were observed at 2 and 3 days. Glucose-6-phosphate dehydrogenase (G6PDH) was used as the reference gene. Data are presented as relative signal intensity of product bands on agarose gel. White columns show sham controls; black columns show severe TBI groups. Statistical significance of changes compared with the sham group was tested with the Mann–Whitney U test. *$p<0.05$ and **$p<0.01$ compared with the sham group at a consistent time point.

Notably, the increase in RIPK3 expression was more prominent in cortex than in hippocampus, independent of the severity of injury, which is consistent with observations regarding NO levels, nitrosative stress and mitochondrial dysfunction. Together, these findings support a role for post-traumatic NO in functional activation of necroptosis after

brain trauma through mitochondrial dysfunction and elevated nitrosative stress. The differences in the RIPK3 expression profiles between moderate and severe TBI (biphasic vs steady increase over time) and underlying mechanisms need to be revealed with further analyses.

4.3.3.2. Tyrosine Nitration and Apoptotic Cell Death after FPI

In order to assess a possible association of post-TBI NO/nitrosative stress to secondary apoptotic cell death in our settings, coronal brain sections of rats after severe TBI were co-immunostained for tyrosine nitration and caspase-3 (Casp-3) activation at consistent time points following LFP injury (**Figure 22a-t**). In the ipsilateral cortex at 4h, when NO levels were already doubled, there was no detectable increase in Casp-3 activation (**Figure 22c, g**). Casp-3 immunoreactivity gradually increased from day 1 to day 3 post-TBI (**Figure 22k, o, s**), in regions close to the lesion site. In detail, at day 1 there was mainly 3NT positive cells but only a few cells were Casp-3 or double positive (**Figure 22i-l**). At day 2, however, many cells positive for both 3NT and a strong Casp-3 staining located in the nuclei were observed (**Figure 22m-p**); suggesting persistent tyrosine nitration might activate apoptotic cell death. At day 3 following severe TBI, the number of 3NT positive cells were greatly reduced while Casp-3 positivity remained (**Figure 22q-t**). It should be noted, however, 3NT-positive cells from day 1 to 3 were not always apoptotic, consistent with our findings regarding the RIPK3 mediated necrotic cells loss. There was not any detectable Casp-3 activity or tyrosine nitration in the contralateral cortex at any time point (**Figure Appendix 3**).

These results indicated that programmed necrotic cell death or apoptosis could be induced by persistent nitrosative stress at the injury site, which in turn contribute to delayed cell loss in injured brain after TBI. These results have been published elsewhere as an original article (Ucal et al., 2016).

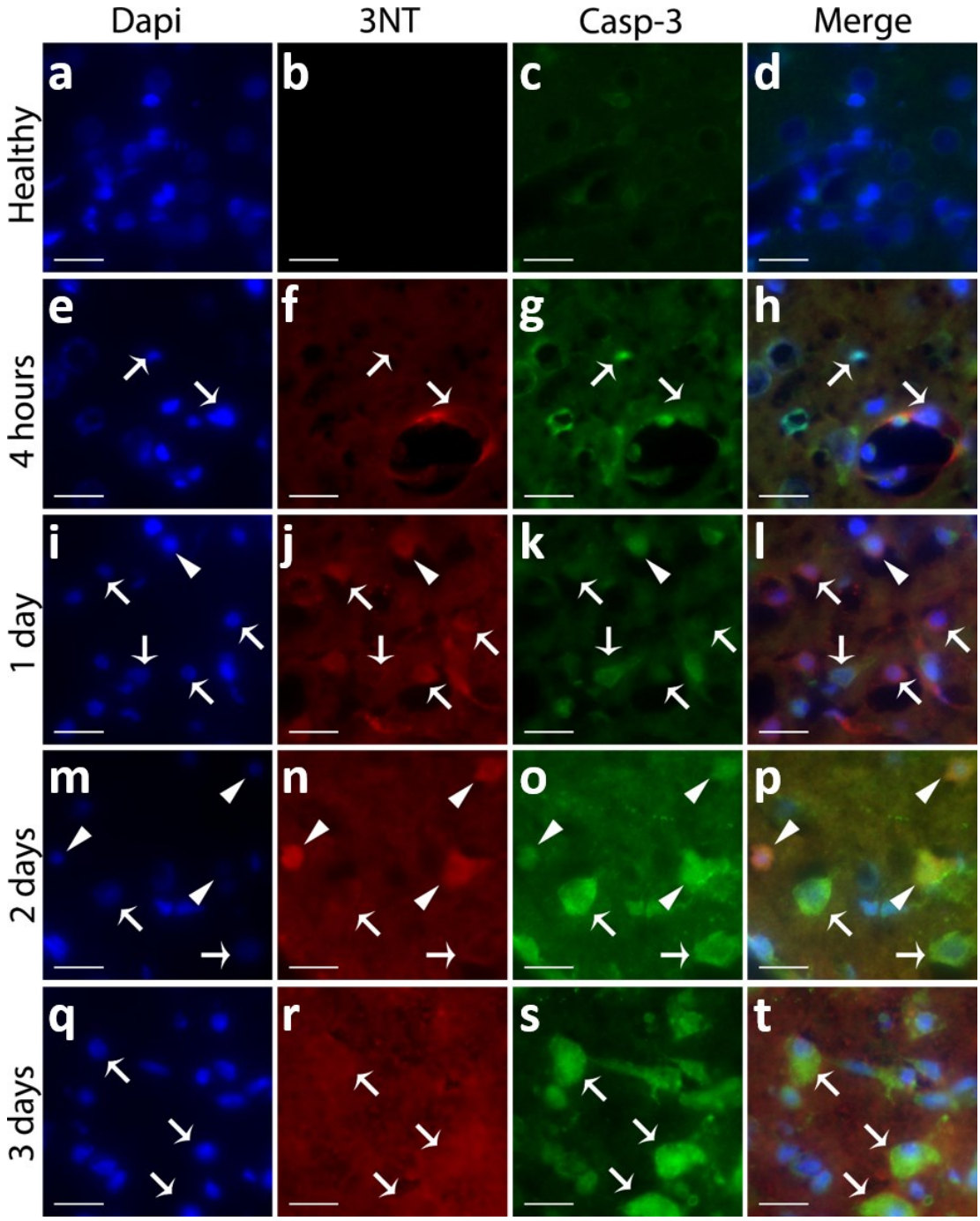

Figure 22: 3NT and Casp3 co-immunostaining following severe TBI.
Co-immunostaining of cortical sections of healthy (**a–d**) and severely injured animals (**e-t**) for 3-nitrotyrosine (3NT) and cleaved caspase (Casp)-3 at 4 h (**e-h**), 1 day (**i-l**), 2 days (**m-p**), and 3 days (**q-t**) after severe traumatic brain injury (TBI) (ipsilateral to injury). Note the presence of both single- and double-positive cells, indicating a possible but not obligate association between nitrosative stress and apoptotic cell loss after TBI. Arrows indicate single-positive cells (either for 3NT or Casp-3); arrowheads indicate double-positive cells. Cell nuclei were stained with DAPI for counterstaining. Scale bars: 10 µm.

4.4. Gene Expression in Brain after TBI

In order to assess whether NO levels observed after TBI could be associated to activation of specific signalling mechanisms, mRNA expression of a number of genes were analysed after moderate and severe TBI. Expression of neuroprotective/neurotrophic (LIF, NT4 and BDNF) and hypoxic signalling (HIF1α, GLUT1) genes were analysed at consistent time points following LFP injury.

4.4.1. Neuroprotective/Neurotrophic Gene Expression after TBI

Leukaemia inhibitory factor (LIF) has been shown to be involved in regulation of neural stem and progenitor cell proliferation, and promotion of neuronal survival (Bauer and Patterson, 2006, Oshima et al., 2007). NO has been shown to be a downstream mediator of such effect by LIF in adult olfactory neuronal progenitors (Lopez-Arenas et al., 2012) and in a mouse model of cerebral infarct (Huang et al., 2012). Cortical expression of LIF was significantly higher in severely injured group at 4h post-TBI (1.13 ±0.10, *p=0.016*) as compared to sham controls (0.57 ±0.10). Average levels of LIF expression in moderate group (1.15 ±0.22) was comparable to severe group, however the increase was not statistically significant compared to sham controls (*p=0.151*). At 1d after TBI significant increases in LIF expression was observed independent of injury severity (**moderate** 0.92 ±0.18, *p=0.032*; **severe** 1.18 ±0.29, *p=0.008*) as compared to sham controls (0.43 ±0.06) (**Figure 23A**). At day 2 and 3, however, cortical LIF expression returned to basal levels. It should be noted that LIF increase and NO elevations in injured cortex correspond to similar time points. In ipsilateral hippocampus, on the other hand, LIF expression appeared to be repressed from day 1 to day 3 (**Figure 23B**) and the decrease was statistically significant at day 3 in the moderately injured group (**moderate** 0.52 ±0.10, *p=0.016*) as compared to sham controls (1.46 ±0.29).

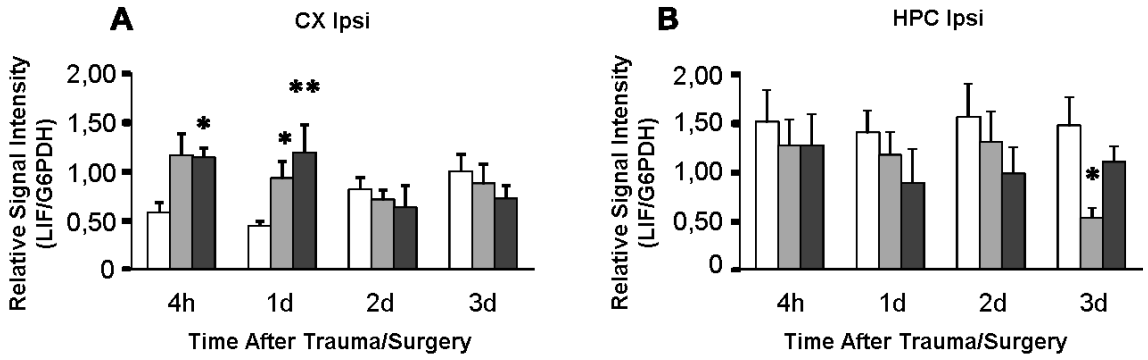

Figure 23: LIF Expression in the cortex and hippocampus following TBI.
mRNA expression levels of leukaemia inhibitory factor (LIF) in the ipsilateral cortex (CX) (**A**) and hippocampus (HPC) (**B**) at 4 h and 1, 2, and 3 days after traumatic brain injury (TBI), determined by reverse transcription polymerase chain reaction (RT-PCR). Significant increases were observed at 4 h and 1 day in cortex, followed by reduction to sham levels, while a tendency to severity-dependent decrease over time was observed in hippocampus. Glucose-6-phosphate dehydrogenase (G6PDH) was used as the reference gene. Data are presented as relative signal intensity of product bands on agarose gel. White columns show sham controls; grey columns show moderate TBI groups; black columns show severe TBI groups. Statistical significance of changes compared with the sham group was tested with the Mann–Whitney U test. *$p<0.05$ and **$p<0.01$ compared with the sham group at a consistent time point.

Various studies have shown that neurotrophic factors such as neurotrophin 4 (NT4) or brain-derived neurotrophic factor (BDNF) promotes neuronal survival via NO activity. Cortical expression of NT4 did not show any significant change as compared to sham controls (**Figure 24A**). Hippocampus, on the other hand, showed remarkable reductions in NT4 expression in a severity-dependent manner (**Figure 24B**). Moderate TBI led to decrease in expression at each time point analysed, although the reduction was statistically significant only at day 3 (**moderate** 0.30 ±0.09, **sham** 1.43 ±0.18, *p=0.008*). Upon severe TBI, however, the reduction in NT4 expression was more prominent than the moderate groups from 4h to 2 days post-TBI (**Figure 24B**). Hippocampal NT4 expressions were significantly lower at day 2 (0.24 ±0.13, *p=0.016*) and day 3 (0.75 ±0.13, *p=0.016*) after severe TBI compared with sham controls (1.43 ±0.18).

There were no major modulations in BDNF expression either in the cortex or hippocampus at any time point as compared to the sham controls, independent of injury severity (**Figure 25A, B**).

