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José Luís Monteiro Alves

NEUROPEPTIDE RESPONSE IN TRAUMATIC BRAIN INJURY

Doctoral Thesis in Health Sciences, Branch of Medicine, supervised by Full Professor Anabela Mota Pinto and Doctor Ana Paula Pereira da Silva Martins, presented to the Faculty of Medicine of the University of Coimbra.

July 2021

Faculdade de Medicina da Universidade de Coimbra

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Dissertação de Doutoramento apresentada à Faculdade de Medicina da Universidade de Coimbra, no âmbito do Programa Doutoral em Ciências da Saúde, para candidatura ao grau de Doutor em Ciências da Saúde, ramo Medicina, sob a orientação da Professora Doutora Anabela Mota Pinto e da Doutora Ana Paula Pereira da Silva Martins.

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List of Abbreviations

Aβ	Amyloid-beta
APCs	Antigen Presenting Cells
AQP4	Aquaporin-4
BBB	Blood-Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
BM	Basement Membrane
Ca^{2+}	Calcium
CGRP	alcitonin Gene-Related Peptide
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CT	Computed Tomography
CTE	Chronic Traumatic Encephalopathy
DAI	Diffuse Axonal Injury
DAMPs	Damage-Associated Molecular Patterns
DG	Dentate Gyrus
GCS	Glasgow Coma Scale
GFAP	Glial Fibrillary Acidic Protein
GJs	Gap Junctions
Glu	Glutamate
GOS	Glasgow Outcome Scale
HMGB1	High Mobility Group Box-1
HSPs	Heat Shock Proteins
ICAM-1	Intercellular Adhesion Molecule 1
ICP	Intracranial Pressure
IL-1	Interleukin-1
iNOS	Inducible Nitric Oxide Synthase
LOC	Loss of consciousness
Mg^{2+}	Magnesium
MMPs	Matrix Metalloproteinases
MRI	Magnetic Resonance Imaging
NAA	N-acetylaspartate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NFTs	Neurofibrillary Tangles
NKs	Natural Killer cells
NO	Nitric Oxide
NPY	Neuropeptide Y
NSPCs	Neural Stem/Progenitor cells
NVU	Neurovascular Unit

PET	Positron Emission Tomography
p-tau	Phosphorylated tau protein
PTE	Post-Traumatic Epilepsy
PTSD	Post-Traumatic Stress Disorder
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SD	Standard Deviation
SEM	Standard Error of the Mean
SP	Substance P
TAI	Traumatic Axonal Injury
TBI	Traumatic Brain Injury
TCE	*Traumatismo Crânio-Encefálico*
TJs	Tight Junctions
TLRs	Toll-Like Receptors
TNF	Tumor Necrosis Factor
TRP	Transient Receptor Potential
UCH-L1	Ubiquitin C-Terminal Hydrolase-L1
ZO	*Zonula Occludens*

Published work

The results presented in this thesis have been published in international peer-reviewed journals (indexed in PubMed) as follows:

International peer-reviewed journals (indexed in PubMed)

Alves JL, Mendes J, Leitão R, Silva AP, Mota Pinto A. **A multi-staged neuropeptide response to traumatic brain injury.** Eur J Trauma Emerg Surg. 2020; DOI: 10.1007/s00068-020-01431-z (online ahead of print). (**Chapter III**)

Alves JL, Rato J, Silva V. **Why does brain trauma research fail?** World Neurosurg. 2019; 130:115-121. (**Chapter IV**)

Alves JL. **Blood-Brain Barrier and Traumatic Brain Injury.** J Neurosci Res. 2014; 92(2):141-147. (**Chapter I**)

Resumo

O traumatismo crâneo-encefálico (TCE) é um importante problema de Saúde Pública, com impacto significativo na vida das suas vítimas e considerável repercussão em termos sociais e económicos. Para além de sequelas neurológicas major, acumula-se a evidência científica sobre a relação entre o TCE, mesmo o de menor intensidade, e posteriores transtornos da função cognitiva, equilíbrio e coordenação motora. Após o traumatismo inicial, o TCE induz diferentes fenómenos patológicos, como excitoxicidade glutamatérgica, perturbação da barreira hemato-encefálica, edema cerebral e neuroinflamação, que por sua vez determinam consequências a longo-prazo no contexto de dano secundário, tais como neurodegeneração e perturbação das funções cerebrais superiores.

De momento, não existem protocolos terapêuticos eficazes no tratamento do TCE e suas consequências, apesar do conhecimento científico estar, no que respeita aos mecanismos celulares subjacentes, em evolução contínua mas ainda com muitas questões por responder. Estudos recentes ligam a resposta multifatorial pós-TCE e inflamação a níveis elevados de Substância P, entre outras moléculas. A Substância P atua via recetores NK-1, promovendo a permeabilidade da barreira hemato-encefálica e modulando a conhecida hipomagnesémia pós-traumática, com influência direta nos recetores N-metil-D-aspartato (NMDA) e respetivas vias de sinalização e indução de excitotoxicidade.

O Neuropeptídeo Y (NPY), um dos neuropeptídeos mais abundantes no cérebro e pouco estudado em relação ao TCE, demonstra aparentes efeitos neuroprotetores em diferentes contextos patológicos, modulando a excitabilidade hipocâmpica glutamatérgica (via recetores NPY Y2), assim como promovendo a atividade pró-neurogénica (via recetores NPY Y1) e pró-migratória, como evidenciado em modelos animais de isquémia. O NPY desempenha um papel importante na resposta inicial a diferentes eventos (acidente vascular cerebral, epilepsia), provavelmente atuando como modulador do ambiente citotóxico e regeneração neuronal pós-agressão. Porém, o papel do NPY na resposta primária e secundária ao TCE ainda não está esclarecido, considerando todos os mecanismos celulares e neurobiológicos que agravam o dano cerebral inicial (**Capítulo I**).

No presente trabalho, colocou-se a hipótese de que o TCE origina uma resposta neuropeptídica faseada, com um aumento imediato da Substância P e posterior incremento compensatório do NPY, com potencial efeito neuroprotector. Do ponto de vista translacional, a "ciência básica" permite responder a questões ao nível celular e molecular como um meio de melhorar a prática clínica e respectivo *outcome* ("*from bench to bedside and back again*"). Assim, este trabalho inclui procedimentos experimentais que poderão interferir com a resposta pós-traumática secundária. De modo a elucidar o papel do NPY e o seu potencial terapêutico numa administração exógena, foi utilizado um modelo animal de TCE (protocolo de traumatismo por "queda de peso") (**Capítulo II**). Com este trabalho foi possível demonstrar o efeito neuroprotetor do NPY numa condição de TCE, prevenindo ou atenuando diferentes consequências deletérias, incluindo disrupção da barreira hemato-encefálica, morte neuronal, ativação das células da glia (astrócitos e microglia) e neuroinflamação.

Relativamente à componente clínica, um abrangente protocolo de colheitas de amostras de sangue em vítimas humanas de TCE permitiu estudar a resposta neuropeptídica faseada (**Capítulo III**), com significativas flutuações temporais nos seus níveis (incluindo um aumento precoce nos níveis de SP e um incremento bimodal nos níveis de NPY), assim como óbvias alterações iónicas e variações nos níveis de S100B, um conhecido biomarcador no TCE.

Finalmente, o **Capítulo IV** inclui uma discussão geral e considerações sobre o estado atual, dificuldades e direções futuras na investigação em TCE, nomeadamente na sua vertente translacional.

Palavras-chave: Glia; Modelos animais; Neuroinflamação; Neuropeptídeo Y; Substância P; Traumatismo Crânio-Encefálico.

Summary

Traumatic Brain Injury (TBI) is a major public health problem, with considerable clinical impact on its victims and a tremendous economic and social burden. Besides significant neurological sequelae, scientific evidence shows that, even in mild cases, TBI can be responsible for long-term deficits and impairments concerning cognitive function, balance and motor coordination. Following initial injury, TCE promotes different pathological events, such as glutamatergic excitotoxicity, Blood-Brain Barrier (BBB) breakdown, brain edema and neuroinflammation, and long-term consequences in the context of secondary injury, with neurodegeneration and impairment of higher functions.

Presently, there is no successful therapeutic protocol for TBI in all its forms, although the knowledge regarding mechanisms underlying cellular damage is continuously evolving but still with many open questions. Recent reports link post-TBI multifactorial response and inflammation to increased Substance P (SP) levels, among several other molecules. SP acts via NK-1 receptors, increasing BBB permeability and modulating the well-known post-traumatic hypomagnesemia, with direct influence on NMDA receptors signalling pathway and excitotoxicity.

Neuropeptide Y (NPY), one of the most abundant neuropeptides in the brain and scarcely studied regarding TBI, has been shown to display neuroprotective effects in different pathological contexts by modulating glutamatergic hippocampal excitability (via receptors NPY Y2), as well as by having a pro-neurogenic (via NPY Y1 receptors) and pro-migratory activity, as shown in ischemia animal models. NPY plays a role in the primary response to different events (stroke, epilepsy), arguably acting as a modulator for both post-aggression cytotoxic environment and neuronal regeneration. However, NPY's role in the primary and secondary response to TBI is yet to be elucidated, considering all cellular and neurobiological mechanisms that further aggravate initial brain damage (**Chapter I**).

In the present study, TBI was hypothesized to lead to a multistage neuropeptide response, with an immediate increase in SP, followed by a compensatory NPY upregulation with a potential neuroprotective effect. From a translational perspective, basic science is essential to clarify the cellular and molecular mechanisms in order to improve clinical practice and outcome ("*from bench to bedside and back again*"). Therefore, this work includes experimental procedures that ultimately interfere with the secondary post-traumatic response. In order to better elucidate NPY's role and its therapeutic potential, an animal model of TBI (*weight drop injury* protocol) was used (**Chapter II**). A neuroprotective effect of NPY was uncovered, since it prevented or attenuated several TBI deleterious effects, including BBB disruption, neuronal death, microglia and astrocyte activation and overall neuroinflammation.

A comprehensive blood-sampling protocol on human TBI victims allowed us to conclude about a multistage neuropeptide response (**Chapter III**), with significant fluctuations in its levels (including an early increase in SP and a bimodal rise in NPY) and revealing timings concerning ionic disturbance and variations in S100B levels, a well-known TBI biomarker.

Finally, **Chapter IV** includes a general discussion and considerations over the current status, obstacles and future directions in TBI research with a translational perspective.

Keywords: Animal models; Glia; Neuroinflammation; Neuropeptide Y; Substance P; Traumatic Brain Injury.

"A physician is obligated to consider more than a diseased organ, more even than the whole man - he must view the man in his world."

Harvey Cushing

CHAPTER I

Traumatic Brain Injury

1.1 Introduction

TBI is a common clinical situation, one of the most frequent trauma events in paediatric and adult patients. It is an unforeseen and rapidly evolving occurrence, with multiple causes (traffic accidents, falls, firearms and others) and potentially devastating consequences for the victims, their families and society,[1] along with significant healthcare costs. Affecting both young and older people, with distinct epidemiological contexts,[2] its clinical presentation ranges from mildly injured victims with no apparent lesions to severely injured, comatose patients, in need of Neuro-Intensive Care intervention and long term rehabilitation.[3]

TBI is classically defined as damage to the brain due to an external mechanical force, such as impact, crush, penetration by a projectile, blast waves or others.[4] The mechanisms of injury can be divided into two types, corresponding to two different and quasi-sequential stages as follows:

1) primary injury, due to mechanical forces inducing tissue deformation at the moment of injury, with immediate/early structural disruption of brain tissue (including contusions, haemorrhages and axonal stretching);[5]

2) secondary injury, starting a few moments after the initial trauma, with a complex cascade of events, such as excitotoxicity, BBB breakdown, hypoxic damage, ischemic phenomena, and others,[4, 6] affecting all Central Nervous System (CNS) components (neuronal and glial cells, neurovasculature) and leading to structural and functional impairment. In theory, and from a therapeutic perspective, it is possible to act on secondary injury events given their prolonged duration (from seconds to several days following initial injury). Primary injury can only be prevented, not attenuated or reversed.

Concerning the clinical dimension of TBI, major deficits and neurological findings (impaired consciousness, motor deficits, seizures) are frequently accompanied by unspecific symptoms (namely headaches and dizziness) and minor cognitive deficits. These findings usually become obvious in the first hours post-TBI[7] and might persist up to 2 weeks, as part of a transitory post-concussion syndrome or as more permanent sequelae of the initial trauma.[8]

Despite contemporary sophistication and accessibility to imaging exams and updated clinical protocols, there is an urgent need for reliable and straightforward therapeutical tools, able to interrupt self-sustained pathophysiology mechanisms and improve long-term neurological-cognitive sequelae.

1.2 Epidemiology

TBI is a major public health issue, with a significant impact on its victims and society, a prevailing cause of long-term disabilities[9] with a non-negligible economic burden.[10] Being a leading cause of death below 45 years of age, TBI accounts for over 10 million deaths or prolonged hospitalization yearly worldwide, significantly affecting more than 50 million people per year (data drastically subject to underestimation).[11, 12]

Public Health authorities are increasingly more active in this field, as TBI is recognized as a "silent epidemic",[13] globally spread but with a predilection for developing countries (nearly three times higher, due to the contribution of road traffic accidents).[14]

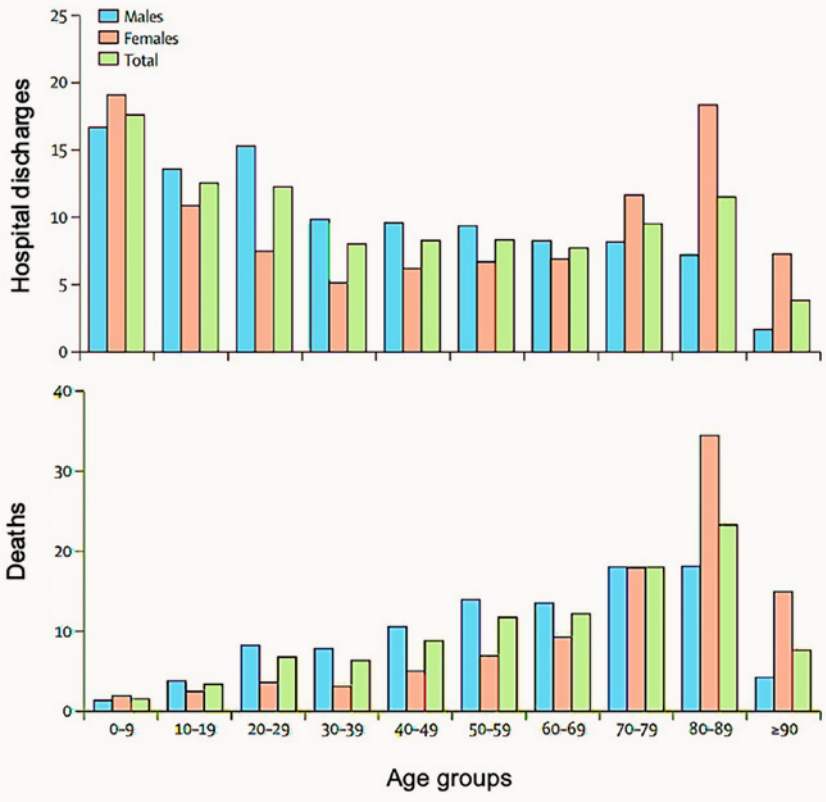


Figure 1.1 - Epidemiology of Traumatic Brain Injury in Europe by age group (hospital discharges and deaths). Recognizable trends are present, including male predominance until age 60-69 and overall increased mortality in older patients (adapted from Majdan et al.,[15] with permission).

Majdan and colleagues,[15] in a report concerning European reality **(Figure 1.1)**, have shown TBI causing 37% of all injury-related deaths, extrapolating it to about 82.000 deaths and about 2.1 million hospital discharges in Europe.[15] Substantial inter-countries disparities were also obvious - for an estimated global age-adjusted mortality of 11.7/100.000, data ranged from 3.6 to 21.8.[15] Dissimilarities in reported cases, hospital discharges and related deaths are most likely due to a combination of factors, such as disparities in data coding and collection procedures, imbalance in the accuracy of data collection and case reporting and different interpretations in administrative coding systems.[2, 15]

Peeters and colleagues[16] undertook a similar epidemiological analysis concerning Europe, although with a distinct methodology that included a meta-analysis based on PubMed Electronic database search. Again, significant variability was evident in reported incidence among different countries (from 47.3 up to 546/100.000/year), severe TBI incidence (from 4.1 to 17.3/100.000/year in some areas of France) and admission for TBI (overall incidence of 262/100.000/year). This type of injury is consistently more prevalent in two age groups: ≤25 years and ≥75 years. A clear shift from road traffic accidents to falls as the main cause for TBI is noticeable, with only 2 recent studies mentioning road traffic accidents as the leading cause for TBI.[16]

Most studies display a similar trend towards TBI-related deaths, mostly in older patients and with an obvious male predominance. Male-female ratio ranged from 1.6 to 4.7 (Latvia), with an overall global average of 2.2.[15] Roozenbeek and colleagues[2] describe a trend towards increasing age and percentage of patients older than 50 years. Absolute incidence of TBI among the elderly rises, as life expectancy increases and higher mortality within old age groups is expected, due to significant morbidities and chronic medication (anticoagulants, platelet aggregation inhibitors).[16, 17] Even so, TBI is a major cause of death or severe disability in the paediatric population[17, 18] and remains the most common cause for disability in childhood.[18] Other authors mention the relative predominance of TBI in lower social-economic groups and its frequent under-reporting.[19]

Regarding Portugal, Santos and colleagues[20] reported an incidence rate of 65/100.000 and a mortality rate of 10/100.000 individuals, both regarding 2014. Importantly, people aged 80 or older presented with a much higher mortality rate (57/100.000 individuals). As in most reports, TBI's incidence and severity were greater in men, displaying higher mortality (2,3:1 compared to females) and number of hospitalizations (1,4:1).[20] Other reports show an overall male-female ratio of 3:1 and a decreased overall incidence in the last decades, accompanied by an increase in TBI's severity and mortality (up to 10,6% in patients admitted to portu-

guese hospitals).[21, 22] Most importantly, all epidemiological studies agree that these numbers most likely underestimate the incidence of TBI and its implications.[2, 15]

Concerning the United States of America, TBI is implicated in 18.4 deaths/100.000/year,[23] as the cause for 30% of all deaths related to traumatic events.[24] Overall mortality due to TBI decreased 8.2% in the last decade, due to an obvious improvement in healthcare and road safety, although slightly counterbalanced by a minor increase in falls.[23]

Two global trends are noticeable: TBI incidence is increasing in low-middle income countries (due to broader use of motor vehicles); TBI episodes due to falls are increasing in high-income countries.[2, 25]

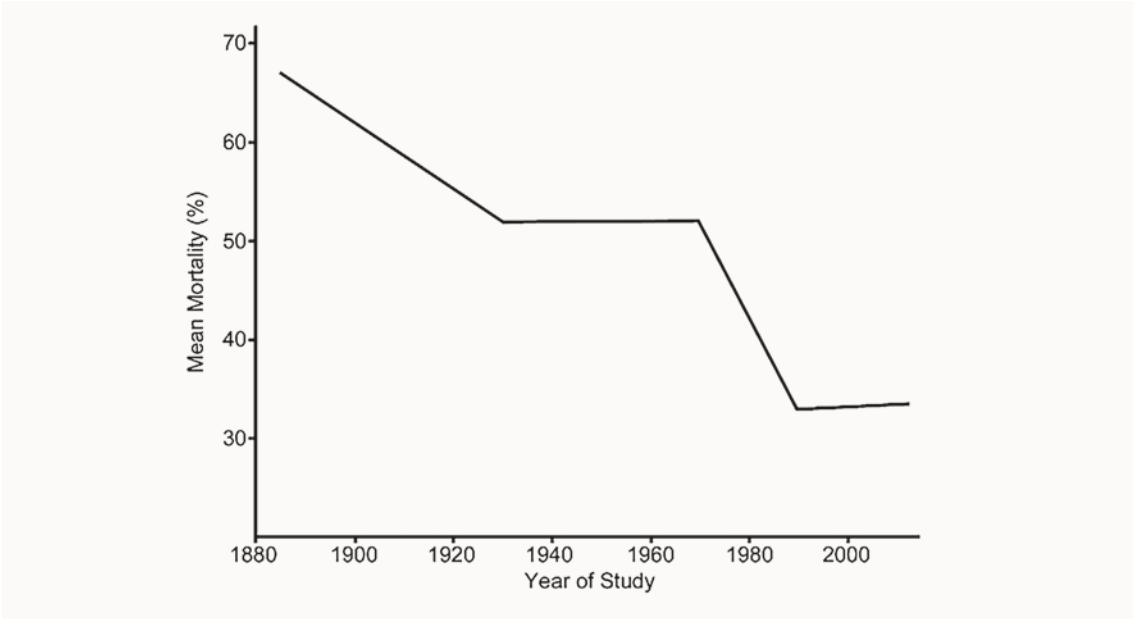


Figure 1.2 - Mortality rates concerning TBI between 1885 - 2006. A decline in overall mortality includes two obvious plateaus (adapted from Stein et al.,[26] with permission).

Stein and colleagues[26] undertook a meta-analysis of 207 case-series (140.000 patients, time span of 150 years until 2006) concerning severe closed TBI **(Figure 1.2)**. The mortality rate in severe TBI victims has significantly decreased over this time period (approximately 50%). However, this decrease in mortality is far from uniform, with a major fall in mortality in the 1970-1990 period and a relatively stagnant curve since 1990 **(Figure 1.2)**.

This comprehensive set of data provides an accurate picture of 3 distinct realities: the introduction of CT scans and advances in Intensive Care as game-changers in

mortality-curve progression (1970-80s); a relatively recent epidemiological shift, as previously depicted, towards an elderly population,[2] withholding any additional advancement in the mortality curve; a lack of impactful advancements in therapeutic or diagnostic modalities in recent decades. A meta-analysis of observational studies concerning severe TBI [300 patients, outcome assessment with Glasgow Outcome Scale (GOS)] also showed no obvious reduction in mortality or unfavorable outcome in recent years.[27]

1.3 Clinical context

The clinical picture in TBI is defined by the mechanism of injury **(Table 1.1)** and consequent macro- and microstructural disruption, as well as neurobiochemical changes (early and late-onset), causing both nonspecific functional impairments and focal, objective findings and deficits (motor and sensory) due to intracranial/parenchymal lesions.

Table 1.1 - Main types of Traumatic Brain Injury, according to mechanism of injury. Legend: TBI, traumatic brain injury (adapted from Logsdon et al.[28]).

TBI Type	Mechanism of injury
Closed Head Impact	Impact from a blunt object
Penetrating injury	Object that fractures the skull, penetration into the parenchyma
Blast injury	Blast energy from exploding device Acceleration/deceleration injury
Fall	Rapid skull impact on the ground (or other)
Concussion	Temporary altered state of consciousness, violent blow
Shaken baby syndrome	Rapid acceleration/deceleration injury, abusive shaking

1.3.1 Initial clinical assessment

Initial clinical assessment and management procedures are based on updated Evidence-Based Medicine guidelines, with evolving but well-defined protocols: initial evaluation, patient stabilization and standard clinical-neurological assessment.[29, 30] According to specific criteria, imaging evaluation ensues (usually, non-contrast CT scan), followed by secondary assessment and possible surgical intervention.[30, 31] Glasgow Coma Scale (GCS) is a consensual tool in providing an overall view of the individual's neurologic condition following trauma **(Table 1.2)**. Upon adding obtained scores in 3 components (best response in each), the GCS score varies from 3 to 15. Besides establishing an objective reference for further neurological assessments, it will provide an overall picture of TBI severity as follows (classification subject to variations and more comprehensive versions)[32]: GCS score 14-15 (mild TBI), representing more than 80% of all TBI cases[33]; GCS score 9-13 (moderate TBI); GCS score 3-8 (severe TBI).[34, 35]

Table 1.2 - Glasgow Coma Scale.