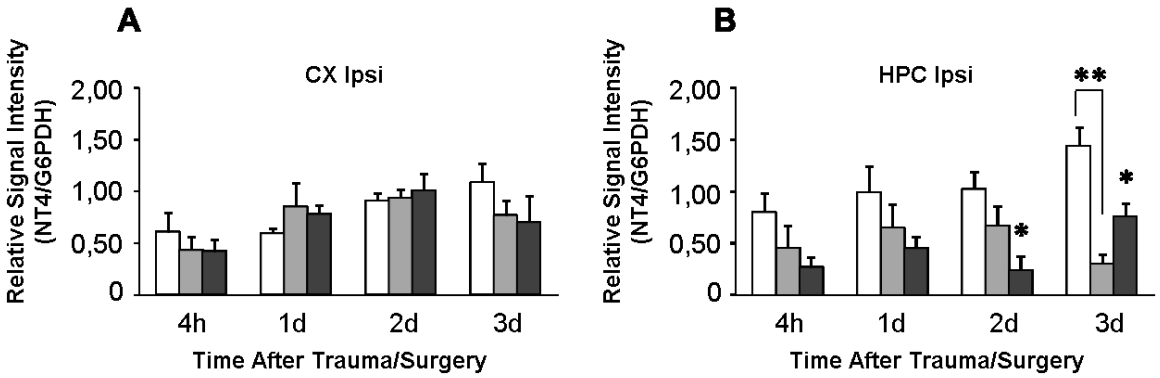

Figure 24: NT4 expression in the cortex and hippocampus following TBI.
mRNA expression levels of neurotrophin-4 (NT4) in the ipsilateral cortex (CX) (**A**) and hippocampus (HPC) (**B**) at 4 h and 1, 2, and 3 days after traumatic brain injury (TBI), determined by reverse transcription polymerase chain reaction (RT-PCR). A severity-dependent repression was observed in hippocampus, while cortical NT4 levels did not change after TBI compared with sham controls. Glucose-6-phosphate dehydrogenase (G6PDH) was used as the reference gene. Data are presented as relative signal intensity of product bands on agarose gel. White columns show sham controls; grey columns show moderate TBI groups; black columns show severe TBI groups. Statistical significance of changes compared with the sham group was tested with the Mann–Whitney U test. *$p<0.05$ and **$p<0.01$ compared with the sham group at a consistent time point.

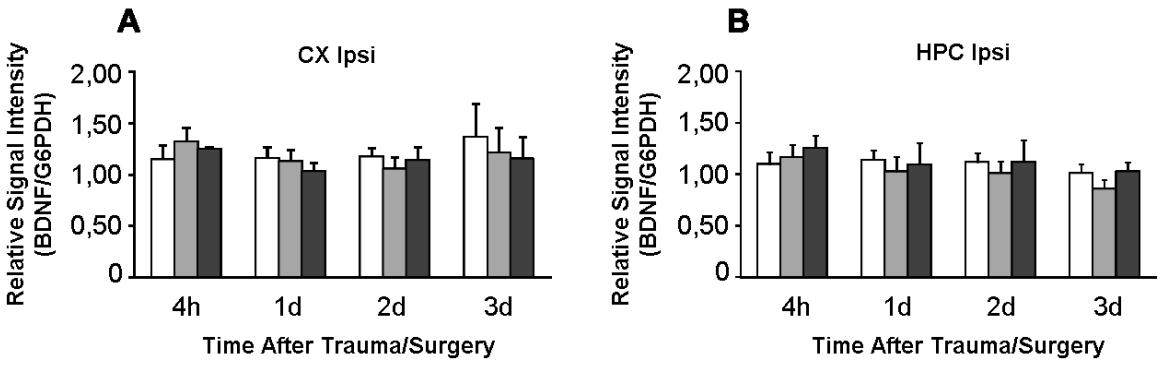

Figure 25: BDNF Expression in the cortex and hippocampus following TBI.
mRNA expression levels of brain-derived neurotrophic factor (BDNF) in the ipsilateral cortex (CX) (**A**) and hippocampus (HPC) (**B**) at 4 h and 1, 2, and 3 days after traumatic brain injury (TBI), determined by reverse transcription polymerase chain reaction (RT-PCR). Neither in cortex nor in hippocampus a detectable change was observed. Glucose-6-phosphate dehydrogenase (G6PDH) was used as the reference gene. Data are presented as relative signal intensity of product bands on agarose gel. White columns show sham controls; grey columns show moderate TBI groups; black columns show severe TBI groups. Statistical significance of changes compared with the sham group was tested with the Mann–Whitney U test.

4.4.2. Hypoxic Signalling after TBI

Hypoxic insult is one of the important components of secondary brain damage after TBI and NO has been shown to regulate hypoxic signalling mediated by HIF1α. Therefore, mRNA expression of HIF1α was analysed early after LFP. HIF1α mRNA expression did not show any difference at any time point after TBI either in cortex or hippocampus (**Figure 26**). Next, mRNA expression of GLUT1, a target gene of HIF1α was checked. Cortical expression of GLUT1 did not show any changes after TBI at any time point analysed (**Figure 27A**). In hippocampus, there was a significant increase in GLUT1 expression in moderately injured group at 3 days post-TBI (0.95 ±0.11, *p=0.032*) as compared to sham controls (0.58 ±0.06), but not in severely injured group (**Figure 27B**).

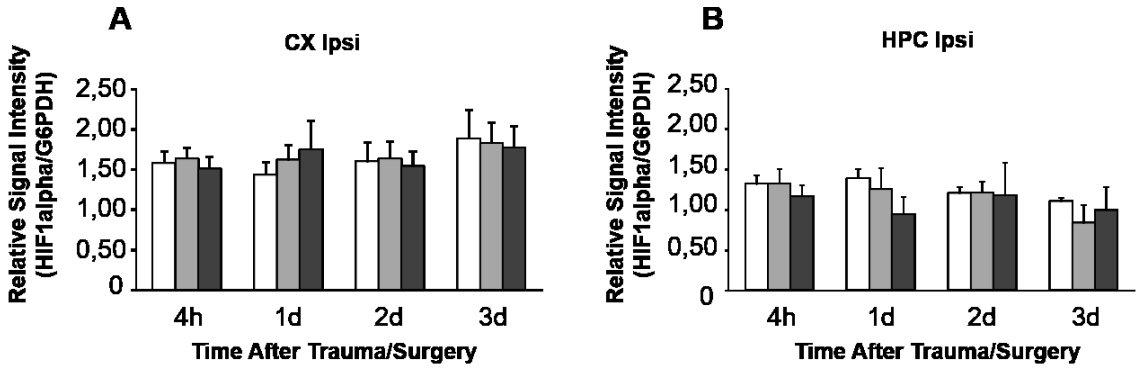

Figure 26: HIF1α Expression in cortex and hippocampus following TBI.
mRNA expression levels of hypoxia inducible factor 1 alpha (HIF1α) in the ipsilateral cortex (CX) (**A**) and hippocampus (HPC) (**B**) at 4 h and 1, 2, and 3 days after traumatic brain injury (TBI), determined by reverse transcription polymerase chain reaction (RT-PCR). Neither in cortex nor in hippocampus a detectable change was observed. Glucose-6-phosphate dehydrogenase (G6PDH) was used as the reference gene. Data are presented as relative signal intensity of product bands on agarose gel. White columns show sham controls; grey columns show moderate TBI groups; black columns show severe TBI groups. Statistical significance of changes compared with the sham group was tested with the Mann–Whitney U test.

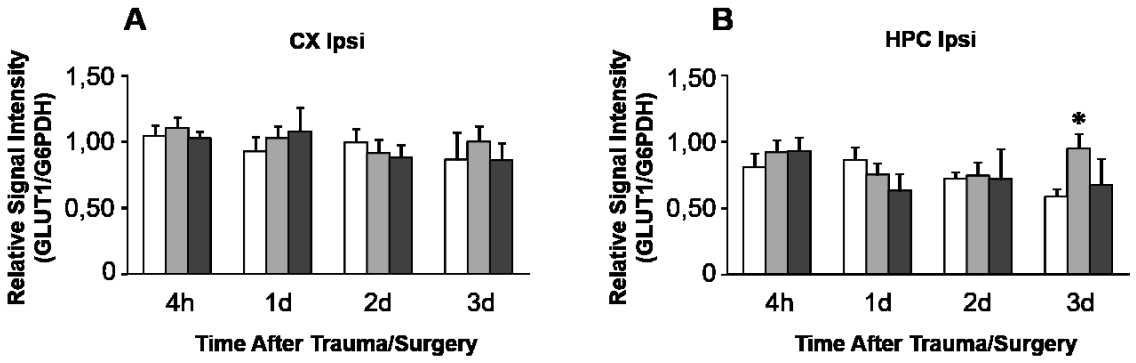

Figure 27: GLUT1 Expression in cortex and hippocampus following TBI.
mRNA expression levels of glucose transporter 1 (GLUT1) in the ipsilateral cortex (CX) (**A**) and hippocampus (HPC) (**B**) at 4 h and 1, 2, and 3 days after traumatic brain injury (TBI), determined by reverse transcription polymerase chain reaction (RT-PCR). Cortical levels of GLUT1 mRNA expression were not affected after TBI, however a significant increase was observed in hippocampus at 3 days post-TBI. Glucose-6-phosphate dehydrogenase (G6PDH) was used as the reference gene. Data are presented as relative signal intensity of product bands on agarose gel. White columns show sham controls; grey columns show moderate TBI groups; black columns show severe TBI groups. Statistical significance of changes compared with the sham group was tested with the Mann–Whitney U test. $*p<0.05$ compared with the sham group at the consistent time point.

4.5. NO levels in distant organs – Liver and Heart

A unilateral cortical injury by LFP in brain induced changes also in distant organs with a profile similar to cerebral cortex. Induction of a moderate TBI induced remarkable changes in NO levels of liver, particularly at 4h post-TBI, although the effect was statistically not significant (F[4,20]=1.819, $p=0.164$). At 4h post-TBI, NO levels noticeably increased in liver (1.98± 0.50) compared to sham controls (1.08± 0.09), which was followed by a gradual decrease to basal levels up to 3 days post-TBI (**Figure 28A**). In heart tissue, however, significant changes in NO levels were observed after TBI (F[4,54]=4.557, $p=0.003$). Post-hoc analysis revealed a significant increase at 4h after moderate TBI in heart (1.48 ±0.10, $p=0.001$) as compared to sham controls (1.02 ±0.04). Within 1 day, NO levels were reduced to basal levels and remained so up to 3 days (**Figure 28B**).

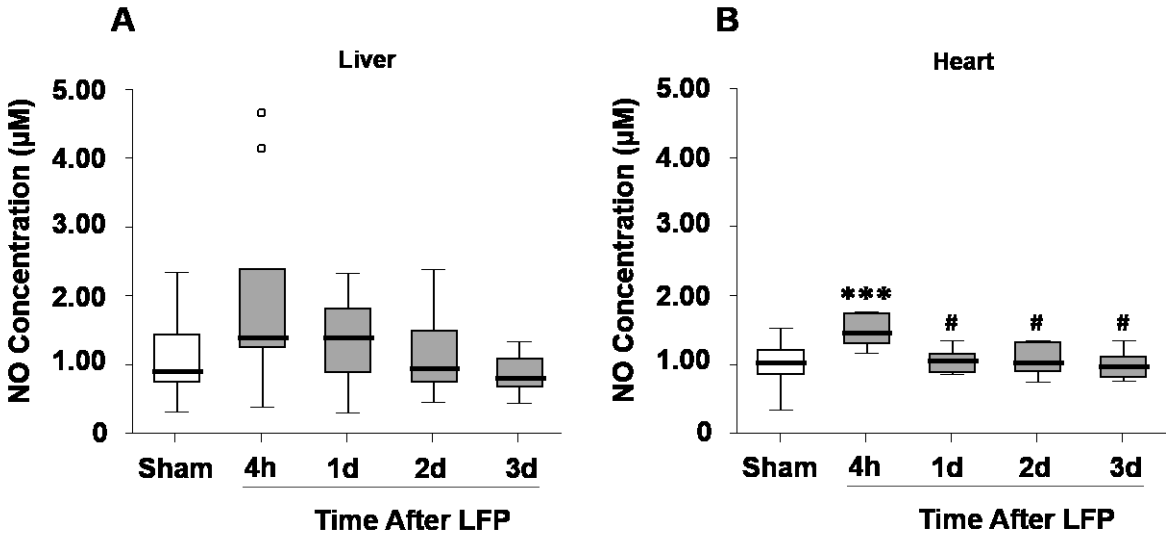

Figure 28: NO Levels in liver and heart following moderate TBI.
Changes in nitric oxide (NO) levels in the liver (**A**) and heart tissues (**B**) at 4 h, and 1, 2, and 3 days following moderate brain injury determined by diethyldithiocarbamate-iron (DETC-Fe) spin trapping and electron paramagnetic resonance (EPR) spectrometry. The profile of changes in NO levels in both organs were remarkable similar to those observed in cerebral cortex, peaking at 4 h. Statistical significance was tested with one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test or Welch's variance-weighted ANOVA followed by Games–Howell test. ***$p<0.001$ compared with sham group. #$p<0.05$ compared to 4 h post traumatic brain injury (TBI) group. Open circles show outliers. LFP, lateral fluid percussion injury.