	Eye response	Verbal response	Motor response
6	-	-	Obeys commands
5	-	Orientated	Localising pain
4	Opens spontaneously	Confused	Withdrawal from pain
3	Open to verbal command	Inappropriate words	Flexion to pain
2	Open to pain	Incomprehensible sounds	Extension to pain
1	No response	No response	No response

While many authors only accept GCS scores of 14 and 15 to be considered mild TBI,[35] others still include a score of 13 in this group.[36, 37] As expected, the GCS

3-8 (severe TBI) group has the highest mortality and morbidity.[36] Other injury severity-based scales are available,[38] with expected strengths and shortcomings. One can also use classifications based on pathoanatomic features (contusions, haematomas, subarachnoid haemorrhage or others), mechanism of injury or pathophysiologic mechanisms (primary vs. secondary injury).[38]

> Initial findings (focal deficits, coma), largely associated with what is classically denominated "primary injury", arise from TBI inflicting a structural disruption due to direct trauma and associated lesions, such as brain contusions and lacerations, intracranial haematomas, skull fractures and intracranial bony fragments.[39] These mechanisms of injury are not mutually exclusive and will be associated with brain edema, increased Intracranial Pressure (ICP), compression of brain structures and brain herniation (see Primary Injury section).[40, 41]

Loss of consciousness (LOC) is also helpful in assessing TBI's severity, with mild TBI presenting with less than 30 minutes of LOC or mental changes.[42] It has been reported that up to 37.5% of patients sustaining blunt head trauma experience LOC.[43] Many mechanistic hypotheses have been described: disturbance in the Reticular Activating System; nerve fibers shearing strains and functional decoupling; dorsal pontine inhibitory cholinergic system activation.[42, 44]

1.3.2 Concussion and post-concussion syndrome

Historically, concussion has been defined as a disturbance in consciousness as a result of non-penetrating TBI and with no underlying macroscopic brain structural lesion, tipically following rapid acceleration/deceleration of the head.[45] The American Academy of Neurology defined concussion as "clinical syndrome of biomechanically induced alteration of brain function typically affecting memory and orientation, which may involve loss of consciousness".[46] This disturbance of consciousness must be brief, although there is no defined consensual time frame, with a disputed definition of concussion in itself, as well in its usefulness.[47, 48] There are no pathognomonic findings in imaging, despite frequent minor brain edema and sulcal effacement for reactive hyperemia.[49] Post-traumatic Magnetic Resonance Imaging (MRI) will display relevant findings in up to 25% of patients with normal CT.[50]

Following TBI, a myriad of often vague symptoms, including depression, irritability, chronic fatigue, headaches, insomnia and post-traumatic stress, can be present[51, 52] This multitude of symptoms are frequently included in a single designation of post-concussion syndrome **(Table 1.3)**.

Table 1.3 - Post-concussion syndrome symptoms (adapted from Maruta et al.[53]).

Cognitive disturbance
Disturbance in judgement
Short and long term mnesic disturbances
Difficulty in focusing
Psychosocial and personal variables
Diminished sexual drive
Chronic fatigue
Personality changes
Irritability
Depression
Emotional lability
Disruption in sleep architecture
Somatic symptoms
Headaches
Vasovagal symptoms
Anosmia
Blurred vision, unspecific visual complaints
Tinnitus
Photophobia
Phonophobia
Dystonia
Hypoacusis
Dizziness

Overall disturbance in consciousness includes confusion, amnesia or obvious LOC. Confusion is a common sign, either immediately post-TBI or of late-onset.[54] LOC is present in a minority of patients, likely due to RAS temporary disruption,[55] associated with memory and spatial-temporal notion impairment. Prolonged LOC or significant memory impairment are an indication of potential brain injury and not just a simple concussion.[46] Electrophysiologic studies have shown cortical spreading depression patterns, another likely contribution for transient mental status changes.[49, 56] Another common symptom is post-traumatic amnesia (retrograde or anterograde), with its length correlating with TBI's severity.[57, 58] All post-concussion symptoms can also be part of more severe forms of TBI.

There is significant controversy surrounding post-concussion syndrome, certainly reinforced by considerable heterogeneity in research protocols and diagnostic criteria and relevant methodological flaws.[59, 60] Even so, a meta-analysis based on 6 studies in post-TBI neurological status and performance (after 6 months) has shown persistence of symptoms in 14-26% of victims.[59, 61]

1.3.3 Epilepsy

Post-traumatic epilepsy (PTE) is a known consequence of TBI, notably difficult to manage in many cases,[62] accounting for up to 20% of all epilepsy cases.[63] Several studies mention a range of estimated incidence of 2.9-50%,[64] from early-onset epilepsy (until 7 days post-TBI) to several years after the initial injury.[65] The risk for PTE is highest in focal/penetrating TBI.[66, 67] Concerning mild TBI,[67] studies have shown a statistically significant risk for PTE.[68, 69] Diffuse injuries have also been mentioned to increase PTE's incidence.[67, 68]

Glutamate homeostasis impairment is arguably involved as an epileptogenic factor.[70, 71] Concerning diffuse TBI, changes in potassium and glutamate transport in astrocytes are likely involved, along with loss of homeostatic functions, by disturbing loco-regional neuronal units and synaptic connections.[67]

Concerning typical clinical picture, PTE is classified as of **immediate onset** (within 24h of trauma, lasting for brief seconds, spontaneous termination), **early-onset** (within the first 7 days, frequently with underlying traumatic findings, recurrence in 25% of cases) or **late-onset** (after 7 days, usually with underlying traumatic findings, recurrence in up to 70% of cases, requiring anticonvulsants).[72, 73]

Apparently, PTE's relative risk is higher in women, patients with a family history of epilepsy and in the first 6 months following injury, although stratified risk models show higher susceptibility up to 10 years later.[73, 74]

1.3.4 Cognitive disturbance

Cognitive and behavioural disturbances are present in 5 to 15% of all TBI victims[75] as a frequent feature of brain trauma.[58, 76] Post-TBI minor cognitive deficits (memory impairment, learning disabilities, attention deficits) are directly related to cortical and hippocampal neuronal loss.[76, 77] Three independent factors were identified as increased risk signifiers - age, educational level and pre-existing psychiatric disturbance.[75]

Post-TBI cognitive disturbance is, in part, explained by structural disruption upon hippocampal neuronal damage and loss,[78] including synaptic signalling impairment and deafferentation of CA1 hippocampal subregion.[79] Long-term changes in potentiation capacity of CA1 subregion, directly involved in learning and memory skills, are therefore implicated in long-term clinical repercussion of trauma.[80] When in the presence of abnormal initial CT, patients consistently underperform in most neuropsychological measures, including learning and episodic memory, and corresponding GOS scores, even at 1 year following trauma.[81] Children and adolescents victims of isolated sports-related TBI also present lower-than-expected neurocognitive performance up to 3.5 years post-TBI.[82] Persistent cognitive and performance impairments, indirectly assessed by academic achievements, are a direct consequence of childhood TBI.[83, 84]

Extensive research in animal models of trauma shows TBI's impact on cognitive and motor performance.[85, 86] Included in overall post-traumatic cognitive disturbance and related to hippocampal function,[76, 87] learning and spatial memory impairment are also shown to be underperforming in post-TBI context, persisting for over a year.[86] In TBI victims, similar findings are well reported in distinct assessment protocols and neuropsychiatric evaluation following head trauma.[7, 88]

Other possible sequelae, including emotion processing impairment, might also be attributed to hippocampal damage, in light of new theories of a complex integration of mnesic-cognitive mechanisms and emotional states of anxiety and avoidance learning.[89, 90]

1.3.5 Neuropsychological symptoms

Neuropsychological symptoms (confusion, irritability, impulsiveness, depressive humour) are frequent and coexist with more serious psychiatric conditions, such as depression, Post-Traumatic Stress Disorder (PTSD) and suicidal ideation.[91, 92] As much as 22% of all patients experiencing mild to moderate TBI develop a psychiatric disorder within a year post-TBI, of varying severity.[93] In severe TBI victims, 62.5% present, at 1 and 6 months post-TBI, with a higher risk for depression (incidence up to 11%),[94, 95] PTSD (incidence as high as 30%),[96] chronic fatigue, insomnia and lesser quality of life.[52, 59]

It is noteworthy that the true incidence of these symptoms is yet disputed.[52, 97] As some studies rely on self-reported symptoms and recovery, this fact might partially explain disparities found in the literature.[98] Cultural and circumstantial differences in medico-legal litigation might also explain disparate reported outcomes.[99]

The subjectivity in appreciating mental/psychiatric issues is unavoidable and will remain an obstacle for those seeking proper validation of therapeutic strategies.[100]

1.3.6 Chronic Traumatic Encephalopathy

Initially described in professional boxers (*dementia pugilistica*),[101] with their typical slurred speech and cognitive-behavioural issues, Chronic Traumatic Encephalopathy (CTE) is a well-defined clinical entity, described in several other contexts and sports and associated with repetitive head impacts.[102, 103] A whole range of symptoms, with varying intensity and significance, is generally divided into motor, cognitive and psychiatric symptoms **(Table 1.4)**.

Table 1.4 - Main symptoms in Chronic Traumatic Encephalopathy (adapted from Fesharaki-Zadeh et al.[103]).

	Motor symptoms	**Cognitive symptoms**	**Psychiatric symptoms**
Early stages	Dysarthria Poor coordination Tremors	Difficulty concentrating	Emotional lability Aggressiveness
Development	Parkinsonism	Memory impairment Declining cognitive performance	Personality changes Paranoid delusion
Late stages	Pyramidal signs Parkinsonism Ataxia	Significant amnesia	Psychosis Disinhibition Kluver-Bucy Syndrome

Nonspecific findings include abnormal cerebral and cerebellar atrophy, *cavum septum pellucidum* and significant neuropathology findings, as follows: neuronal/axonal loss; cortical and subcortical neurofibrillary tangles (NFTs); B-amyloid deposits (diffuse plaques or deposits in vessels walls, as amyloid angiopathy); hippocampal sclerosis; corticobasal degeneration; neuronal and astrocytic aggregates or cell processes around a vessel, generally at the base of cortical sulci.[104, 105] CTE displays chronically activated microglia and abnormal deposit of phosphorylated tau protein (p-tau) and TDP-43 (TAR deoxyribonucleic acid), suggesting a chronic neuroinflammatory response.[105, 106] Features considered to be

supportive of this diagnosis are p-tau pretangles and cortical NFTs, subpial astrocytic p-tau, "dot-like" p-tau neurites, hippocampal CA2/CA4 tangles and NFTs.[107]

1.3.7 Intracranial Pressure

Normally, ICP values are situated in the range of 5-15 mmHg, with values persistently above 20mmHg (considered a threshold for intracranial hypertension) being associated with increased risk for severe disability and death in the setting of impaired brain perfusion.[108] Three primary intracranial constituents - blood, Cerebrospinal Fluid (CSF) and parenchyma - must reach a dynamic equilibrium among their volumes (classical Monro-Kellie hypothesis), compensating for one's increase with a decrease in the other two components and preventing an undesirable increase in ICP.[109] These compensatory mechanisms are able to accommodate small increases in ICP but, beyond a certain threshold, will no longer compensate for all abnormal processes taking place. Compression of blood vessels within brain parenchyma will further compromise tissue perfusion and ensuing loco-regional ischemia exacerbates cell injury and death, with neurogenic inflammation reinforcing classical inflammation and progressing brain tissue dysfunction in a vicious circle **(Figure 1.3)** (see Pathophysiology of Traumatic Brain Injury section).[110, 111]

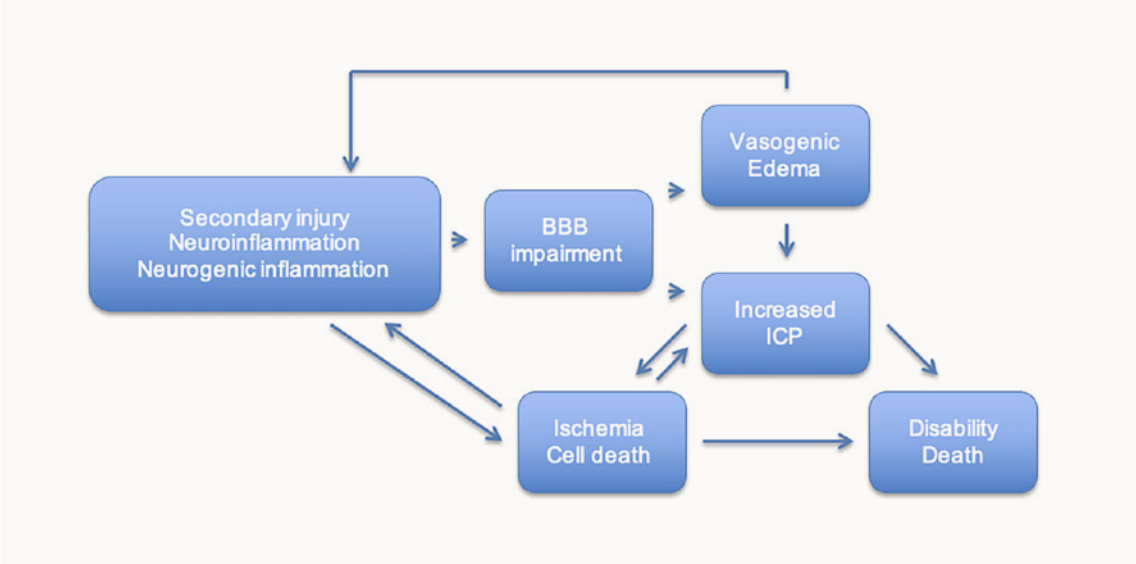


Figure 1.3 - Events leading to increased intracranial pressure. Secondary injury induces overall inflammation and ensuing BBB impairment. Consequently, reinforced brain edema and increased ICP will aggravate loco-regional ischemia and further promote secondary phenomena and inflammation. Thus, a vicious circle of pathological pathways and damage leads to progressing secondary injury and non-controllable ICP increase. **Legend**: BBB, blood-brain barrier; ICP, intracranial pressure.