4h post-TBI seems to be a critical time point for NO metabolism in both brain and distant organs, such as liver and heart. Therefore, in order to check whether it was a severity dependent response also in distant organs, NO levels at 4h after severe TBI were also quantified in liver and heart and analysed together with 4h post-surgery sham and 4h post-TBI moderate groups (**liver** $F[2,13]=4.726$, $p=0.028$; **heart** $F[2,20]=6.954$, $p=0.005$). Indeed, severe TBI induced significant NO elevations in liver (2.44 ±0.31, $p=0.026$) as compared to sham group 4h post-surgery (1.33 ±0.18), while increase in moderately injured group was not statistically significant (1.98 ±0.50, $p=0.403$) (**Figure 29A**). Similarly, there was a significant increase in NO levels in heart tissue after a severe TBI at 4h (1.62 ±0.07, $p=0.004$) as compared to sham group 4h post-surgery (1.23 ±0.07). In this second analysis, however, the increase in heart NO levels of moderately injured group was not statistically significant (1.48 ±0.10, $p=0.099$), probably due to higher inter animal variance in 4h post-surgery sham group. (**Figure 29B**). Taken together these results indicate that changes in NO metabolism in liver and heart were also modulated by the severity of the injury at the cerebral cortex, similar to that seen in brain sub regions.

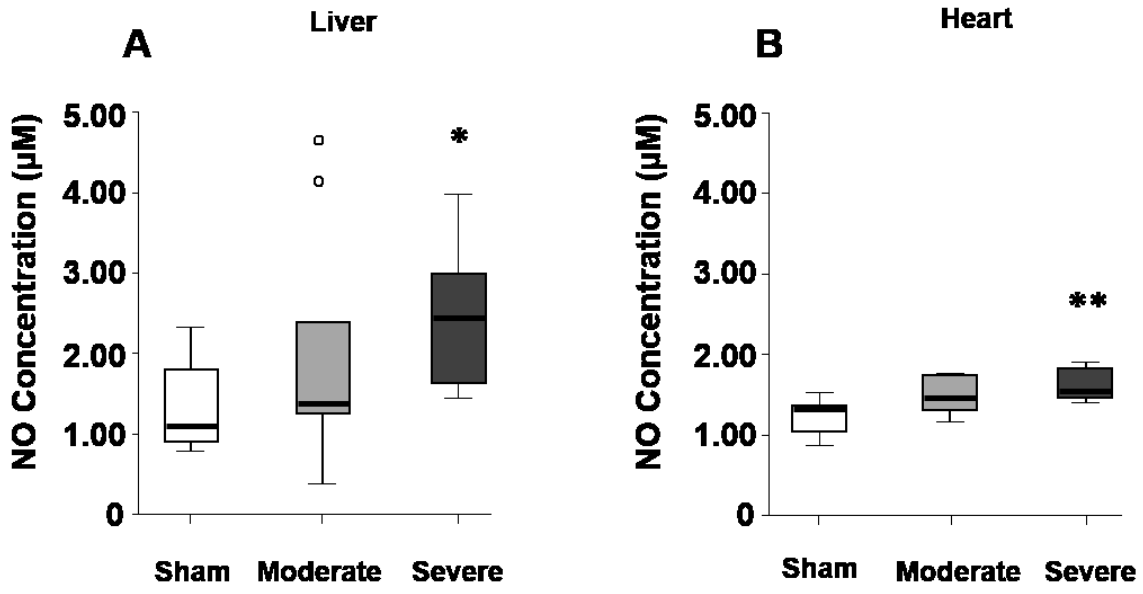

Figure 29: Severity effect on 4h post-TBI NO levels in liver and heart
Changes in nitric oxide (NO) levels in the liver (**A**) and heart tissues (**B**) at 4 h following moderate or severe brain injury determined by diethyldithiocarbamate-iron (DETC-Fe) spin trapping and electron paramagnetic resonance (EPR) spectrometry. A severity-dependent response was observed in both tissues. Statistical significance was tested with one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test or Welch's variance-weighted ANOVA followed by Games–Howell test. *$p<0.05$ and **$p<0.01$ compared with sham group 4 h after surgery. Open circles show outliers.

5. DISCUSSION

In this study, we analyzed moderate and severe TBI-induced NO changes in different brain sub regions as well as in two injury-distant organs, liver and heart. We determined specific NO-associated changes and their potential impact on the injured brain to identify whether TBI-induced NO changes have detrimental or beneficial effects. We showed for the first time by direct quantification that basal levels of NO differ significantly among brain sub regions. In response to severe TBI, NO levels were almost doubled in all regions, albeit with detrimental effects, such as tyrosine nitration, RIPK3-associated cell death, and/or apoptosis, only in the ipsilateral cortex. Our findings clearly indicated NO to contribute to the injury-associated mitochondrial dysfunction by the impairment of glutamate and pyruvate utilization. NO changes in liver and heart tissues also showed a similar profile with brain.

5.1. NO levels in brain

Cerebral NO levels were assessed by direct quantification using spin trapping followed by detection of mononitrosyl-iron complex (MNIC) via electron paramagnetic resonance (EPR) spectrometry.

Our results first of all demonstrated basal NO levels to differ significantly between cerebral sub regions. Despite considerable amount of research on the role of NO in brain including injury-associated changes, basal concentrations of average NO levels in different brain regions remained undetermined. Therefore, our study is the first to provide this information best to our knowledge. Among the three regions subjected to the analysis, cerebellum showed the highest concentration of basal NO, which was almost 3-fold of what was observed in the cerebral cortex. Hippocampal formation, on the other hand, had the lowest NO concentration with almost half of those measured in cortex. These findings are in line with a previous study, which shows nNOS to be expressed highest in the cerebellum and lowest in the hippocampus among these three regions (Greenwood et al., 1997). A direct comparison of the concentrations defined in our study with previous studies using EPR was not possible since in those studies control NO levels were either provided as arbitrary units of EPR signal amplitude (Ziaja et al., 2007) or remained undetermined (Fujii and Berliner, 1999). This is also true for studies using electrodes inserted into the brain to measure momentary changes in NO evoked currents (Cherian et al., 2000, Cherian and Robertson, 2003). Due to the method in the latter, basal levels were

calibrated to zero, therefore not evaluated; while injury associated changes were represented as deviations from the zero point.

5.2. Trauma Associated Changes in Brain NO levels

Our results indicated a rapid but transient elevation in cerebral NO levels following a unilateral trauma of cerebral cortex.

TBI-induced NO elevations manifested by our findings correspond to the increases observed in injured cerebral hemispheres of mice brains at 72 hours following controlled cortical impact (CCI) injury (Bayir et al., 2005). It should be noted that in another study using EPR, a reduction of NO levels in injured cerebral hemispheres was observed at 2-24 hours after focal penetration injury in rats (Ziaja et al., 2007). Direct measurements using electrode systems also reported decreases of NO at 3 or 4 hours after TBI in rat models of CCI or FPI at the injury site (Cherian et al., 2000, Cherian and Robertson, 2003, Ahn et al., 2004). However, these studies reflect changes in extracellular local NO concentrations with high temporal resolution at the injury core. Therefore, these findings might not reflect average NO levels of specific brain regions. Taken together, differences in injury types, organisms and measurement methods appear to give rise to considerable variability among the few studies measuring direct NO following brain trauma, including ours.

Nonetheless, our findings were underlined by several other studies measuring NO metabolites in human patients (Carpenter et al., 2008, Sohrevardi et al., 2013, Hlatky et al., 2003, Zhou et al., 2000, Yang et al., 2002) or experimental rat models of various types of traumatic injury (Sakamoto et al., 1997, Yuksel et al., 2013, Valable et al., 2010, Di Pietro et al., 2014, Ansari et al., 2014).

NO upregulation in our findings was severity-dependent since severe TBI led to prolonged elevations in the cortex compared to the moderately injured group. A similar temporal difference in nitrite/nitrate levels of cerebrospinal fluid was shown between moderate and severe groups of human patients (Zhou et al., 2000). In another clinical study, on the other hand, nitrite/nitrate levels of micro dialysate samples from injured area showed no correlation to the injury severity after diffuse brain injury (Hlatky et al., 2003). Perhaps injury type or cohort heterogeneity partially account for these discrepancies. In accordance with the latter presumption, using a rat model of diffuse injury Di Pietro et al showed that tissue levels of nitrite/nitrate to be significantly higher after severe injury from

6 hours to 5 days as compared to mild group, although both had peak levels at 12 hours post-TBI (Di Pietro et al., 2014). A similar difference was also reported for plasma levels of nitrite/nitrate in adult human patients with acute moderate or severe cerebral injury (Yang et al., 2002). Our findings using fluid percussion model of rats showed that severe TBI did not induce NO levels higher than the moderate group, but in fact induced a prolonged and statistically significant production of NO, at levels comparable to moderately injured cortex. It should be noted that, higher levels of NO metabolites after severe injury could also result from such temporal difference, as more NO metabolites would accumulate following a protracted upregulation compared to a shorter one, although NO levels at a given time were not necessarily higher.

Our results pointed towards a severity-dependent expansion of NO upregulation to the uninjured brain sub regions including contralateral regions and cerebellum, whereas moderate TBI led to an induction only in the ipsilateral cortex and hippocampus. Our study is one of the few that take both severity and distance into consideration at the same time. In most of the studies so far, researchers have been interested only in the injury site or injured cerebral hemisphere, therefore other regions remained undetermined. There is one early study underlining our observation where a rat model of moderate diffuse TBI using Marmarou's weight drop injury showed elevated levels of NO at 6h in cortex, diencephalon and brain stem (Sharma et al., 1998). It appears that effect on distant regions depend on the presence and strength of concussion in the model utilized. Although FPI is superior to CCI or other focal injury models in modelling concussion, Marmarou's weight drop model produces concussion relatively better than FPI (Xiong et al., 2013). Furthermore, unlike Marmarou's model, craniotomy applied prior to fluid percussion injury precludes generation of increased cranial pressure, thereby impeding spread of damage to the regions distant to the injury site. Such details between models should at least partially account for the strong variability in studies overall, including clinical cohorts and experimental models.

5.3. Trauma Associated Changes in Expression of NOSs

Analyses of mRNA expression of inducible form of nitric oxide synthase (iNOS) showed a strong upregulation at 4 hours in the ipsilateral cortex and hippocampus, independent of injury severity. These findings correspond to post-TBI iNOS increases in mRNA or protein levels reported in various studies (Gahm et al., 2000, Gahm et al., 2002,

Hall et al., 2012, Abdul-Muneer et al., 2013, Jung et al., 2014). mRNA expression of nNOS, on the other hand, did not show any significant changes after TBI, in line with previous studies performed with human patients (Gahm et al., 2002) and a CCI model of mice (Hall et al., 2012) reporting no change in nNOS expression. Moreover, even a reduction of nNOS positive cells was reported in rats at day 2 following weight drop injury (Gahm et al., 2000). In addition, enzymatic activity of nNOS was shown to undergo significant reductions from 1 to 7 days in a rat model of FPI following a rapid increase at 5 to 30 min post-TBI (Wada et al., 1998a). Taken together these results strongly indicate the primary role of iNOS for NO elevations observed at 4 hours and 1 day post-TBI in our study in the ipsilateral cortex and hippocampus.

In the contralateral regions, however, there was not any detectable change in either iNOS or nNOS mRNA expression levels at 4 hours, although NO levels were almost doubled at this time in the contralateral cortex and hippocampus. These results suggest that the contralateral cerebral, also perhaps cerebellar NO elevations, differ from injury site in terms of NO source. Despite the lack of an expression change in nNOS mRNA, activity of resident nNOS enzymes were not determined in our study. Indeed, in a mouse model of CCI injury, genetic ablation of iNOS could not totally prevent NO elevations, although reduced significantly (Bayir et al., 2005), which indicates the role of other NOS isoforms. To further support this possibility, previous studies have provided the link between enzymatic activation of nNOS and Ca^{2+} influx due to glutamate-mediated NMDA receptor activation (Christopherson et al., 1999). Given the fact that post-TBI elevation of glutamate levels is one of the well-documented early pathophysiological sequelae after TBI (Werner and Engelhard, 2007), nNOS-mediated local NO production in these regions might have well been through glutamate-NMDAR-Ca^{2+}-nNOS pathway due to post-traumatic increase in glutamate levels.

Likewise, Hall et al observed increases in cortical eNOS protein expression after CCI, although this was not supported by a noticeable increase in mRNA levels (Hall et al., 2012). The fact that the 3NT signals were detected only in some capillaries in the contralateral cortex in our study is also suggestive of a contribution by eNOS in these regions. Nevertheless, the consistency of both concentration levels and temporal characteristics of NO between ipsi- and contralateral portions post-severe TBI, severity modulated nature of NO changes in injury-distant regions, and finally preservation of regional differences in NO concentration after a TBI (i.e. lowest in hippocampus, highest

in cerebellum) altogether are rather supportive of a possibility of a local enzymatic production of NO in these regions, possibly due to the concussion-dependent increase in local glutamate levels of the contralateral and injury-distant sub regions. Whether diffusion from the ipsilateral sides or synthesis from inorganic sources, like nitrite and nitrate, contribute to NO elevations in uninjured regions need further experiments in order to be proven.

5.4. Post Traumatic Tyrosine Nitration

Transient NO elevation induced by TBI was associated with persistent nitration of tyrosine residues. 3NT formation was mainly restricted to regions adjacent to the lesion site in the ipsilateral cortex, but not in injury-distant regions as well as hippocampus, cerebellum or contralateral side of the cortex. These findings were supported by various studies using animal models of CCI, FPI or closed head injury, where 3NT was not detectable by immunohistochemistry in the contralateral sides of the injured brain (Hall et al., 2004, Bayir et al., 2005, Lau et al., 2006, Ansari et al., 2008).

It was for the first time clearly demonstrated by our findings that the lack of tyrosine nitration in remote regions was not due to the lack of NO bioavailability. Indeed, tyrosine nitration is a marker of peroxynitrite (ONOO-)-mediated damage, which is produced via diffusion limited reaction of NO with O_2^-. This phenomenon was underlined by the observation of increase in tyrosine nitration at day 1 following mitochondrial dysfunction observed at 4 hours post-LFP. Furthermore, neither remarkable increase of tyrosine nitration nor mitochondrial dysfunction was observed in the ipsilateral hippocampus, despite elevated NO levels after both moderate and severe injury. Finally, this presumption was supported by the absence of a nitrosative stress in the cerebellum, although this region had the highest levels of NO after severe TBI (double as cortex). Taken together, these results might indicate critical role of oxygen radicals in nitrosative stress in addition to NO.

5.5. Post Traumatic Mitochondrial Dysfunction

Mitochondrial dysfunction after brain trauma has been well described. Numerous clinical and experimental studies documented injury-induced reductions in activity or expression of mitochondrial enzymes, disruptions in mitochondrial ultrastructure, and

fluctuations in respiration metabolites as well as functional disturbances in mitochondrial respiration in the injured brain.

Various studies using either pyruvate (Singh et al., 2006, Opii et al., 2007, Pandya et al., 2007, Mbye et al., 2008, Deng-Bryant et al., 2008, Pandya et al., 2009, Mustafa et al., 2010, Gilmer et al., 2009, Sauerbeck et al., 2011, Kilbaugh et al., 2011, Readnower et al., 2011, Sauerbeck et al., 2012, Miller et al., 2015) or glutamate (Xiong et al., 1997a, Xiong et al., 1997b, Verweij et al., 1997, Xiong et al., 1998, Xiong et al., 1999, Robertson et al., 2006, Watson et al., 2013) for the respirometric assessment showed significant decreases in the substrate-dependent oxygen consumption rates in tissue homogenates or isolated mitochondria of injured brain at various time points after CCI in adult mouse or rat models. To our knowledge, our study is the first one, which has tested multiple targets in TCA cycle and revealed dynamics of the changes in the substrate-dependent oxygen consumption in a time course following LFP. Significantly decreased oxygen consumption with either of these substrates in our results is consistent with the previous studies. However, it should be noted that mitochondrial dysfunction persisted up to 7 days in some studies (Xiong et al., 1997a, Xiong et al., 1999, Watson et al., 2013), which contrasts to spontaneous recovery observed at 24 hours in our LFP model. An early study using FPI (Vink et al., 1990) did not show any change in glutamate utilization apart from a non-significant reduction in ATP synthesis efficiency at 4 hours following severe injury. The discrepancy between our results and findings of Vink et al could be explained by differences in experimental settings. First, we used injured portion of cortex but Vink et al used the whole cerebral hemisphere, thus in their case the observed effect might have been diluted by the reduced representation of injury core in the whole sample. Second, Vink et al utilized isolated mitochondria, in which the disrupted and destroyed mitochondria are eliminated during preparation thereby the actual extent of mitochondrial damage is hindered. Nevertheless, these findings together suggest that a more pronounced mitochondrial dysfunction is induced by CCI than that is observed after FPI. This supposition was further substantiated by the lack of any significant change in Complex II activity assessed by succinate-dependent oxygen consumption rates in our settings. However, various studies reported significant reductions after CCI injury in mice or rats (Opii et al., 2007, Mbye et al., 2008, Gilmer et al., 2009, Watson et al., 2013, Miller et al., 2015). Nonetheless, there is data showing reduced enzymatic activity of succinate dehydrogenase (SDH), a TCA cycle enzyme catalysing succinate to fumarate conversion

and thereby electron transfer to FAD, in various regions of brain following mild, moderate or severe brain injury by LFP (Jiang et al., 1999, Jiang et al., 2000). Although SDH activity was not assessed in our study, impeding a direct comparison of the results, these data might be suggestive of other mechanisms that sustain substrate-dependent respiration in spite of a reduction in the activity of the enzyme catalysing the respective substrate. A similar phenomenon was observed in our laboratory where a significant reduction of 2-oxoglutarate dehydrogenase complex (OGDHC) activity after TBI was not reflected by a concomitant reduction in 2-oxoglutarate-dependent state 2 or state 3 respiration rates (**preliminary results of an ongoing study**).

5.6. Role of NO in post-TBI mitochondrial dysfunction: glutamate node

Our findings clearly demonstrated the causative role of NO in mitochondrial dysfunction following TBI. Indeed, 3NT has been frequently used as an oxidative/nitrosative damage marker in the isolated mitochondria after TBI (Singh et al., 2006, Opii et al., 2007, Mbye et al., 2008, Gilmer et al., 2010, Mustafa et al., 2010, Miller et al., 2015), while the functional location of damage remained, however, unassessed in these studies. On the other hand, several mitochondrial proteins were shown to be targeted for modification mediated by NO or its derivatives in numerous *in vitro* studies (Poderoso et al., 1996, Costa et al., 1997, Poderoso et al., 1998, Clementi et al., 1998, Beltran et al., 2000, Orsi et al., 2000, Riobo et al., 2001, Chinta and Andersen, 2006, Carreras and Poderoso, 2007, Davis et al., 2010). Nevertheless, whether these findings have pathophysiological correlates in TBI remained as an open question mainly due to the obscurity of NO levels in traumatized brain. Our results indicated the glutamate node of TCA cycle to be targeted by NO-mediated damage after TBI while pyruvate-dependent respiration might be repressed due to the factors other than NO. It should be noted that our *in vitro* results might not be directly comparable with post-TBI tissue since a higher NO level (~20 μM) was applied to tissue homogenates in a shorter time (10 minutes) while *in vivo*, perhaps a smaller NO concentration (sub micromolar levels) should have been effective for an extended time (hours)[1]. Nevertheless, it is indicative of a higher vulnerability of glutamate utilization than that of pyruvate at a certain level of NO, a

[1] Note that our measurement detected NO concentration that is accumulated from 3 h to 4 h after TBI, See Materials and Methods Section for details.

difference that was also observed in our post-TBI results. Finally, absence of an impairment in succinate-dependent respiration showed that Complex II was not affected in our settings. This also showed that Complex IV activity to be intact after TBI, since it is the final electron acceptor and inhibition of this protein would have been detected with oxygen consumption using any of the three substrates. NO-mediated reversible inhibition of Complex IV (CcO), which was shown in *in vitro* studies [reviewed in (Cooper and Giulivi, 2007)], did not appear to have a major contribution in TBI pathophysiology.

Glutamate is converted to 2-oxoglutarate in order to be utilized in TCA cycle either by glutamate dehydrogenase (GDH) or glutamate oxaloacetate transaminases (GOTs). Repression of each would reduce glutamate utilization for respiration. However, Watson et al has reported that GDH protein levels were not affected, despite a significant reduction in glutamate-dependent respiration at 7 days post-TBI in a rat model of CCI (Watson et al., 2013). Neither a change in enzymatic activity of GDH was observed at 4 hours following severe brain injury by LFP in our settings (**preliminary results of an ongoing study**). Mitochondrial isoform of GOT (GOT2), on the other hand, was shown to undergo significant reductions in both protein expression and enzymatic activity in cerebral cortex of rats at 1 to 6 hours following exposure to blast injury, and recovered to basal levels at 24 hours (Arun et al., 2013). The time course of GOT2 disturbance in this study was notably consistent with our observations of reduction and spontaneous recovery of glutamate utilization after LFP.

It should be noted that a reduction in 2-oxoglutarate-dependent state 2 or state 3 respiration rates was not observed at 4 hours post-LFP in our experiments, suggesting that it was not downstream mechanisms to be impaired by post-TBI NO but exclusively the catabolism of glutamate itself. Paradoxically, there was a significant reduction in OGDHC enzymatic activity at the same time point after LFP (**preliminary results of an ongoing study**). In fact, there is data showing OGDHC inactivation by peroxynitrite-mediated tyrosine nitration (Shi et al., 2011), suggestive of a role of post-TBI NO in OGDHC inactivation. Together with aforementioned findings, these data might be indicative of a link between OGDHC and conversion of glutamate. Early studies provided data suggesting a complex formation by GOT and OGDHC, where GOT provides 2-oxoglutarate to OGDHC (Fahien et al., 1988, Smith et al., 1992). It is interesting to speculate that post-traumatic repression of either GOT2 or OGDHC (or both) might have led to disruption of complex formation and thereby impaired glutamate utilization. It

appears, however, this deficiency was surpassed during assessment of 2-oxoglutarate-dependent respiration rates due to the substrate availability in excess amounts. Nevertheless, these assumptions need further experiments to be proven and a better understanding of the exact mechanism of repressed glutamate utilization after TBI and the exact role of NO in this process require further study.

Having established causative role of NO in post-TBI repression of mitochondrial glutamate utilization in the ipsilateral cortex, it appears that the deficiency may not be exclusively NO-dependent. Lack of a mitochondrial dysfunction in the ipsilateral hippocampus despite the elevated NO levels with severe TBI supports this presumption. Furthermore, it should be noted that recovery of glutamate utilization in the injured cortex at 24 hours post-TBI while NO levels were still high, albeit not significant, is indicative of the role for additional factors in mitochondrial recovery. Assessment of the cerebellar and contralateral cortical mitochondrial dysfunction after LFP would provide valuable insights to answer these questions in detail since similar or higher NO levels were observed there at 4 hours after TBI. Nevertheless, NO-related detrimental outcome, such as tyrosine nitration and co-localized apoptosis induction, were not observed in these regions. These findings might suggest that brain trauma induces NO elevations, perhaps as part of a protective response, but at the same time leads the injury site to be particularly susceptible for that, while in relatively distant regions equally high (contralateral cortex) or higher levels of NO (cerebellum) were not associated with detrimental outcomes such as nitrosative stress, mitochondrial dysfunction or cell death.

5.7. Post Traumatic Cell Death and NO

Caspase activation and apoptotic cell death after fluid percussion injury in rats has been well documented (Rink et al., 1995, Keane et al., 2001, Knoblach and Faden, 2002, Shojo et al., 2010, Knoblach et al., 2002) and associated with increased nitrosative stress (Lau et al., 2006, Zhang et al., 2002b). Our observation of increased active caspase-3 staining at day 1 and day 2 corresponds to these findings. Further, co-localization of 3NT positivity and caspase-3 activation at day 1 and day 2 in the ipsilateral cortex is indicative of apoptotic cell death being induced by nitrosative stress. This observation was further supported by the clearance of double positive cells at day 3 in the injured cortex as well by the lack of caspase-3 activation in remote regions where 3NT positivity was not observed, such as hippocampal formation and the contralateral cortex.