In TBI, primary and secondary injury mechanisms contribute to a potentially sustained and detrimental increase in ICP. As the swollen brain structures are hypoperfused, arterial blood pressure is increased as part of an inefficient compensating mechanism (Cushing reflex), further increasing ICP.[108, 112] Brain herniation will ensue, with brain tissue moving down pressure gradients and compressing adjacent structures, including blood vessels (further reinforcing brain ischemia) and cardiorespiratory centres within the brain stem.[113] Intracranial hypertension will progressively compromise cerebral perfusion pressure and cerebral blood flow,[111, 114] leading to further brain injury, namely in patients with an already compromised cerebral autoregulation capacity.[114]

Nonspecific treatment protocols [diuretics, hyperosmolar therapies, Cerebrospinal Fluid (CSF) diversion procedures, decompressive craniectomy] are effective in improving outcome to a certain degree,[115, 116] although not influencing basic pathophysiological mechanisms.[117]

1.3.8 Other consequences

Many other direct and indirect consequences of TBI are relevant. Dementia and Alzheimer's Disease are closely associated with TBI, as reported in epidemiological reports and anatomopathological studies displaying deposition of amyloid-beta (Aβ),[105, 118] p-tau[119] and TAR DNA binding protein 43 (TDP-43).[120] A long-lasting state of neuroinflammation is another likely contributing factor for neurodegenerative diseases.[121, 122]

The reported incidence of hydrocephalus is highly variable, with some reports mentioning up to 29% in severe TBI victims, whether in its acute obstructive form or in communicating hydrocephalus, in which CSF reabsorption via arachnoid villi is impaired due to blood products.[123, 124] A specific type, similar to idiopathic Normal Pressure Hydrocephalus, is also a possible consequence of TBI, displaying the classic triad of gait disturbance, urinary incontinence and cognitive impairment.[124]

Other possible long-term consequences of TBI include bladder/bowel control impairment, multiple endocrinopathies and hypopituitarism, hypogonadotropic hypogonadism and overall personal and social impairment.

1.4 Pathophysiology of Traumatic Brain Injury

"The most complex disease in the most complex organ."

Wheble et al.[125]

Besides the macroscopic consequences of brain trauma (including intracranial bleeds, brain contusions, hydrocephalus, post-traumatic atrophy), several events are discernible at a cellular and molecular level, with obvious repercussions in disease progression and prognosis. Significant events include vasogenic and cytotoxic edema, coagulopathy, neurodegeneration and neuronal death,[126] classical and neurogenic inflammation,[12, 127] excitotoxicity,[128, 129] membrane transport disruption and BBB breakdown.[130, 131]

Primary injury includes mechanical deformation of neural tissues, implying neuronal depolarization and glutamate/aspartate spilling and inducing a significant influx of calcium (Ca^{2+}).[39] TBI also increases several transcription factors and inflammatory mediators (cytokines and chemokines) that will reinforce brain edema, BBB impairment and cell death, including apoptosis.[126, 132] Primary damage from direct brain lesion is followed by secondary cellular/biochemical deregulation, in which innumerous injury pathways overlap, reinforcing brain edema following microvascular permeability and early/late cell death.[12, 133] Theoretically susceptible to pharmacological intervention, TBI's secondary injury results in worsening major neurological deficits and other clinical findings (as previously discussed) in the context of structural disruption, early and late-onset neuronal/astrocyte damage and death, neurometabolic impairment and overall synaptic disturbance, among others.[85]

1.4.1 Primary injury

Primary injury represents the direct consequence of injury to the cranial vault and brain, derived from mechanical harm.[39] Primary insult is necessarily of short duration but will damage several structures, from bone to cerebrovascular structures and brain parenchyma itself. Common primary injuries, with diverse clinical and therapeutic implications, include[39, 134]:

- Skull fractures;
- Epidural haematomas, commonly from laceration of the middle meningeal artery;
- Subdural haematomas, usually derived from venous injury (cortical veins, subdural bridging veins);
- Intracerebral haemorrhages, a consequence of brain parenchyma contusion or laceration, typically located in frontal and temporal lobes, occipital poles and opercular region;
- Subarachnoid haemorrhages (the most common form of vascular injury after TBI);
- Cranial nerve injuries;
- CSF leak (nose, ear), a consequence of dural tear;
- Diffuse Axonal Injury (DAI).

In the presence of severe TBI, typically more than one injury type will be present, as in the usual association between brain contusions and subarachnoid haemorrhages.[39, 135] Two widely mentioned Computed Tomography (CT) scan-based classification schemes, Marshall score and Rotterdam score, frame these post-traumatic findings in objective grading systems.[136, 137]

A common sub-type of lesions is coup-contrecoup injuries **(Figure 1.4)**, a consequence of a sudden and violent impact, followed by movement of the brain back and forth within and against the boundaries of the cranial vault.[39, 138]

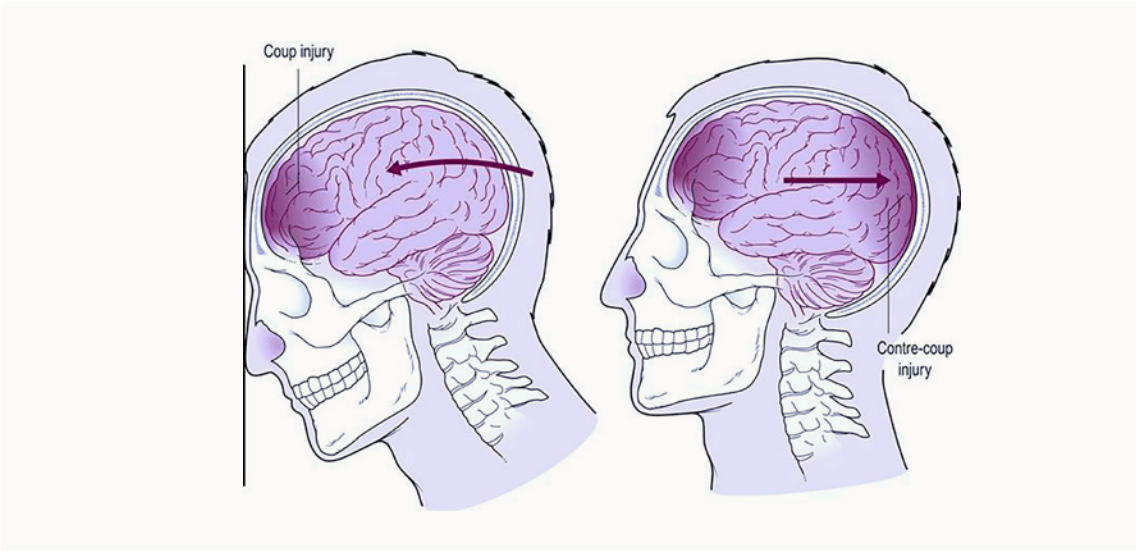


Figure 1.4 - Traumatic brain injury with coup and contrecoup injuries, causing brain contusions (adapted from Klima et al.,[140] with permission).

Typical brain contusions, a combination of edema, injured tissue and local blood derived from damage in small blood vessels, are recognizable in this setting as haemorrhages underlying sites of impact accompanied by obvious contrecoup contusions, in opposite location to initial injury location. Peri-contusional areas display many features of secondary injury, with a varying predominance of specific phenomena, such as vacuolation, microglial activation, dystrophy and cytoskeleton abnormalities.[139]

As expected, early clinical findings and neurological deficits (motor, speech) are dependent on primary injury location and extension and the presence of relevant mass effect **(Table 1.5)**.

Table 1.5 - Main pathological events in TBI concerning primary injury

Primary mechanisms	Primary events
Mechanical forces	Axonal shearing Cellular damage
Laceration Microvessels disruption	Haemorrhages Pia-arachnoid membranes disruption
Early edema Vascular dysregulation and injury	Increased intracranial pressure
Early edema Vasospasm	Ischemia

1.4.2 Secondary injury

Several studies highlight crucial roles by other CNS components, including glial cells (astrocytes, microglia), endothelial cells, perivascular mural cells, among others, forming the so-called Neurovascular Unit (NVU).[141, 142] Several components of this complex entity, including BBB, are interconnected in their functions, regulating the exchange of metabolites and ensuring local energetic supply. Equally important, this multimodal CNS injury evolves upon an acute setting but persists for years after the initial trauma. In fact, microglial activation, hippocampal neuronal degeneration and myelin loss are present up to 1 year after moderate to severe TBI in animal models, showing that TBI should not be viewed as a static, acute disorder.[143]

Secondary damage is derived from complementary mechanisms, as inflammation, loss of adequate homeostasis, calcium metabolism imbalance, energy depletion and mitochondrial dysfunction **(Figure 1.5)**,[144, 145] affecting different components of an integrated response **(Table 1.6)**.

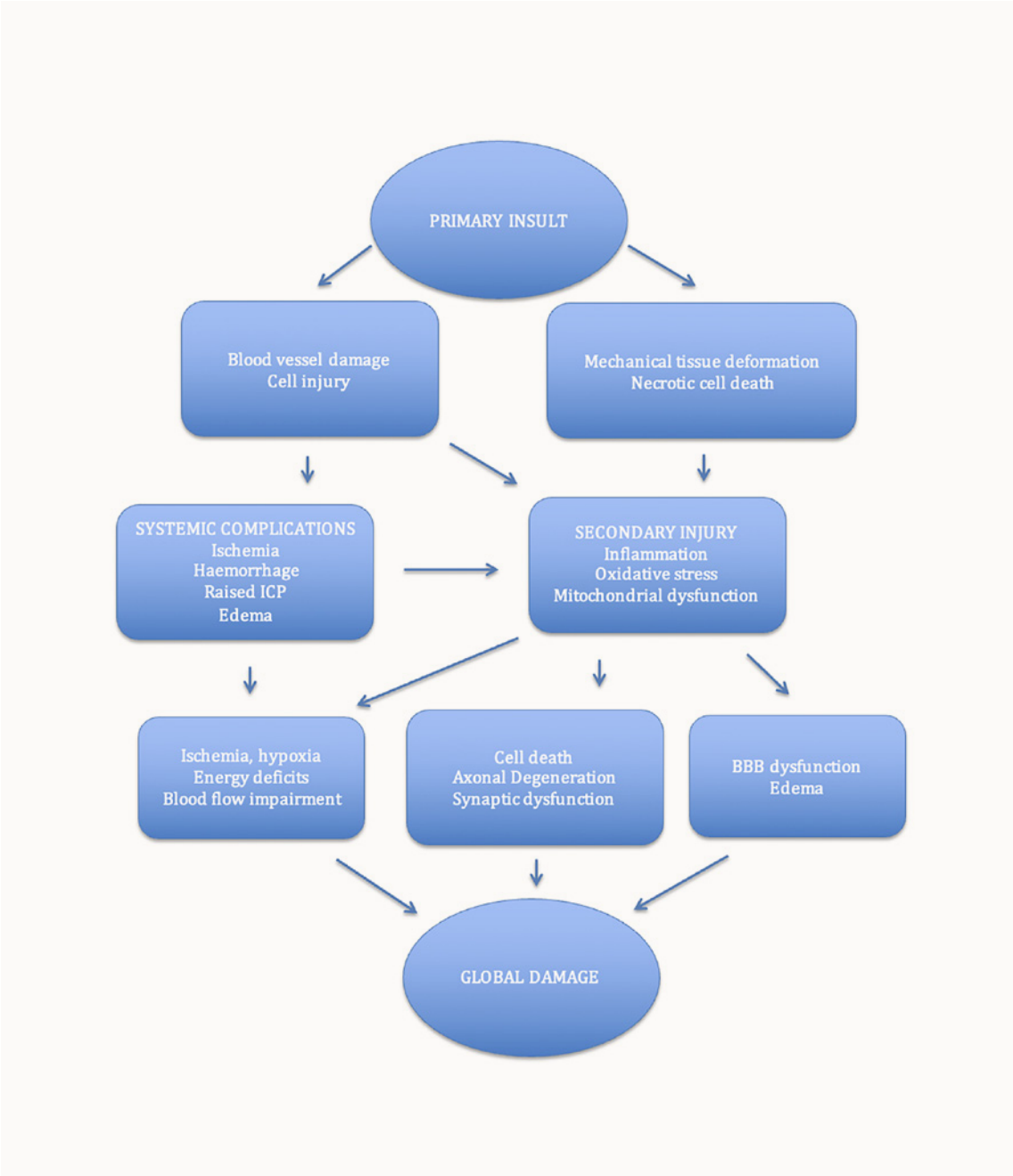


Figure 1.5 - Primary and secondary injury following TBI. A complex and multifactorial response is triggered by TBI's primary injury, involving distinct mechanisms and pathways, leading to ensuing secondary injury and global damage. **Legend**: BBB, Blood-Brain Barrier; ICP, Intracranial Pressure (based on Kaur et al.[39]).