On the other hand, programmed necrosis has also been implicated in ROS/RNS mediated cell death (Davis et al., 2010, Higgins et al., 2012, Kim et al., 2007). Inhibition of the regulators of programmed necrosis, RIPK1 and RIPK3 (Cho et al., 2009b, Zhang et al., 2009, Li et al., 2012), has been shown to improve neurological and histological outcome after experimental TBI in mice (You et al., 2008) and reduce oxidative damage, inflammation and injury in neonatal hypoxia-ischemia (Northington et al., 2011). Furthermore, a recent study showed necroptotic neuronal death was induced by ischemic insult through upregulation of endogenous RIPK3 (Vieira et al., 2014). Consistent with these findings, our results showed an increase in the expression of RIPK3 in both cortex and hippocampus following fluid percussion injury. It should be noted that RIPK3 upregulation was more pronounced in the cortex than it was in the hippocampus, independent of injury severity. On the other hand, a severity dependent difference was observed in time course of RIPK3 upregulation in the ipsilateral cortex. A biphasic upregulation following moderate injury was in contrast with the gradual increase observed after severe TBI. Such difference might possibly suggest different pathophysiological procedures in which RIPK3 should be involved after TBI. In fact, RIPK3 was implied in inflammatory processes independent of its role in necrosis. RIPK3 requirement has been reported in formation of NLRP3 inflammasome (Vince et al., 2012) which in turn mediates the cleavage and maturation of interleukin 1 beta (IL-1β) and has been reported to be involved in inflammatory brain diseases (Malhotra et al., 2015, Hoegen et al., 2011). However, increases in IL-1β expression has been reported to occur quite rapid and rather earlier than 24 hours in the experimental models of TBI (Ciallella et al., 2002, Shojo et al., 2010, Fan et al., 1995). Notably, sustained increase in RIPK3 expression at day 3 in both moderate and severe TBI were suggestive of involvement of RIPK3-mediated programmed necrosis in TBI. This might as well partly explain the clearance of 3NT positive cells in the ipsilateral cortex after TBI that were not apoptotic. Nevertheless, a more complete and conclusive understanding of the roles of RIPK3 in inflammation or cell death after TBI requires further studies.

5.8. Gene Expression after TBI

5.8.1. LIF expression after TBI

Expression of LIF has been shown to undergo fast and transient upregulation after cortical laceration injury (Banner et al., 1997), perinatal hypoxia/ischemia (Covey and

Levison, 2007) or peripheral nerve injury (Dowsing et al., 2001). Cortical increase of LIF mRNA expression at 4 hours and 1 day post-TBI in our study is in line with these findings. Post-injury LIF has been associated to activation of astrocytes and microglia (Sugiura et al., 2000, Kerr and Patterson, 2004). It might also be associated to NO elevations in traumatized brain through JAK/STAT signalling since cytokine-induced activation of this pathway induced iNOS expression in cultured astroglial cells (Dell'Albani et al., 2001). In fact, activation of JAK/STAT pathway has been revealed in a rat model of contusion (Zhao et al., 2011) and LIF was also shown to activate this pathway after neuronal injury (Rajan et al., 1995, Covey and Levison, 2007). Recently, administration of exogenous LIF was shown to induce NO levels in a mouse model of cerebral infarction and promoted neuronal stem cell proliferation in a NO-dependent manner (Huang et al., 2012, Lopez-Arenas et al., 2012). Taken together, these results point towards a possible causal association between increases in LIF expression and NO levels observed early after TBI in our study and this association could be mediated by JAK/STAT signalling. However, this needs to be validated with further analyses like LIF expression in contralateral regions as well as expression of JAK/STAT signalling cascade components early after TBI in regions close to or distant from the injury site.

Unlike cortex, increase of NO levels in hippocampus was not accompanied by an upregulation in LIF expression. Instead, there were remarkable severity-dependent decreases in LIF expression from 1 to 3 days post-TBI suggesting that endogenous LIF activity in hippocampus should be independent of its inflammatory actions. Various studies reported stimulatory actions of LIF in the adult neural stem cell (NSC) self-renewal (Bauer and Patterson, 2006, Bauer, 2009), progenitor cell proliferation (Covey and Levison, 2007, Deverman and Patterson, 2012) and promotion of neuronal survival (Moon et al., 2009, Suzuki et al., 2005). Sub granular zone (SGZ) of dentate gyrus in hippocampus is one of the two main locations that sustain adult neurogenesis. Injury-induced suppression of LIF expression in hippocampus, therefore, might indicate disturbance of these events early after trauma, which was also in line with the concomitant repression of hippocampal neurotrophin expression.

5.8.2. Neurotrophin expression after TBI

In vitro and *in vivo* studies have shown involvement of neurotrophins in survival, proliferation and migration of neural cells as well as synaptic plasticity (Gao et al., 1995,

McTigue et al., 1998, McAllister et al., 1999, Yuan and Yankner, 2000, Zhang et al., 2003).

Severity-dependent significant reductions were observed in hippocampal expression of neurotrophin 4 (NT4) mRNA after TBI. These results are in line with reported vulnerability of hippocampal neurons in response to a brain trauma (Lowenstein et al., 1992, Maxwell et al., 2003). A previous study has showed a transient increase in the hippocampal NT4 protein levels at 1 day post-FPI (Royo et al., 2006), however, protein levels of NT4 were not assessed in our study. Noteworthy, authors of the study showed that genetic ablation of NT4 worsens the outcome after trauma and post-TBI supplementation with recombinant NT4 reduced hippocampal cell death, concluding NT4 should have a protective role after TBI. Further, a similar transient increase in cortical NT4 protein levels was also observed in the same study. In our results, however, there was a slight increase at day 1 and a remarkable decrease at day 3 mRNA levels, neither of which was statistically significant.

Another neurotrophin, brain-derived neurotrophic factor (BDNF), has been shown to undergo fast and transient upregulation in different sub regions of hippocampus following TBI in various studies (Yang et al., 1996, Hicks et al., 1997, Wang et al., 2014). These acute BDNF increases were attributed to be part of a protective response to TBI and this assumption was also underlined by experiments using genetic ablation of BDNF (Gao and Chen, 2009).

Expression of BDNF mRNA did not change at any time point analysed in our study at the injury site of cerebral cortex. These findings correspond to previous work reporting cortical BDNF levels altered only at remote regions of cortex with respect to the injury site, but not at or near the impact site (Truettner et al., 1999, Kobori et al., 2002). Perhaps, differences in brain activity at the injury site and remote regions account for these differences. Previous research documenting injury-induced cellular depolarization (Katayama et al., 1990, Tavalin et al., 1995), spreading depression of cortical activity after trauma (Strong et al., 2002) or repressed neuronal firing assessed by depth electrodes in the cerebral cortex in a rat model of FPI (Alves et al., 2005) support this presumption. Likewise, it could also explain the lack of a change in hippocampal levels of BDNF mRNA in our results. Findings of a recent study revealed reductions in BDNF levels of hippocampus in regions closer to the cortical injury site (Rostami et al., 2014). Notably,

another study demonstrated hippocampal BDNF mRNA to be significantly upregulated following mild TBI while severe TBI-induced changes were almost negligible (Shindo et al., 2006). Finally, possible regional variations of BDNF expression among different sub regions of hippocampus were not assessed in our settings, where hippocampal hemispheres (ipsi- or contralateral) were analysed as a whole.

Several studies have indicated a close and complex relationship between neurotrophins and NO (**See Section 1.2.3.5**). Cortical and hippocampal expression of NT4 and BDNF after TBI in our settings did not show any clear correlation to the changes of NO levels in these regions, suggesting that neurotrophin-NO interactions may not be of considerable relevance in the traumatized brain. Nevertheless, it is not possible to exclude such interactions based on our findings. Perhaps, a more detailed analysis of spatial distribution of post-TBI NT4 and BDNF expressions, i.e. with immunohistochemical analysis, would provide more information on this subject.

5.8.3. HIF1α and GLUT1 expression after TBI

Our PCR analyses did not show any significant change in mRNA expression of HIF1α either in the cortex or hippocampus, ipsilateral to the injury. These results contradict with the previous work reporting TBI-induced increases of HIF1α in mRNA or protein levels (Anderson et al., 2009, Huang et al., 2010, Li et al., 2013, Schaible et al., 2014). Nevertheless, a detailed comparison of published results reveals model-dependent differences, which reflect the divergence of experimental TBI types in modelling vascular damage.

Focal injury models, like CCI or Feeney's weight drop injury, resulted in more robust increases in HIF1α mRNA as early as 6-12 hours (Huang et al., 2010, Schaible et al., 2014) and in protein levels at 24-48 hours (Li et al., 2013). In contrast, a diffuse injury model showed a slight increase at 6h (Umschweif et al., 2013) and penetrating ballistic-like brain injury (PBBI) resulted in significant decreases at 1, 3 and 7 days in protein levels of HIF1α, albeit latter with higher mRNA at day 1 (Cartagena et al., 2014). Likewise, in another study using FPI, any HIF1α was not detectable in the cortex at 24 hours post-TBI (Park et al., 2009). These studies correspond to our results concerning mRNA expression in cortex.

It should be noted that the lack of a change in transcriptional activity of HIF1α does not exclude a possible increase in protein levels via stabilization against prolyl hydroxylase mediated degradation; as well increased mRNA expression does not necessarily indicate active HIF1 signalling. Accordingly, Schaible et al showed the cortical expression of GLUT1, together with several other HIF1 target genes, remained unchanged after CCI despite significant increases in HIF1α levels (Schaible et al., 2014). Further, Umschweif et al showed increase only in 55 kDa GLUT1 (vascular endothelial isoform), but not 45 kDa (astroglial isoform) after a mice model of closed head injury (Umschweif et al., 2013, Leino et al., 1997, Yu and Ding, 1998). A rat model of diffuse brain injury also did not induce a change in astroglial GLUT1 expression (Hamlin et al., 2001). These findings underline our results showing no change in cortical GLUT1 expression. On the other hand, there was a significant increase of GLUT1 expression in the ipsilateral hippocampi of moderately injured group at day 3 post-LFP, despite the lack of a change in HIF1α expression. These results correspond to the findings of an earlier FPI study which showed an increase of HIF1α protein levels in hippocampus, but not in cortex, after FPI (Park et al., 2009). Taken together our and others' results suggest that the hippocampus could be more prone to the post-traumatic hypoxia in the acute phase than the injured cortex and post-FPI activation of hypoxic signalling appears to take place possibly via HIF1α stabilization rather than increased transcriptional activity at the mRNA level.

It is interesting to note that, mitochondrial ROS generation is required for HIF1α stabilization under hypoxic conditions (Chandel et al., 1998, Herr et al., 2007). Mitochondrial dysfunction observed at 4h after LFP in our settings presumably resulted in an increase of mitochondrial ROS production. However, isochronous elevation of NO levels might have attenuated a potential activation of hypoxic signalling via scavenging mitochondrial ROS. Supporting this presumption, NO was shown to attenuate both hypoxia-induced and ROS-induced HIF1α stabilization in the earlier studies (Agani et al., 2002, Zhou et al., 2006, Kohl et al., 2006). It should be noted that under normoxia or without ROS challenge, NO was shown to promote stabilisation of HIF1α (Zhou et al., 2006, Thomas et al., 2004). This second phenomenon was not possible to observe in the injured cortex in our settings since NO production returned to basal levels within 1 day post-TBI together with the recovery of mitochondrial respiration. Nevertheless, the ipsilateral hippocampus did not show a significant change in mitochondrial dysfunction at 4h post-severe TBI, and a change in HIF1α expression was not detectable in this region

despite significantly increased NO levels, excluding a NO-mediated transactivation of hypoxic signalling.

5.9. Post-Traumatic NO Changes in Distant Organs: Liver and Heart

There is a great body of literature on NO regulation in the liver and particularly in the heart, and the influence of NO on the physiological functioning of these organs. However, whether and how a brain injury influences NO metabolism in these organs remained largely overlooked. Our study provided remarkable results with respect to the systemic reaction of the body in terms of NO changes in response to a traumatized brain.

Best to our knowledge, our study is the first one that has measured NO levels in the heart tissue after a brain trauma. NO changes in the heart tissue showed a profile similar to which was observed in the cerebral tissue. A significant and transient increase of NO levels was observed at 4 hours following LFP and this effect was modulated by the severity of brain injury. Later, NO levels were comparable to basal levels within 24 hours up to 3 days post-TBI. Although less prominent, similar results were observed in the liver. There has been only one study measuring NO levels in the liver after TBI and authors have shown a rather biphasic response, the first being a non-significant slight increase 2h-to-6h followed by a reduction until 24h and the latter being a more distinguished elevation from 2 to 7 days after focal penetration injury (Ziaja et al., 2011). It should be noted that brain NO findings of the same model (Ziaja et al., 2007) also showed similar differences when compared to our results of brain NO levels, suggesting that the differences between trauma models might account for the discrepancy of results. Nevertheless, both their and our work principally show elevated NO levels in the liver in response to a brain trauma. Our results further showed changes in the heart NO levels and interestingly, both liver and heart NO increases at 4h were modulated by the severity of the damage, similar to our observations in brain. Investigation of sources of NO elevations in these organs is still in progress. However, in our preliminary analyses, mRNA expression of either eNOS or iNOS in cardiac tissue did not show a significant increase at 4h (**preliminary results of an ongoing study, Figure Appendix 5**).

It should be noted that causes and consequences of observed post-TBI NO elevations in the heart and liver were not assessed in our study. Nevertheless, our findings together with the current knowledge allow for some speculations.

The trauma of the cerebral cortex should be communicated to the internal organs via neural innervation or blood, and mechanisms leading to the observed NO elevations in the liver and heart could be based on signalling via either path. Cardiac or hepatic NO changes observed in our study could well be due to the modulated neural activity of the autonomous control regions. However, whether this modulation is due to the trauma, inflammation, cathecholamin release or NO elevation, or a mixture of all, is far from being clear.

In cardiac tissue, NO was shown to reduce NA release and facilitate ACh release, which in turn inhibits the cardiac responses to sympathetic nerve stimulation but facilitate the negative chronotropic action of vagal stimulation [reviewed in (Sears et al., 2004)]. Therefore, the transient elevation of cardiac NO levels could be part of a feedback response to the elevated sympathetic nerve activity (sympathetic storm) and a reaction to counterbalance the effect of cathecholamines. On the other hand, heart – and liver tissue as well, were shown to produce NO via iNOS in response to several inflammatory cytokines including TNFα, IFNγ, IL-1β, and IL-6 (Song et al., 2000, Muntane et al., 2000, Pinsky et al., 1995, Kinugawa et al., 1994), which were also detectable in serum early after injury in human patients and various models of experimental TBI (Woodcock and Morganti-Kossmann, 2013). Therefore, post-TBI release of inflammatory cytokines could also be responsible for the hepatic and cardiac NO elevations observed in our study. Although an increase in the iNOS mRNA expression was not observed in our settings, comparison of post-LFP serum cytokine profiles to the observed cardiac and hepatic NO profiles would be helpful for a better understanding.

The similarity of the heart and liver NO changes to those observed in brain was noteworthy. Inhibitory effect of NO in brain regions that control the sympathetic nerve activity, and its excitatory effects in the parasympathetic control centers in the CNS are known (**For details and references, see Section 1.3.4 and Table Appendix 1**). Cardiac NO also has effects in the same direction. Therefore, NO changes observed in the brain and the internal organs might comprise two parts of the same protective response – central and peripheral – to restore the disturbed homeostasis through attenuating the sympathetic overflow caused by the cathecholamine release after brain trauma. In this view, NO appears to have an important and a central role as a protective mediator. However, whether these post-TBI transient NO disturbances in the liver and heart are detrimental or beneficial to the patient to cope with TBI remains as an open question, which needs further

studies to be answered. Further, post-TBI changes in the cardiac and hepatic NO metabolisms might also have important implications for organ transplantation from brain-dead donors. Accordingly, a recent study provided evidence for the association of the increased iNOS activity in the left ventricle to the donor heart dysfunction (Bulcao et al., 2010).

6. CONCLUSIONS & FUTURE PERSPECTIVES

In conclusion results of this study showed for the first time that different sub regions of the brain have varying average NO levels. Although a close relationship between NO production and traumatic brain injury has been shown in previous studies, our findings shed light in dynamics of the changes in brain NO levels and revealed a severity- and distance-dependent effect for the first time. In addition, this study showed for the first time a close relationship between the brain injury and NO metabolism in peripheral organs, liver and particularly heart. It is noteworthy that changes in liver and heart NO levels after moderate TBI were more pronounced than that of the contralateral cerebral regions or the cerebellum. These findings clearly identified NO to have a central role in TBI pathophysiology not only in brain but potentially in whole body homeostasis. Some of the results presented in this thesis were published elsewhere as an original article (Ucal et al., 2016).

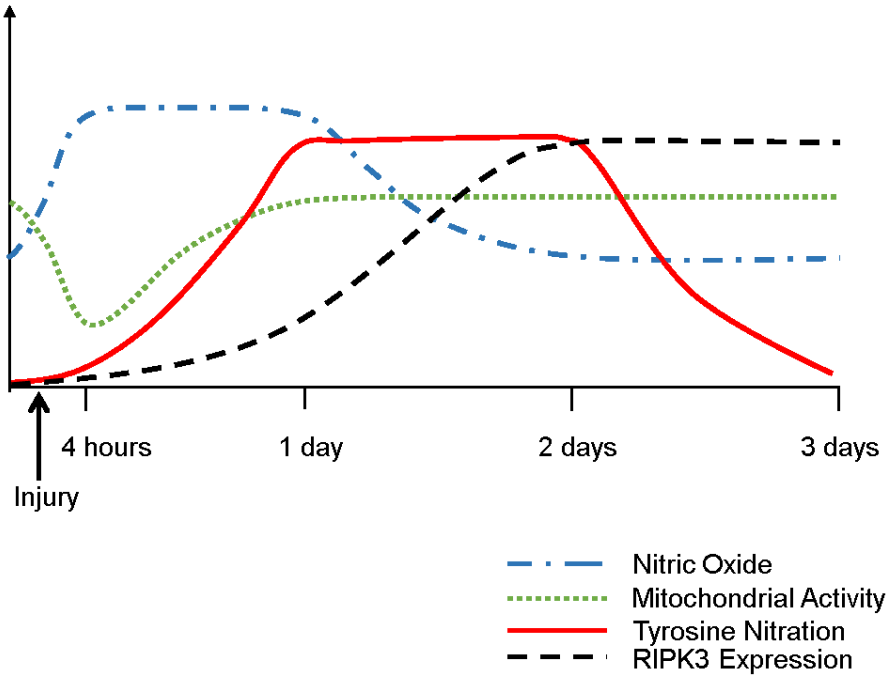

Figure 30: Schematic representation and overview of the dynamics of changes in nitric oxide (NO) levels and related stress markers in the post-traumatic brain.
NO levels increased as early as 4 h after traumatic brain injury (TBI) in the brain in a site-specific-, severity-, and distance-dependent manner with a concomitant reduction in mitochondrial activity restricted to the cortex. 3-nitrotyrosine (3NT) positivity persisted up to day 3 in the ipsilateral cortex despite the recovery of NO and mitochondrial activity to the basal levels. Cell death followed later, with consistent upregulation of receptor-interacting serine/threonine-protein kinase 3 (RIPK3) expression in the cortex (ipsilateral) and caspase-3 activation in the cortex in the injury vicinity.

The results presented here have significant contributions to the resolution of a long-standing controversy on whether NO is protective or detrimental in the brain injury. Our findings confirmed that it, indeed, has detrimental effects in the injury core (summarized in **Figure 30**). Nevertheless, despite similar NO levels in the ipsi- and contralateral regions after severe TBI, confinement of NO-associated detrimental effects (like mitochondrial dysfunction, nitrosative stress and cell death) to the cortical injury site only, principally proves that post-TBI NO elevation may not necessarily be toxic by itself. It should be noted, however, that beneficial effects (like inflammatory modulation, support of cell survival, cerebral blood flow regulation) were not addressed in this study. Therefore, further research is necessary to find out whether it is part of a protective response in the contralateral regions. Given the ability of NO to support cell survival through PI3K/Akt pathway, analysis of this pathway, for instance, in the ipsi- and contralateral cortex at consistent time points with NO elevations would help better in understanding of beneficial mechanisms. Similarly, NO elevations in the contralateral cortex or hippocampus did not accompany an increase in either iNOS or nNOS; but post-TBI eNOS expression in these regions was not assessed in this study. Nevertheless, 3NT formation was only detected in few capillary-like structures in the contralateral regions suggesting that a considerable portion of NO production after injury could come from endothelial sources. Indeed this might be indicative of beneficial effects through regulation of CBF, despite resulting in nitrosative stress-induced vasoconstriction in a small portion of cerebral capillaries. These could be few of many further research possibilities guided by the data provided in this study. Similarly, the nature of the NO elevations in the liver and heart has to be prospectively analysed in detail, in terms of both causes and consequences, in order to identify the exact role of NO in these vital organs after TBI. From our data, it is not clear whether observed effects refer to NO as a mediator of post-TBI damage in these organs or as part of a protective response to restore homeostasis by counterbalancing the sympathetic overflow and the effect of cathecholamines. Cytokine-induced iNOS activation in cardiac myocytes has already been shown and the resultant NO production has usually been associated to detrimental effects. Absence of an increase in iNOS mRNA expression in the post-TBI heart tissue in our study, already provides a clue in favour of a protective response. Nevertheless, NO-associated detrimental effects in the post-TBI heart, e.g. nitrosative stress, mitochondrial dysfunction, should be addressed as a first step in further work to determine whether the observed NO elevations have any detrimental outcome.

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8. APPENDICES

8.1. APEENDIX A: Supplemental Figures

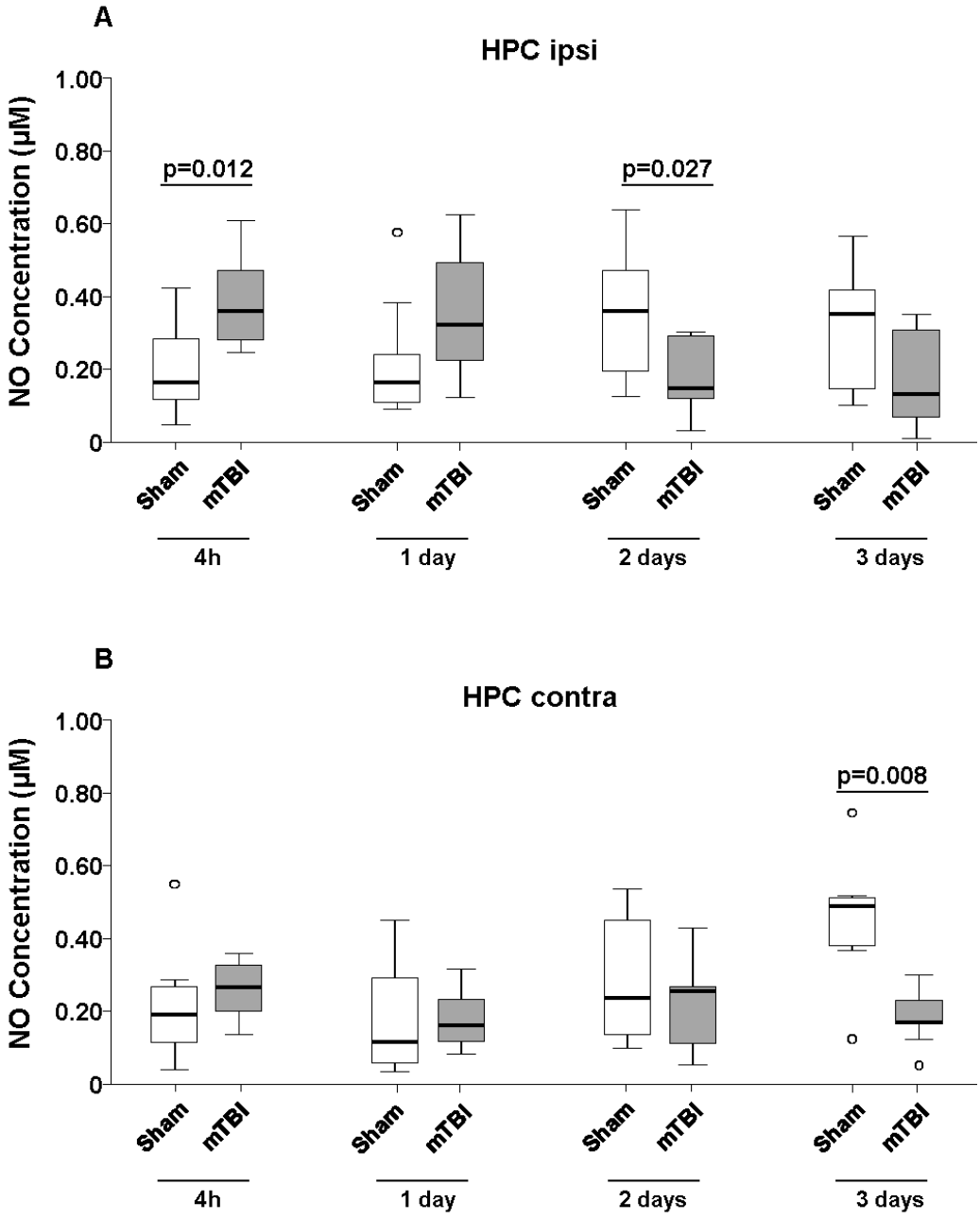

Figure Appendix 1: Nitric Oxide Levels in Hippocampus after Moderate TBI.
Changes in nitric oxide (NO) levels in the hippocampus at the ipsilateral (**A**) and contralateral sides (**B**) at 4 h, and 1, 2, and 3 days following moderate brain injury (**grey boxes**) determined by diethyldithiocarbamate-iron (DETC-Fe) spin trapping and electron paramagnetic resonance (EPR) spectrometry. A significant increase in NO levels was detected in the ipsilateral hippocampus at 4 h, which decreases to basal levels within 1 day. Statistical significance was tested with Mann-Whitney U Test. Open circles show outliers. HPC, hippocampus; mTBI, moderate traumatic brain injury.