Table 1.6 – Main pathological events in TBI. Legend: AQP4, aquaporin-4; Ca^{2+}, calcium ion; NADPH, nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species.

Cellular alterations	Secondary events
Decreased glutamate uptake Cell death	Excitotoxicity
Proinflammatory status	Neuroinflammation
Proinflammatory status Neuropeptides upregulation Cellular debris, neurotoxic factors	BBB disruption
Caspases activation Excitotoxicity	Apoptosis
Calpain kinase activity P35 cleavage	Protease activation
ROS production (Mitochondrial) Ca^{2+} imbalance NADPH oxidation	Oxidative Stress
Unfolded proteins Cytokines upregulation (Intracellular) Ca^{2+} triggering	Endoplasmic Reticulum stress

It is important to mention that, upon CNS injury, neuroinflammation and associated phenomena (as microglial activation) are not limited to the cerebral cortex and deeper structures like the hippocampus. Instead, these post-aggression response mechanisms have also been described in the cerebellum[146] and meninges.[147]

Cellular alterations

Injuries arising from TBI are highly heterogeneous in their nature and mechanisms, depending on injury characteristics, the severity of inflicted lesions and anatomical locations of damage.[148] A relevant distinction to be made, both on clinical and mechanistic grounds, is between focal and diffuse injury.[148]

Distinct cell populations react differently to mechanical forces and strains involved in TBI **(Figure 1.6)**. Axons are considered rigid structures within an elastic surrounding environment, making them susceptible, as astrocytes and other glial cells are, to mechanical stress.[148, 149]

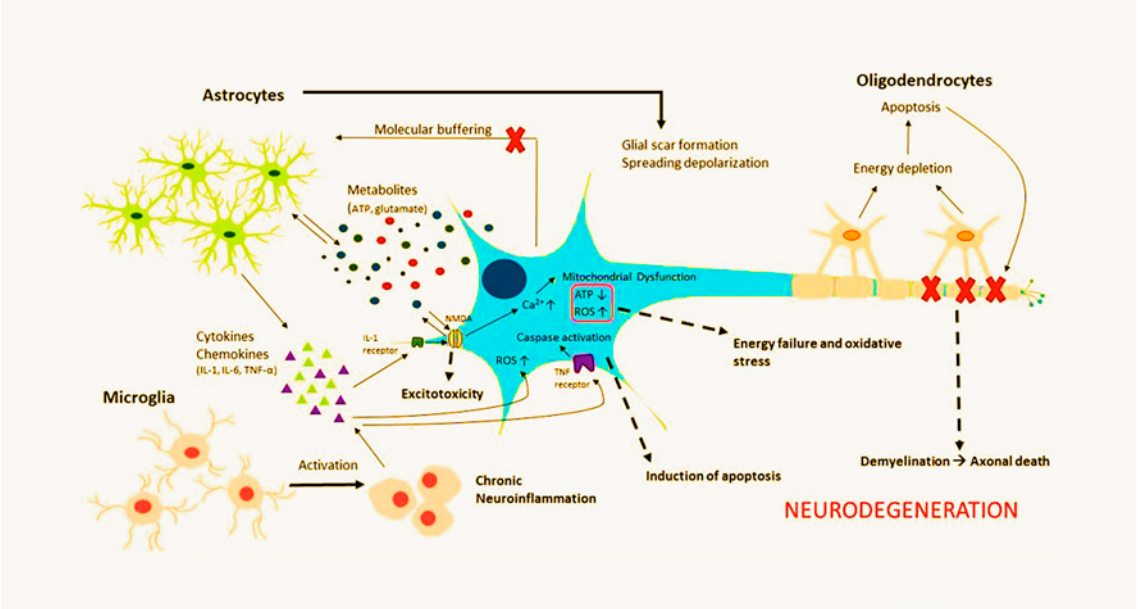


Figure 1.6 - Glial cells and secondary injury. Following primary injury, distinct cell populations will initiate different pathological pathways, contributing to cumulative damage. Legend: ATP, adenosine triphosphate; Ca^{2+}, calcium ion; IL-1, interleukin-1; IL-6, interleukin-6; ROS, reactive oxygen species; TNF, tumor necrosis factor (adapted from Sajja et al.,[145] with permission).

While susceptible to membrane distortion, astrocytes display mechanosensitive ion channels contributing to a rapid influx of extracellular Ca^{2+} and sodium upon injury.[148, 150] Other astroglial responses were shown, in *in vitro* studies concerning mechanical stress, including protein kinase signalling, ATP release, secretion of vasoactive molecules (endothelin-1, isoprostanes) and matrix metalloproteinases (MMPs), including MMP-9.[151, 152] Additionally, Gap junctions (GJs), consisting of transmembrane connexin hemichannels (connecting adjacent cells and allowing passage of ions and metabolites), are needed for astrocytic networks involving synapses and blood vessels.[145, 153] GJs, namely Cx43, are suspected of allowing the spread of noxious and cellular death components and events (inflammatory cytokines, extracellular ATP release, NMDAR activation).[154, 155] GJs were reported to influence post-traumatic outcomes in spinal injury and TBI,[156, 157] by upsetting intercellular Ca^{2+} signalling within astrocytic networks, promoting further neuroinflammation and cell death.[158]

Post-traumatic apoptosis is a major mechanism of secondary damage and cell death following primary necrosis, directly influencing neurological changes and cognitive impairment.[159] Increased apoptotic phenomena, following even mild TBI, peak at 48h but persist for several days.[132, 160] Cell death might also occur via secondary excitotoxicity-induced necrosis, depending on initial injury and target cell populations.[161]

Neurons

Neurodegeneration is an early consequence of trauma, arguably extending for months.[162, 163] Traumatic events produce direct neuronal injury, with axonal stretching and shearing and dendritic injury.[164, 165] Inertial forces with rapid head acceleration-deceleration (and additional rotation) induce DAI, with significant clinical and prognostic implications. Typical findings include axonal tearing injuries, swelling, microbleeds and disconnection, along with cytoskeletal defects.[166, 167] Axons will retract and develop axonal retraction bulbs.[167, 168] Another typical finding is secondary axotomy, with delayed axonal swelling and disconnection.[165] Increased membrane permeability, activated cysteine proteases and mitochondrial swelling may also be involved in neuronal damage.[163] Downstream axonal segments undergo Wallerian degeneration, still present several months post-TBI.[169, 170] As mentioned, white matter injury involves not only DAI but also myelin disruption.[167, 171] In animal models of TBI, significant axonal injury is usually present, starting in specific areas (cingulum, external capsule) and immediately following TBI.[172] This degenerative response is accompanied by apoptotic neuronal death, particularly in the hippocampus, thalamus and cingulum, peaking at 24h post-injury.[171]

A pronounced reduction in the number of intact functional neurons from 24 h to 1 week after injury, namely calbindin-reactive CA2/CA3 hippocampal neurons,[162, 173] is followed by an expected return to baseline levels between 7 days and 1 month post-TBI.[163, 173] Animal models with focal injury display earlier neuronal loss, namely at a cortical and hippocampal level, with more pronounced focal findings.[174, 175] At 8-10 weeks post-TBI, there is still a selective reduction in specific populations of inhibitory neurons in the somatosensory cortex and hippocampus.[163] Similar findings were reported by other authors, with an observable neuronal loss at 2 weeks and up to 6 months post-TBI.[175, 176]

Some degree of functional recovery follows structural/cellular endogenous repair mechanisms, namely in the hippocampus, displaying nestin-expressing Neural Stem/Progenitor Cells (NSPCs), mainly in Dentate Gyrus (DG), able to un-

dertake post-TBI NSPCs activation and injury-related neurogenesis.[177, 178] Regular post-natal neurogenesis takes place in two well-identified neurogenic niches: the subventricular zone (on the outside wall of lateral ventricles) and the subgranular zone of hippocampal DG.[178]

Glial Cells

Glial cells are described as the structural framework for the brain. Astrocytes, microglia and oligodendrocytes provide a supporting role for neuronal activity but also interact with neurons, influencing synaptic function and interfering with neuroregeneration and plasticity.[145] Despite an initial beneficial effect, chronic activation of glial cells will have a detrimental impact on neuronal function. Post-TBI neural-glial and glial-glial interactions are influenced by a complex post-traumatic glial dysfunction leading to harmful consequences such as loss of homeostasis and unbalanced neurotransmitter action, axonal degeneration and cell death.[145, 179]

Despite fully inter-connected, glial elements studied in the present thesis are now discussed separately. Although vulnerable to most phenomena observed in TBI,[180] oligodendrocytes are beyond the scope of this work.

Microglia

Microglial cells, composing about 10% of glial cells, are specialized immune CNS, continuously scanning and regulating the brain's environment while adapting its morphology and activity.[181] These cells also regulate neuronal activity and circuits, as well as neurotransmitter signalling/synaptic transmission.[182, 183] They are the first line of response to brain injury, although a more permanent state of activation might represent, in TBI models, a promoter of long-term neuroinflammation and impaired function.[184, 185]

Under normal physiological conditions, microglia cells adopt a "surveying" phenotype, with a ramified morphology characterized by compact cell bodies and elongated processes.[181] Upon CNS aggression and changes in surrounding microenvironment, microglia rapidly transitions into its activated state, with purposed migration to the lesion site, shortening and thickening of processes and enlargement of their cell bodies, ultimately adopting a so-called amoeboid morphology.[181] Activated microglia cells are able to present antigens, produce inflammatory cytokines and chemokines[186, 187] and remove cellular debris by phagocytosis.[188] Classically, three phenotypes were described concerning microglial cells

as follows: 1) resting state; 2) activated but non-phagocytic status, similar to Antigen Presenting Cells (APCs); 3) reactive, with phagocytic behaviour **(Figure 1.7).**[189, 190] A similar activated chronic state has been shown in several neurological conditions usually associated with some degree of chronic neuroinflammation, including Parkinson's, Alzheimer's and Huntington's diseases.[118, 188] "Resting state" microglia plays a major role in synaptic and structural plasticity regulation, particularly during learning and memory.[191, 192] Importantly, upon CNS injury, microglia cells are responsible for ensuring immune cell-based regulation of astrocytes and oligodendrocytes activity.[187]