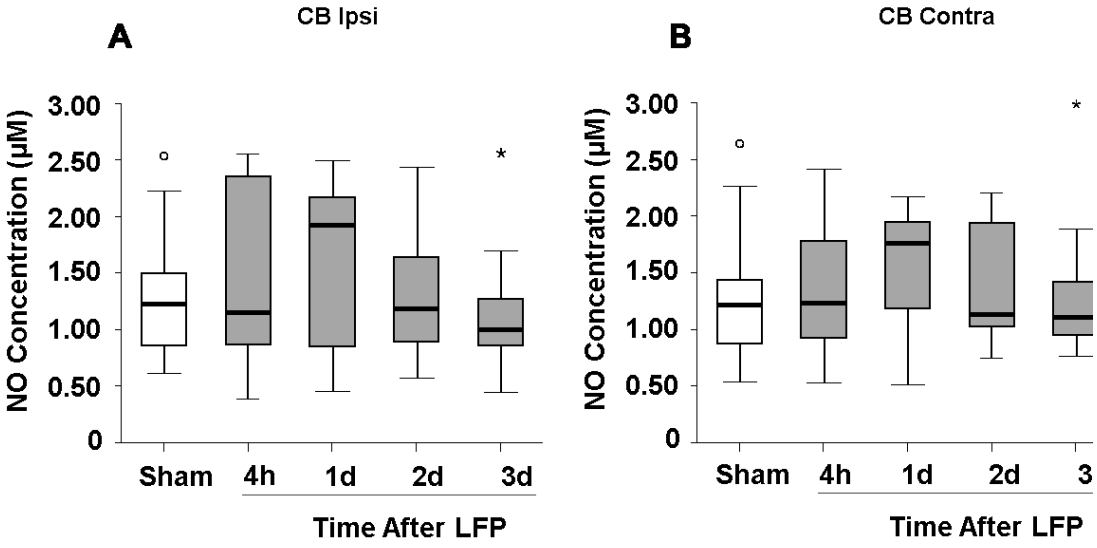

Figure Appendix 2: Nitric Oxide Levels in Cerebellum after Moderate TBI.
Changes in nitric oxide (NO) levels in the cerebellum at the ipsilateral (**A**) and contralateral sides (**B**) at 4 h, and 1, 2, and 3 days following moderate brain injury (**grey boxes**) determined by diethyldithiocarbamate-iron (DETC-Fe) spin trapping and electron paramagnetic resonance (EPR) spectrometry. Although a remarkable increase in average NO levels were observed at 1 day after TBI in both cerebellar hemispheres, changes were not statistically significant. Statistical significance was tested with one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test or Welch's variance-weighted ANOVA followed by Games–Howell test. Open circles show outliers. Stars show extreme outliers. CB, cerebellum; LFP, lateral fluid percussion injury.

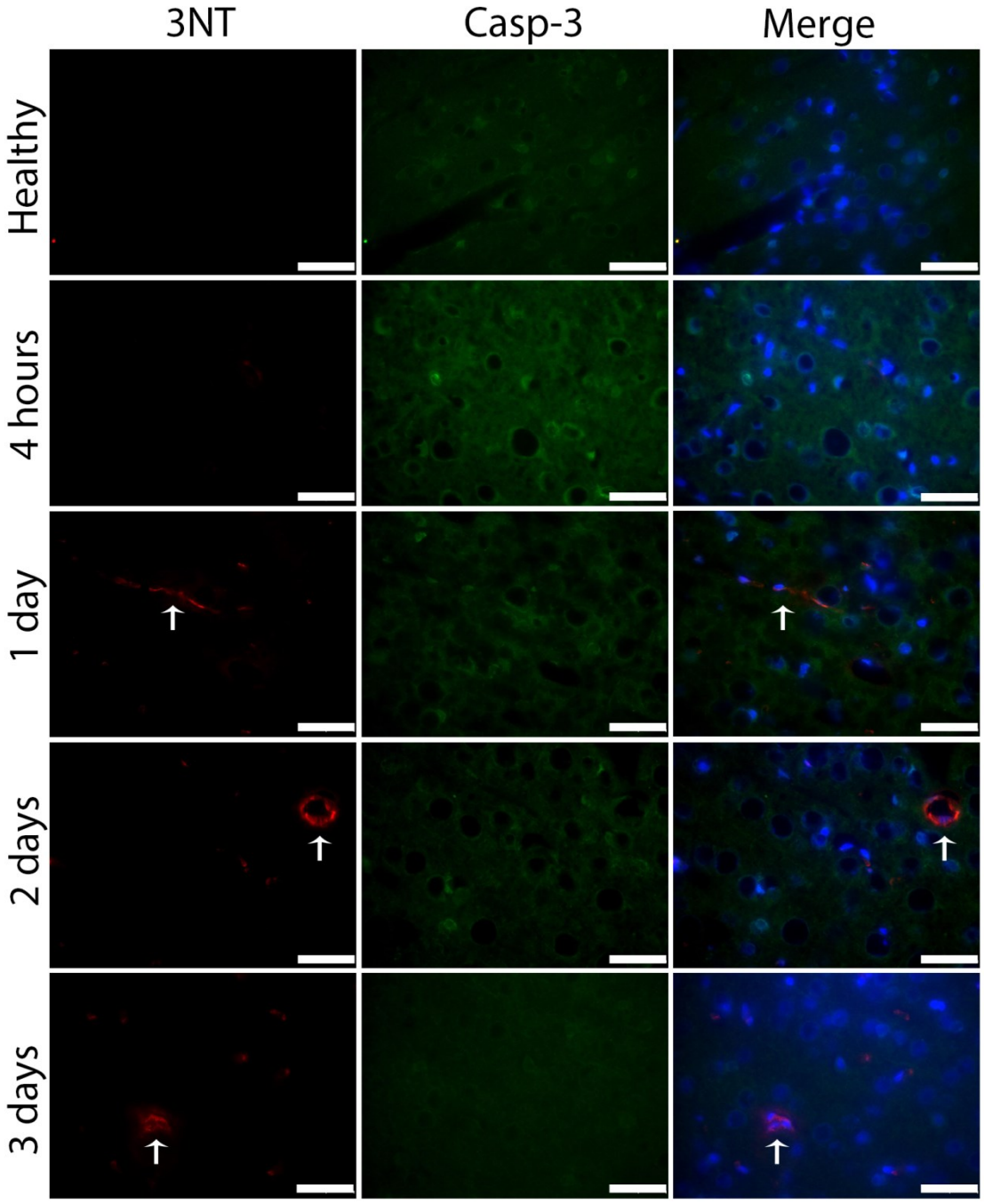

Figure Appendix 3: 3NT and Casp-3 co-immunostaining after severe brain injury.
Co-immunostaining of cortical sections of healthy and brain injured animals for 3-nitrotyrosine (3NT, red) and cleaved caspase (Casp, green)-3 at 4 h, 1 day, 2 days, and 3 days after severe traumatic brain injury (TBI) (contralateral to injury). A major increase in 3NT positivity was not observed at any time point, except some capillary-like structures. There was also no detectable Casp-3 positivity at any time point. Cell nuclei were stained with DAPI (blue) for counterstaining. Scale bars: 10 μm.

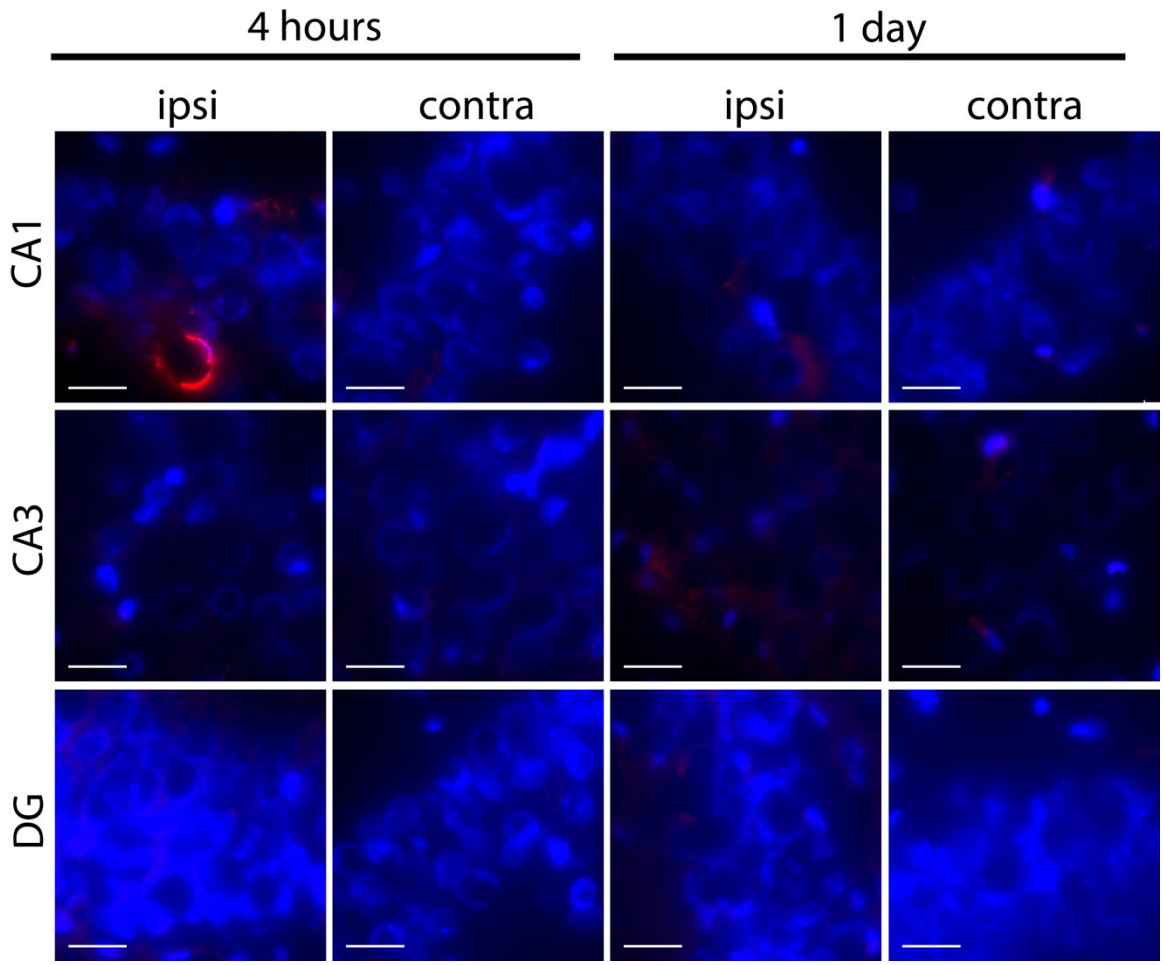

Figure Appendix 4: 3NT staining in hippocampus after severe brain injury.
Immunostaining of hippocampal sections of brain injured animals for 3-nitrotyrosine (3NT, red) at 4 h and 1 day after severe traumatic brain injury (TBI). A major increase in 3NT positivity was not observed at any time point, except some capillary-like structures. Cell nuclei were stained with DAPI (blue) for counterstaining. Scale bars: 10 µm. ipsi: ipsilateral to the injury; contra: contralateral to the injury; CA1 and CA3: cornus ammonis 1 and 3; DG: dentate gyrus.