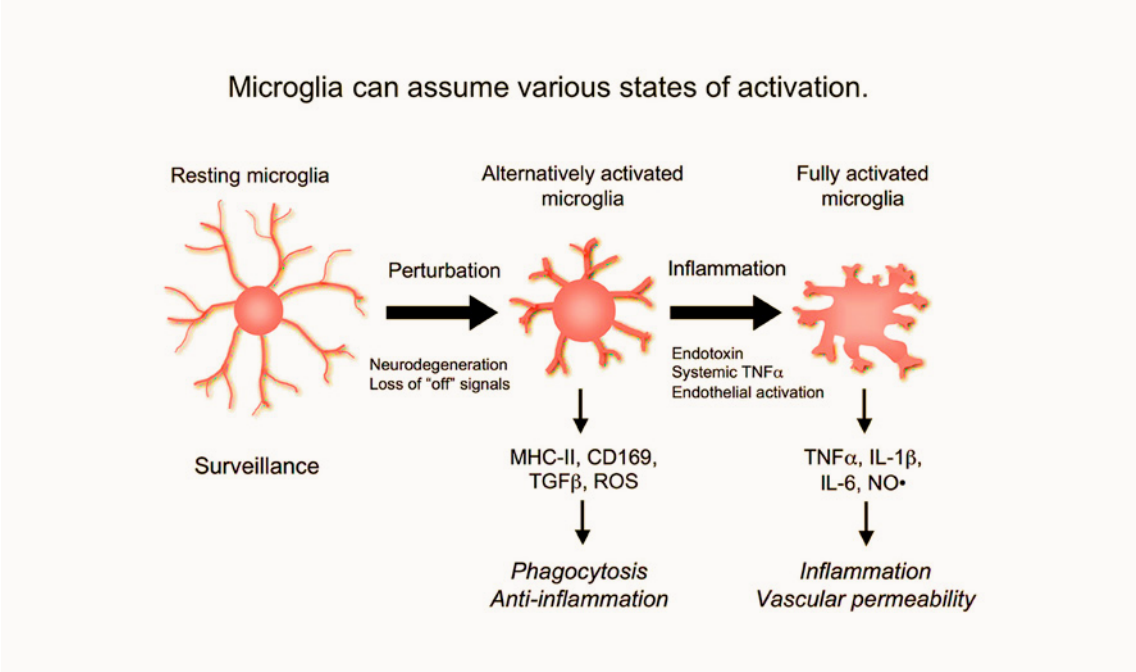


Figure 1.7 - Schematic representation of microglia morphologies. Distinct stimulus can trigger microglia response, which acquires an alternative activated state, eventually culminating in a fully activated state with significant repercussion in overall inflammation and vascular permeability. **Legend**: CD169, sialoadhesin; IL-1, interleukin-1; IL-6, interleukin-6; MHC, major histocompatibility complex; NO, nitric oxide; ROS, reactive oxygen species; TGF, transforming growth factor, TNF, tumor necrosis factor (adapted from Dr. Steven Abcouwer's work, with permission).

Microglial cells possess the ability to display, as classically described, a proinflammatory (M1) phenotype in opposition to an anti-inflammatory (M2) phenotype, promoting debris removal.[193] Activated M1 subtype, upon response to proinflammatory molecules (LPS, IFNγ), is known to secrete proinflammatory cytokines and oxidative molecules (IFN-γ, TNF, IL-1β, IL-12) (**Figure 1.7**) and reinforce cell-mediated immunity.[194, 195]

Activated M2 microglia sub-type displays different functional features, namely by clearing cellular debris and producing higher amounts of anti-inflammatory cytokines and IL-4, IL-10, arginase-1 and TGF-β,[189] also secreted by other types of cells (astrocytes, T-cells).[189, 196] Other states are also mentioned as follows: M2a-like activation state with anti-inflammatory, tissue remodelling and matrix deposition properties; an intermediate M2b phenotype upon stimulation of Toll-Like Receptors (TLRs); a functionally deactivated M2c (in response to TGFβ and IL-10), regulating inflammation resolution.[190, 195]

M1 and M2-like polarized microglia are thought to function in a complementary fashion, especially in the acute phase.[190] M1 phenotype appears to be a more chronic, persistent phenotype (months to years) following TBI,[197, 198] in response to the perilesional microenvironment and loco-regional redox signalling.[190, 199] A specifically activated microglia M1-like profile (e.g., following LPS-induced inflammation) impairs hippocampal neurogenesis and reduces NSPCs survival, impeding differentiation.[200] Proinflammatory cytokines (IL-1β, IL-6, IFNγ, TNF) also suppress neurogenesis.[201, 202]

Neurotoxic NOX2 expression was associated with M1-like phenotype, with ensuing cortical and hippocampal degeneration.[190, 203] This component of CNS injury may prove to be another therapeutic target, as inhibition of NOX2 activity alters M1-/M2-like balance towards M2-like phenotype, reducing oxidative damage and attenuating neurodegeneration.[190, 203] Microglia will also play an essential role in regulating a delicate balance in cytokine expression dictating injured neurons fate (survival or death) in the so-called traumatic "penumbra region", a concept initially described in stroke.[204, 205] Persistent microglial activation might still, in the chronic stage, promote brain repair via Brain-Derived Neurotrophic Factor (BDNF) and others.[189, 206]

Concerning TBI, post-traumatic microglial activation is well documented in several reports.[207, 208] Trauma activates resident microglia, induces cytokines production, and causes an influx of peripheral immune cells,[185] followed by chronic activation of resident microglia and astrocytes, in a true state of neuroinflammation as an integral part of secondary injury mechanisms.[209, 210] Upon TBI, there is a 10 to 20-fold increase in microglia compared to peripheral macrophages, suggesting a predominantly central response instead of peripheral.[211] In fact, a transiently increased expression of M2-like microglia (in the acute phase, up to 24h) is apparently soon overcome by a chronic M1-like phenotype predominance at 7 days post-TBI.[201] Again, relatively unknown underlying dynamic aspects appear to regulate microglia/macrophage phenotypes and corresponding effects following TBI, reinforcing persistent neuroinflammation and structural/functional impairment. Remote microglial activation potentially promotes tissue repair by producing neurotrophic factors, namely BDNF.[189, 212] Cytoskeleton actin

dynamics drives specific activated microglia motility behaviours, with a specific motility/locomotion intent at engaging dead/dying cells.[213] This state of reactive microglia progresses from injury locum to close-by white matter tracts, eventually reaching contralateral hemispheres.[214] Remarkably, microglial activation was described weeks to years following TBI in animal models and post-mortem studies.[197, 215] TBI patients display higher levels of CR3/43, CD68 and Major Histocompatibility Complex (MHC) Class II molecules in activated microglia.[198, 216]

Microglial activation will reinforce existing signalling networks with other cells, including astrocytes and neurons, in the injured area and in distant locations.[217, 218]

Astrocytes

Astrocytes are the predominant CNS cell type, displaying regulatory functions on metabolism, synaptic plasticity neural networks and remyelination.[219] They are responsible for maintaining homeostasis/osmotic balance, neuronal transmission and neurotransmitter recycling, keeping a close spatial relationship to synapses.[220] Astrocytes project cellular processes exhibiting terminal footplate protuberances, simultaneously supporting and being part of BBB,[221] regulate cerebral blood flow, are part of the glymphatic system and mediate neuro-glial signalling interactions.[222] These cells interfere with leukocyte infiltration and neurodegenerative processes and act as regulatory elements for neurotransmitter excess and neurovascular coupling.[223, 224] Astrocytes also play a role in synaptic plasticity and neural circuit reorganization and remyelination.[223, 225] Astrocytes are crucial in the development and maintenance of functional synapses,[148, 226] secreting different molecules such as thrombospondins and hevin that stimulate excitatory synapses.[227]

Astrocytes respond swiftly to pathological stimuli, transitioning into a hypertrophic state and increasing expression of intermediate filaments markers, namely Glial Fibrillary Acidic Protein (GFAP).[228, 229] Astrocytes also influence and adapt the perineuronal network, a synapse-stabilizing structure composed of extracellular matrix and cell adhesion proteins,[230] altering its protein expression and spatial organization in order to accommodate post-injury stimuli and abnormal connections/axonal remodelling.[231]

Morphologically complex and highly heterogeneous, astrocytes present with significant molecular variability and specific sub-types, including fibrous and protoplasmic.[222]

Upon cell death and liberation of cellular debris and neurotoxic factors, astrocytes are activated.[216, 232] This activation comprises increased gene expression,

increase in astrocyte number, changes in morphology and scar formation, with both deleterious and positive effects (e.g., promotion of synapse formation).[222, 233] Astrogliosis consists on cell hypertrophy, increased expression of intermediate filaments (GFAP, nestin, vimentin) and proliferation.[145] Its activation is dependent on complex signalling interactions, including ion channel and GJ's, purinergic receptors, excitotoxicity and specific neurotransmitters and Ca^{2+} homeostasis upset.[222] It further interacts with microglia via several mediators including TNF, IL-1**β** and complement 1q component.[234]

Astrocytes display pro- and anti-inflammatory properties, also designated as A1/A2 profiles, respectively, in interesting parallelism to microglia.[234] The A1 sub-type, following cytokine exposure (TNF, IL-1α), is characterized by impairment of astrocytic homeostatic functions (including phagocytosis) and becomes neurotoxic to surrounding cell populations.[235] A2 sub-type might display a "protective/repairing" profile, expressing synaptogenic and axonal progression factors.[236, 237]

Astrocytes are involved in a global response to TBI.[148, 238] It has been studied as a therapeutic target, with reported neuronal survival and improved cognitive outcome upon its modulation.[239, 240] Following TBI, astrocyte cells enlarge and proliferate, upregulating intermediate filament proteins in direct relation to injury severity.[148, 241] Trauma induces direct secretion of GFAP and S100B from astrocytes, making them ideal serum and plasma biomarkers for TBI (see Biomarkers section).[242] Even mild impacts can result in significant astrogliosis (both in ipsi- and contralateral hemispheres) and dysregulation of AQP4 expression, namely in its typically polarized pattern in the endfeet of reactive astrocytes.[243, 244]

ATP release (via connexion hemichannels) from either viable, injured or dying cells, induces an increase in cytoplasmic Ca^{2+}, involved in the polarization of astrocyte processes towards injury and recruitment of microglia.[148, 245] Simultaneously, late post-traumatic astrogliosis is intended to protect unaffected brain areas from secondary damage.[207] The site of injury is surrounded and covered by layers of astrocytes, with long intertwined processes, forming a protective scar-tissue formation.[148, 246]

Human and animal studies show a role for astrocytes in post-traumatic epileptogenesis, namely upon homeostatic functions upset, functional impairment and inclusion in epileptogenic lesional scars.[67, 246] Fluctuating GFAP expression and downregulation of astrocyte coupling (necessary for a proper syncytium development) are probable causal factors in PTE. Confronted with an energy crisis, the brain is forced to use alternative metabolic pathways, namely lactic acid and pyruvate, with reactive astrocytosis regulating the synthesis of lactic acid, free fatty acids and ketone bodies.[247, 248]

Excitotoxicity

Glutamate is the main endogenous CNS's excitatory neurotransmitter and a crucial element in neuroplasticity and maintenance of cognitive functions,[249] along with lactate and aspartate.[250, 251] Clinical and animal model studies, resorting to microdialysis[252, 253] and spectroscopy,[254, 255] document a significant post-traumatic increase in extracellular brain levels of glutamate. Studies concerning glutamate and CSF analysis in TBI patients displayed a similar increase.[256] Post-TBI changes concerning glutamate and lactate are the consequence of a much broader neurometabolic shift in the injured brain, including emergent lactate oxidative metabolism.[257] This increase in glutamate is evident at 6h post-TBI and reaches its peak after 2 days.[128, 258] Excitotoxicity leads to neuronal damage and death following over-activation of NMDA and AMPA ionotropic receptors, mitochondrial disruption, Reactive Oxygen Species (ROS) upregulation, cytoskeleton disturbance and ionic homeostatic imbalance.[259, 260]

Previous studies in primary cultures of human brain endothelial cells have also shown a role for glutamate in post-TBI BBB's increased permeability,[261, 262] mainly through metabotropic glutamatergic receptors.[263] Imaging studies on human TBI victims display a chronically disrupted dynamic between glutamate and inhibitory transmitter GABA.[256, 264]

Metabolic dysfunction and oxidative stress

Overall brain metabolism is impaired in TBI victims, with the metabolic rate being considered a prognostic factor.[265, 266] Mitochondria dysfunction is followed by attenuation of the nicotinic co-enzyme pool, decreased ATP and reduced respiratory rates,[267, 268] with an obvious cerebral blood flow/metabolism uncoupling.[269] As expected, studies have confirmed a relationship between excitatory amino acids levels (in serum and microdialysis) and the degree of secondary injury.[270]

Disruption of regular energetic mechanisms leads to decreased glucose utilization and lactic acid accumulation.[39, 271] Experimental studies show an initial increase (30m post-TBI) in glucose uptake and metabolism, followed by a more sustained decrease in glucose uptake and significant glycolysis, lasting for several days.[272] This decrease in glucose is present even in mild TBI, as documented in studies with [18F] fluorodeoxyglucose Positron Emission Tomography (PET).[265, 273] Post-TBI increased levels of lactate (brain tissue, CSF, serum) are also discernible,[266, 274] with direct impact on acidosis equilibrium, membrane damage and neuronal vulnerability to secondary ischemic insults. Glutamate-dependent activation of glycolysis should stimulate glucose-fuelled astrocyte production

of lactate, which should be efficiently transported from astrocytes (where it is mainly produced) to neurons (preferred site for breakdown) ("astrocyte-neuron lactate shuttle" hypothesis).[275, 276] Upon excessive neuronal damage, lactate accumulates in the extracellular compartment, which explains its increased levels in TBI microdialysis studies.[266] Following severe TBI, lactate brain uptake and metabolism indexes are correlated with outcome.[275, 277]

Oxidative stress is classically described as resulting from an imbalance between free radical production and the physiologic ability to counter their damaging effect.[39] Post-traumatic excitotoxicity and simultaneous depletion of endogenous antioxidant components (superoxide dismutase, glutathione peroxidase) is one example of this deleterious imbalance.[39, 278] Oxidative damage and its two main free radicals [Reactive Nitrogen Species (RNS) and ROS], included in broader post-TBI metabolic stress, are responsible for a myriad of deleterious events, (protein oxidation, vascular systemic peroxidation, DNA cleavage), promoting early and delayed apoptotic programs.[39, 278]

Blood-brain barrier and edema

Blood-brain barrier

BBB is an anatomical and functional structure composed of distinct elements **(Figure 1.8)** and playing a fundamental role in CNS homeostasis. BBB is formed

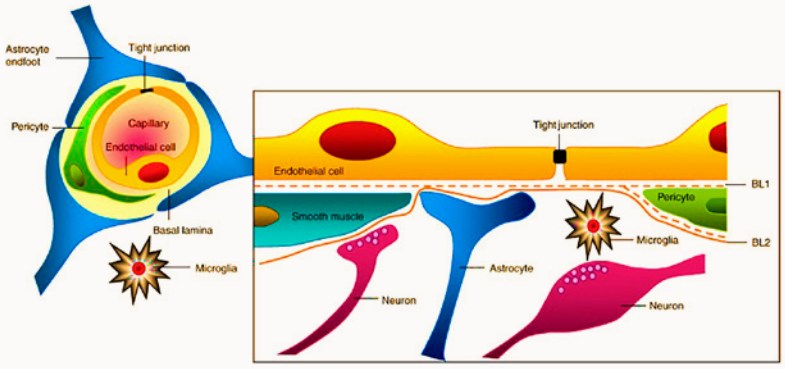


Figure 1.8 - Schematic illustration of cell associations and different components in BBB. Tight junctions between adjacent cells regulate the paracellular diffusion pathway. Perivascular pericytes partially surround endothelial cells and both are enclosed by and contribute to the local basement membrane. Astrocytic endfoot processes reinforce this complex network. Blood flow is regulated via vasoactive peptides and neurotransmitters. **Legend**: BL1, basal lamina 1, perivascular extracellular matrix; BL2, basal lamina 2, extracellular matrix of glial endfeet (in relation to parenchyma) (adapted from Abbott et al.,[281] with permission).

by microvascular endothelial cells and specific subpopulations of brain cells (astrocytes, pericytes), together with an extracellular matrix component, the Basement Membrane (BM), underneath endothelial cells.[279, 280]

While endothelial cells build up BBB as a functional unit by forming Tight Junctions (TJs) in the intercellular space by limiting the paracellular pathway and transcytosis (transcellular transport),[280] pericytes act as mural cells covering capillaries.[282, 283] Astrocytes interact with pericytes and endothelial cells via their endfeet **(Figure 1.9)**.[280, 284] Concerning TJs, specific structuring proteins regulate paracellular permeability between adjacent endothelial cells, being formed by transmembrane proteins (claudins, occludin, adhesion molecules) and accessory cytoplasmic membrane proteins [Zonula Occludens (ZO)].[4, 285]

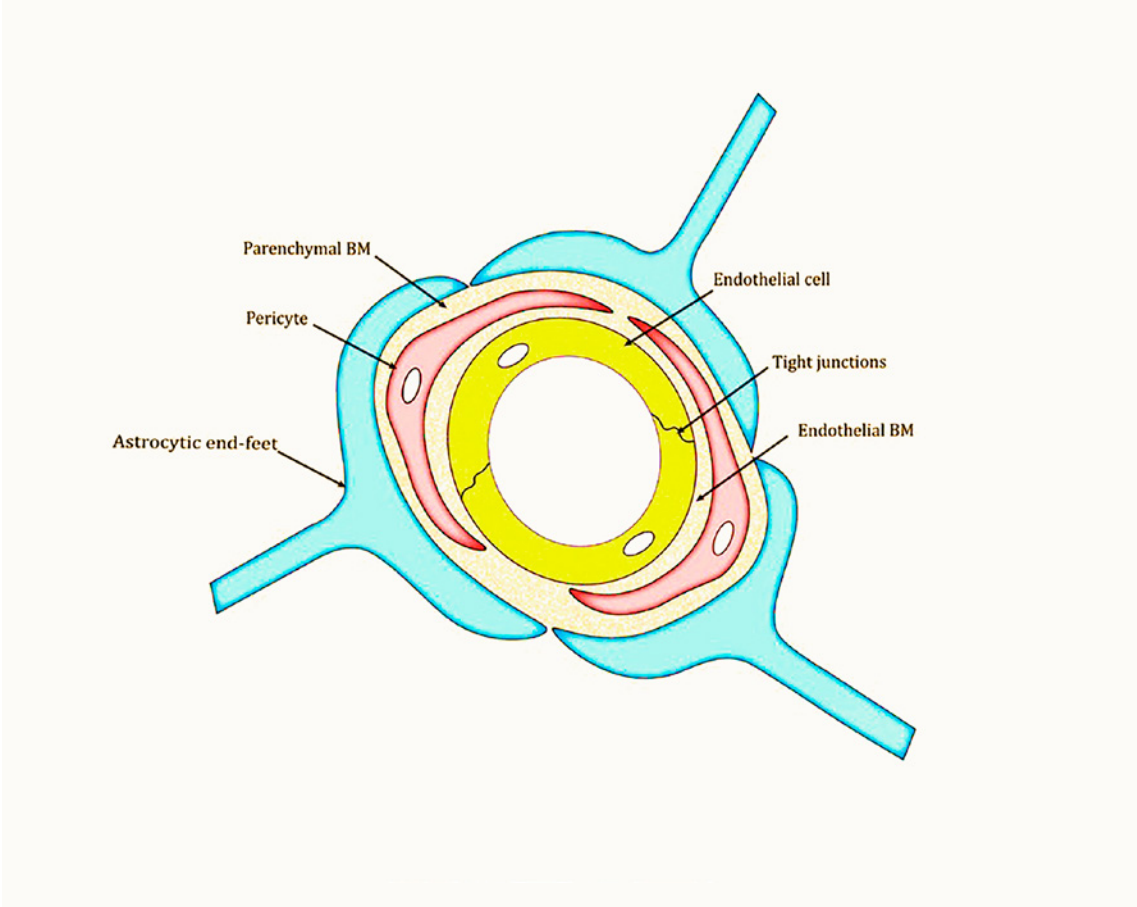


Figure 1.9 - Schematic illustration of Blood-Brain Barrier and Basement Membrane. Basement membrane structurally and functionally reinforces other components of BBB/NVU. **Legend**: BM, Basement membrane (adapted from Xu et al.,[280] with permission).

BBB's function is dependent on its paracrine interactions among endothelial cells and their relation to glial components **(Figure 1.10)**. The concept of NVU is more physiologically accurate and is currently considered the best way to describe and contextualize all underlying cellular and molecular phenomena.[286] Movement of solutes across BBB is due to gradient-driven passive phenomena, eventually facilitated by passive/active transports in the endothelial membrane.[281, 286]

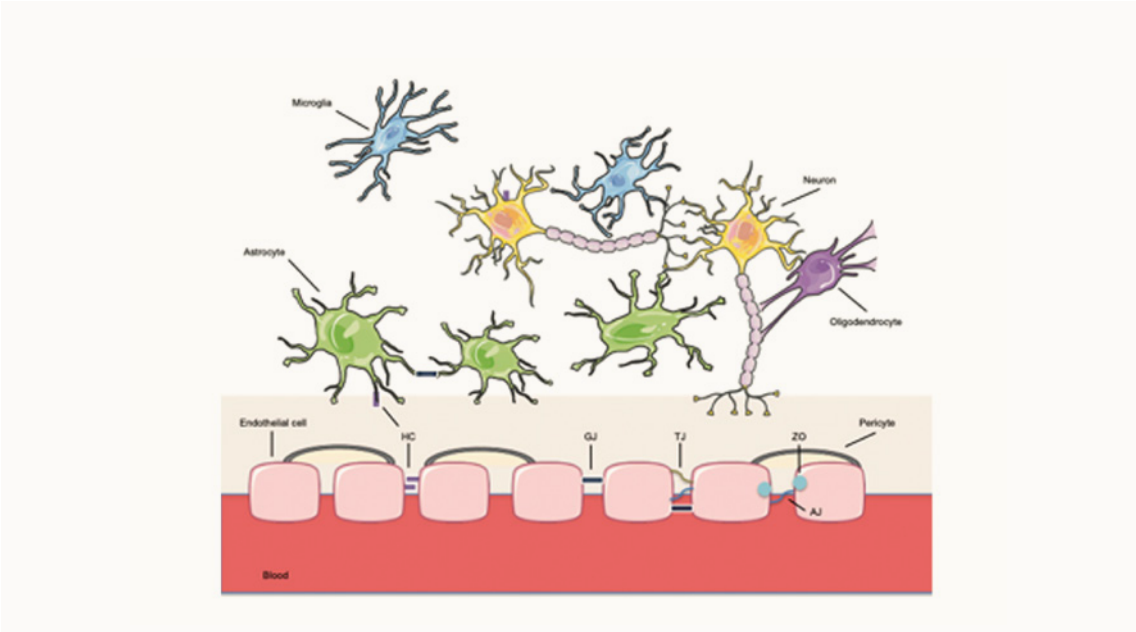


Figure 1.10 - Schematic interpretation of the neurovascular unit. As a functional unit, NVU comprises distinct elements at a cellular and molecular level. **Legend**: AJ, adherens junctions; GJ, gap junctions; HC, hemichannel; TJ, tight junctions; ZO, Zonula occludens.

Astrocytes are a fundamental cellular component of BBB, with astrocytic endfeet enveloping endothelial cells and strengthening structural support.[287, 288] Pericytes also play diverse roles in BBB, including regulation of capillary haemodynamics, clearance of toxic metabolites, angiogenesis, neuroinflammation and BBB's permeability.[289, 290]

Basement membrane (BM) is another relevant structural component in BBB/NVU, as part of the extracellular matrix **(Figure 1.9),** providing structural support and allowing signalling transduction.[280, 291] It is formed by four major proteins: collagen IV (the most abundant), laminin, nidogen and perlecan.[280, 292] As expected, diverse neuropathological contexts (acute and chronic) are found to display significant changes and disrupt ultrastructural BM composition.[293, 294] The hippocampus is a particularly fragile region concerning BBB breakdown, even in healthy ageing individuals.[295, 296]

BBB's upset in TBI has been well documented,[130, 131] with a varying behaviour depending on the type of injury: diffuse TBI induces earlier BBB upset comparing to a biphasic response in focal TBI.[297, 298] Studies show that MMP's expression is involved in delayed BBB opening post-TBI, involving TJs and basal lamina breakdown and recruitment of inflammatory cells.[286, 299]

Ultrastructural studies show an immediate increase in endothelial caveolae in relation to loss of BBB integrity.[300] Caveolae mediate distinct molecules transportation across endothelial cells (insulin, albumin, transferrin, cytokines, chemokines) via respective receptors within caveolae coats.[301] Previous reports mention a decrease of ZO-1 and claudin-5 in cerebral endothelial cells following SP administration.[302] SP is also thought to promote caveolae-dependent transcytosis. NK1 receptor (NK1R) (preferred SP's binding receptor) is also localized within endothelial caveolae and its stimulation promotes caveolae internalization, the first step in transcytosis.[303]

Concerning brain trauma, several questions subsist concerning many aspects of post-traumatic BBB deregulation, supposedly based on transport mechanisms deregulation, increased microvascular permeability (with excessive leakage of proteins and plasma fluid), neuroinflammation and post-traumatic vasogenic edema.[28, 304] Non-selective entry of different blood factors (albumin, fibrinogen), following BBB's mechanical disruption and increased permeability, will guide microglia cells migration to injured brain tissue.[305] BBB's disruption is, therefore, a potential therapeutic target,[306, 307] either by blocking undesired increased permeability or using BBB's impairment to improve drug administration.