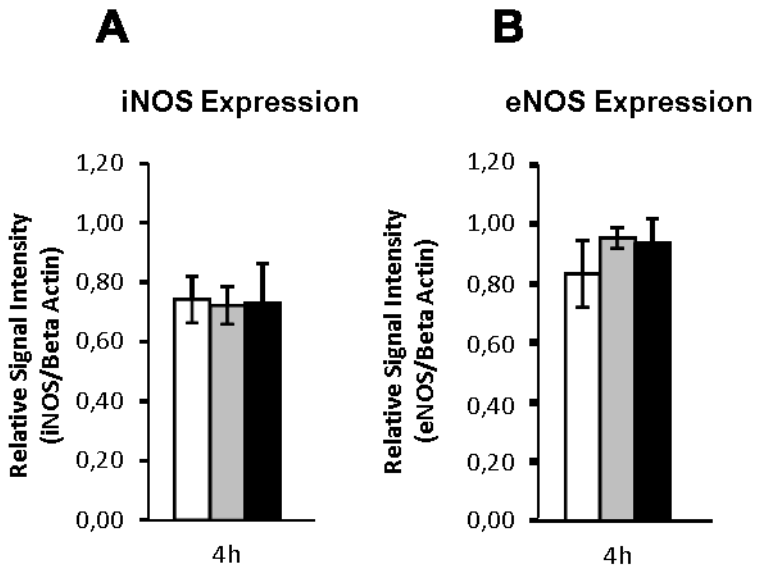

Figure Appendix 5: Expression of iNOS and eNOS in heart after TBI.
mRNA expression levels of inducible nitric oxide synthase (iNOS) (**A**) and endothelial nitric oxide synthase (eNOS) (**B**) in the heart tissue at 4 h after traumatic brain injury (TBI), determined by reverse transcription polymerase chain reaction (RT-PCR). Despite significant increases of cardiac NO levels at 4 h after TBI, a significant change in iNOS or eNOS mRNA expression levels was not observed. Beta actin was used as the reference gene. Data are presented as relative signal intensity of product bands on agarose gel. White columns show sham controls; grey columns show moderate TBI groups and black columns show severe TBI groups. Statistical significance of changes compared with the sham group was tested with the Mann–Whitney U test.

8.2. APPENDIX B: Supplemental Tables

Region/Neurons	NOS expression	NO based experiments	References
Vagus	$nNOS^+$ vagal afferents	neuronal NO facilitates vagally induced bradycardia via a presynaptic modulation of neurotransmission	(Lin et al., 1998), (Choate et al., 2001)
DMNV	$nNOS^+$	NO donors or L-Arg increases the firing rate of DMNV neurons	(Lin et al., 2000b), (Dun et al., 1994), (Travagli and Gillis, 1994)
SPNs	$nNOS^+$		(Blottner and Baumgarten, 1992)
RVLM	$nNOS^+$	NO as a sympathoinhibitory agent in RVLM	(Patel et al., 1996), (Zanzinger et al., 1995b)
RVMM	$nNOS^+$ cell populations that project to NTS and nucleus ambiguus		(Babic et al., 2008)
Caudal Raphe Nuclei	$nNOS^+$ cells		(Dun et al., 1994)
PVN	$nNOS^+$	nNOS inhibition increases the basal firing activity of PVN neurons; inhibitory feedback loop for sympathetic outflow is mediated by NO	(Li et al., 2003), (Bains and Ferguson, 1997), (Latchford and Ferguson, 2003), (Li et al., 2002), (Li et al., 2004), (Ferguson et al., 2008)
CVLM	$nNOS^+$	L-Arg application mediates inhibition of CVLM, which leads to disinhibition of RVLM and potentiates sympathoexcitation.	(de Castro et al., 2012), (Sun and Guyenet, 1985), (Sved and Gordon, 1994), (Phattanarudee et al., 2013)
Medullary LTF	$nNOS^+$	$nNOS^+$ neurons were activated during stimulation of cardiac sympathetic afferents	(Maqbool et al., 1995), (Guo and Longhurst, 2003)
Area Postrema	$nNOS^+$	L-Arg injection did not show significant cardiovascular effects, but $nNOS^+$ neurons were activated in response to ADM. Ablation of this area led to a reduction in activation of $nNOS^+$ neurons in other centres, like PVN	(Rodrigo et al., 1997), (Tseng et al., 1996), (Shan and Krukoff, 2000)
Parabrachial Complex	$nNOS^+$	$nNOS^+$ neurons were activated in response to stimulation of cardiac afferents; ADM acts in the LPBN to increase MAP through mechanisms dependent on activation of ionotropic glutamate receptors, NO synthesis via nNOS and eNOS, and L-type calcium channel activation.	(Dun et al., 1994), (Guo et al., 2005), (Geambasu and Krukoff, 2008)
Locus Coeruleus	$nNOS^+$, sGC^+	NOS inhibitors and haemoglobin enhanced the excitatory postsynaptic potential (EPSP) evoked by focal electrical stimulation of the slice, the effect was reversed by L-Arg; NO activates LC neurons through cGMP pathway; intra-LC administration of NMDA increases NO signal	(Xu et al., 1994), (Matsuoka et al., 1992), (Pineda et al., 1996), (Desvignes et al., 1997)
Midbrain PAG	specific distribution of nNOS expression mainly in dlPAG (dlPAG receives projections from many forebrain nuclei, which are largely glutamatergic, NOS^+ and $NMDAR^+$)	NO donors inhibit neuronal activity in PAG; within the dl-PAG NO potentiates the synaptic release of GABA, which presynaptically inhibits glutamate release through $GABA_B$ receptors on glutamatergic terminals and sustains tonic inhibition of dlPAG neurons through $GABA_A$ receptors.	(Onstott et al., 1993), (Rizvi et al., 1991), (Rizvi et al., 1992), (Alvin J. Beitz, 1991), (Onstott et al., 1993), (Albin et al., 1990), (Lovick and Key, 1996), (Xing et al., 2008) (this is similar to PVN system)

Continued

Region/Neurons	NOS expression	NO based experiments	References
NTS	$nNOS^+$	NO-donors or L-Arg: cardiodepressor; NO-inhibitors: cardiopressor and lead to increase in RSNA	(Ruggiero et al., 1996), (Lin et al., 2000a), (Lin et al., 2000b), (Dun et al., 1994), (Jimbo et al., 1994), (Zanzinger et al., 1995a), (Vitagliano et al., 1996), (Tseng et al., 1996), (Lin et al., 1999), (Harada et al., 1993)
Nucleus Ambiguus	$nNOS^+$	NO donors: decrease in HR	(Dun et al., 1994), (Ruggeri et al., 2000)
Insular Cortex	$nNOS^+$	NO modulates unitary IPSPs (uIPSP) here depending on the presynaptic cell type (enhancement or suppression)	(Wehby and Frank, 1999), (Huh et al., 2000), (Kim, 2005), (Yamamoto et al., 2015)

Table Appendix 1: Regions that regulate autonomous control of sympathetic and parasympathetic nervous system.
Autonomous control of cardiovascular system is achieved via several brain regions and neurons. Action of NO has been implicated in many of them. DMNV: dorsal motor nucleus of Vagus, SPNs: sympathetic preganglionic neurons, RVLM: rostral ventrolateral medulla, RVMM: rostral ventromedial medulla, PVN: paraventricular nucleus of hypothalamus, CVLM: caudal ventrolateral medulla, LTF: lateral tegmental field, PAG: periaqueductal grey, dlPAG: dorsolateral PAG, NTS: solitary tract nucleus, LPBN: lateral parabrachial nucleus, LC: locus coeruleus, RSNA: renal sympathetic nerve activity, EPSP: excitatory postsynaptic potential, IPSP: inhibitory postsynaptic potential, ADM: adrenomedullin, MAP: mean arterial pressure, GABA: gamma aminobutyric acid, NMDA: N-methyl D-Aspartate, L-Arg: L-Arginine.

A

	Group	Mean FPI Pressure (atm)	SEM	Mean weight (g)	SEM	Nr. of animals
		Group Statistics of Animals for NO Measurement post-TBI				
Sham	4h post-surgery	-	-	296,11	15,32	9
	1d post-surgery	-	-	284,89	11,84	9
	2d post-surgery	-	-	301,67	17,67	9
	3d post-surgery	-	-	323,56	8,61	9
Moderate TBI	4h post-TBI	1,89	0,07	296,09	15,91	11
	1d post-TBI	1,91	0,07	307,11	16,89	9
	2d post-TBI	1,89	0,05	279,22	11,26	9
	3d post-TBI	2,01	0,06	282,33	10,70	9
Severe TBI	4h post-TBI	2,67	0,02	277,00	11,82	9
	1d post-TBI	2,66	0,03	279,56	20,59	9
	2d post-TBI	2,63	0,02	313,67	18,93	9
	3d post-TBI	2,67	0,02	326,33	16,57	9

B

		Cortex		Hippocampus		Cerebellum	
		Group Statistics of Samples Measured for NO (number of samples)					
		ipsi	contra	ipsi	contra	ipsi	contra
Sham	4h post-surgery	7	8	8	8	8	8
	1day post-surgery	7	9	9	9	9	9
	2 days post-surgery	9	9	9	9	9	9
	3 days post-surgery	9	9	9	9	9	9
Moderate TBI	4h post-TBI	9	9	8	8	9	8
	1 day post-TBI	9	9	7	7	9	9
	2 days post-TBI	9	9	9	9	9	9
	3 days post-TBI	9	9	9	9	9	9
Severe TBI	4h post-TBI	8	8	8	8	8	8
	1 day post-TBI	9	9	-	-	-	-
	2 days post-TBI	9	9	-	-	-	-
	3 days post-TBI	9	9	-	-	-	-

Table Appendix 2: Group statistics of the animals and the samples for NO measurement.
Details of the number and weight of the animals used for NO measurements, along with trauma strengths (**A**); and number of samples in which NO was quantified (**B**) are provided. Uneven group sizes of animals and measured samples were due to technical problems such as destruction of the tissue during snap freezing or during preparation for measurement. TBI: Traumatic brain injury; LFP: lateral fluid percussion; SEM: standard error mean; g: weight in grams; atm: pressure in unit atmospheres.

Group Statistics for Mitochondrial Activity Measurement

	Group	Mean LFP pressure (SEM) (atm)	Mean weight (SEM) (g)	Nr. of animals
Sham	**4h post-surgery**	-	383,70 (40,53)	10
TBI	**4h post-TBI**	2,54 (0,05)	501,86 (20,93)	7
	1d post-TBI	2,47 (0,05)	239,00 (3,76)	6
	3d post-TBI	2,52 (0,04)	235,33 (3,35)	6

Table Appendix 3: Group statistics of the animals used for mitochondrial activity measurements.
Details of the number and weight of the animals used for mitochondrial activity measurements, along with trauma strengths are provided. TBI: Traumatic brain injury; LFP: lateral fluid percussion; SEM: standard error mean; g: weight in grams; atm: pressure in unit atmospheres.

	Group Statistics for PCR in Hippocampus and Cortex			
	Group	**Mean LFP Pressure (SEM) (atm)**	**Mean Weight (SEM) (g)**	**Nr. of animals**
Sham	**Day of Surgery**	-	229,80 (4,50)	5
	1d post-surgery	-	302,20 (15,78)	5
	2d post-surgery	-	290,60 (7,01)	5
	3d post-surgery	-	327,40 (22,08)	5
Moderate TBI	**Day of Trauma**	2,15 (0,08)	336,80 (22,30)	5
	1d post-TBI	2,37 (0,07)	247,80 (13,81)	5
	2d post-TBI	2,10 (0,04)	284,00 (35,25)	5
	3d post-TBI	2,26 (0,08)	295,40 (14,75)	5
Severe TBI	**Day of Trauma**	2,66 (0,06)	366,60 (24,49)	5
	1d post-TBI	2,82 (0,08)	340,80 (49,72)	5
	2d post-TBI	2,64 (0,04)	289,60 (29,60)	5 (4 for hippocampus)
	3d post-TBI	2,73 (0,05)	290,20 (3,35)	5

Table Appendix 4: Group statistics of the animals used for RT-PCR.
Details of the number and weight of the animals used for PCR analyses, along with trauma strengths are provided. TBI: Traumatic brain injury; LFP: lateral fluid percussion; SEM: standard error mean; g: weight in grams; atm: pressure in unit atmospheres.

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For Figure 7:

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