Importantly, post-traumatic BBB's disruption leads to the accumulation of toxic blood-borne substances (fibrinogen, inflammatory cytokines) in the brain parenchyma, namely in the presence of co-morbidities that further impair BBB.[308] This will lead to subsequent neuronal and white matter damage and activation of the monocyte/macrophage system.[309] Microglial activation, loco-regional migration of circulatory immune cells and increased levels of NO and ROS are events that potentially interfere with BBB function.[261, 310]

Albumin, a plasmatic protein usually excluded from contact with brain tissue, increases Ca^{2+} concentration in microglial cells and directly promotes microglial proliferation, activating MAPK pathways, promoting NO production via ERK signalling and inducing IL-1 synthesis.[311, 312] Post-traumatic increase in BBB's permeability to albumin and other macromolecules, a likely consequence of increased paracellular permeability and deregulated TJs expression/distribution/function, is, therefore, a significant event in complex BBB-microglial interaction and post-traumatic response.[313, 314]

Edema

Despite its complexity in nature and mechanisms, brain edema is classically divided into two main categories, cytotoxic and vasogenic edema, with distinct processes and preferred sites of fluid accumulation **(Figure 1.11)**.[12, 315] Another sub-type, transependymal (interstitial) edema, is not relevant in the post-TBI context. Evolving research on the subject highlights contributions from various ionic pumps, oncotic gradients, BBB disruption and overall inflammatory response.[315, 316]

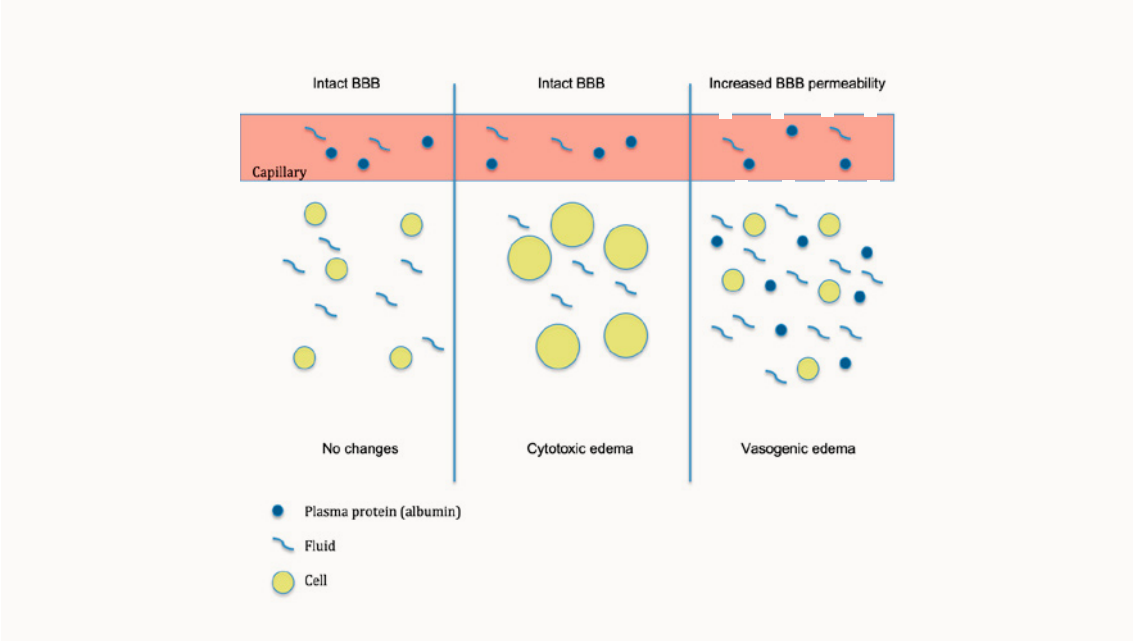


Figure 1.11 - Main features in cerebral edema. Cytotoxic edema: failure of Na^+/K^+ ATPase leads to Na^+ and consequent fluid cellular influx, culminating in cell swelling; Vasogenic edema: BBB breakdown leads to plasma proteins and abnormal fluid accumulation in extracellular space. **Legend**: BBB, blood-brain barrier.

Cytotoxic edema develops with intracellular accumulation of fluid (especially in grey matter),[317] following ionic pump failure and selective activation of ionic channels [e.g., ASIC (Acid Ensing Ion Channel), GLUT (Glucose Transporter) 1/2],[315, 318] further complicated by the loss of homeostatic ionic gradients and impairment of ATP production, a "bioenergetics crisis" and consequent cell death.[317, 319] This phenomenon will affect all CNS cell types due to an inability to maintain necessary transmembrane ionic gradients, leading to intracellular accumulation of sodium and an osmotic gradient driving water into the intracellular compartment.[320, 321] Cellular swelling and rupture will occur, reinforcing loco-regional inflammation in a self-sustained cycle.

On the other hand, vasogenic edema is derived from BBB disruption, with water and proteinaceous fluid overall influx into the interstitium as a consequence of complementary phenomena previously addressed.[114, 208, 315] Accumulation of specific molecules in extracellular space forces a change in its osmotic pressure, driving water from the intravascular compartment into brain parenchyma.[315] Considering that cytotoxic edema is based on a water shift from extracellular into intracellular locations, with no transit between compartments as in vasogenic edema, the latter is in theory responsible for most of the increase in brain volume and ICP.[322] Vasogenic edema is, therefore, a main mortality driver in the first week following TBI or stroke.[317, 323]

Concerning TBI and post-traumatic edema, the contribution and timings of its sub-types have been the subject of controversy and conflicting data.[114, 130] Vasogenic edema is consistently portrayed as an earlier phenomenon, later reinforced by long-lasting cytotoxic edema (significantly from 2-3 days following TBI and up to 2 weeks).[130, 315] Vasogenic edema, however, was shown to be persistent for 3-4 days.[324] Interestingly, a second peak in vasogenic edema may occur after 5 days, possibly mediated by microglial activation.[114, 325] BBB is maximally permeable 4-6h following TBI while, at 7 days, it is much more differentially permeable and only for smaller molecules.[130, 326] The notion of a rigid distinction between "cytotoxic" and "vasogenic" edema, despite its convenience, is rightfully disputed.[114, 315] Post-TBI edema, in all its forms, will then significantly impact ICP.

Astrocytic water channel AQP4 has been implicated in cerebral edema pathogenesis.[327] Its expression is significantly increased in TBI,[327, 328] making it a potential therapeutic target.[329] Mainly expressed in perivascular endfeet processes and *glia limitans,*[330, 331] AQP4 is thought to promote water movement into affected astrocytes.[332, 333] Additionally, AQP4 is theorized to participate in vasogenic edema's resolution, following different types of injury.[243, 333] Following TBI, AQP4's polarized location is shifted and its expression is therefore mainly localized in astrocytic soma and processes, with significant upregulation in astrocyte-based glial scar and milder global AQP4 upregulation, peaking at 7 days.[243, 334]

Inflammation

Neuroinflammatory response and activation of immune response

Within minutes of injury and following cellular damage, release of Heat Shock Proteins (HSPs) and HMGB1 (High Mobility Group Box-1) [a typical Damage-Associated Molecular Patterns (DAMPs)], is followed by its binding to transmembrane

TLRs and activation of nuclear factor-κB (NFκB) and MAPK pathways, leading to release of proinflammatory factors (IL-1β, IL-6, chemokines, immune receptors).[335, 336] These signalling molecules (ATP, HSPs, HGMB1 - so-called "alarmins") are released from damaged meninges, *glia limitans* and brain parenchyma.[337, 338] Alarmins will then induce microglial activation and generation of IL-1β. NFκB will translocate to cell nuclei and promote cellular proliferation and proinflammatory amplifiers release. IL-6, TNF and other similar components peak at 2 days following TBI, with a more extended period of normalization of its values.[338, 339] This cytokine-based response is involved in reactive astrocytosis, microglial activation and migration (as well as axonal dysfunction).[133, 194] Activation of resident CNS cells and recruitment of peripheral leukocytes are therefore dependent on inflammatory mediators.[340, 341] Distinct proinflammatory factors are later counterbalanced by upregulation of anti-inflammatory molecules and neurotrophic factors (IL-4, IL-10, TGF-β).[336]

Hippocampal mRNA levels of CD11b/Iba1 and GFAP/S100B (microglial and astrocytic markers, respectively) are increased in the aged hippocampus.[342] This indicates a hippocampal "pseudo-activation" in the elderly, potentially associated with age-related progressive neurodegeneration.[342] Aged hippocampus display increased cytokines/chemokines expression and a distinct astrocyte phenotype (thickened processes, hypertrophied soma), most likely in relation to reduced neuronal protection and regeneration.[342, 343] The notion of "inflammaging" as a global, low-grade chronic inflammatory state in the elderly is another issue to be accounted for concerning the injured brain and respective prognosis.[344, 345]

The role of this "classical" inflammatory pathway is ambiguous in its purpose and undoubtedly dependent on the timing of assessment.[133] Microglia and astrocytes' role in clearing cell debris and releasing anti-inflammatory cytokines are necessarily beneficial. Contrastingly, microglia phenotypic interchanging (M1 and M2 gene expression) accentuates and lengthens the deleterious inflammatory effect (see Microglia section).[193] Proinflammatory molecules will also play a supplementary role in BBB disruption, as demonstrated by increased BBB permeability dependent on IL-1β, related to loss of occludin/ZO-1 and TJs redistribution.[346]

Neutrophils are the first peripheral cells to become noticed in the brain following TBI, upon chemokines recruitment[347] and peaking at 48-72h post-TBI,[211] followed by macrophages/microglia migration and astrocytes activation.[138] Neutrophils are the major phagocytes of cell debris and are able to release neurotoxic products while increasing endothelial permeability[211, 348] and production of ROS, proteolytic enzymes and cytokines.[337, 338] T lymphocytes, Natural Killer cells (NKs) and dendritic cells are also recruited.[201, 349] Autoreactive $CD4^+$ T cells display a neuroprotective role concerning injured axons, eventually derived from

its ability for IL-4 release (promoting neurotrophin signalling and neuronal recovery).[350, 351] Immune system signalling in injured CNS takes place partially via DAMPs and Pathogen Associated Molecules Patterns (PAMPs), triggering an inflammatory cascade.[352]

Neurogenic inflammation

Neurogenic inflammation was initially described in peripheral tissues (skin, lungs) as an inflammatory process triggered by a noxious stimulus activating peripheral sensory unmyelinated neurons.[353, 354] It leads to increased microvascular permeability, vasodilation and peripheral fibrosis.[354, 355] It also plays a role in secondary injury pathways in the CNS (TBI, stroke).[356, 357] Both SP and Calcitonin Gene-Related Peptide (CGRP) are involved in BBB's disruption, vasogenic edema and neuronal injury. Transient Receptor Potential V1 (TRPV1), co-localized with SP and CGRP, is also involved in neuronal injury by facilitating neurogenic inflammation and BBB's dysfunction.[358, 359]

Despite similar and synergistic actions, distinct neuropeptides display different roles: CGRP mainly promotes vasodilation; SP acts primarily upon capillary permeability and plasma extravasation,[360] a paramount characteristic concerning TBI. SP, acting on NK1 receptors (NK1R), promotes vascular permeability and interstitial leakage of osmotically active molecules and water.[297] Other peptides, including CGRP, play similar roles, namely in complex brain blood-flow self-regulation.[361, 362]

Following moderate to severe TBI, early disruption in NVU is present, likely susceptible to SP's interference.[363] Endovascular endothelial glycocalyx, an endoluminal complex of glycoproteins and proteoglycans acting as an NVU extension, probably plays a role.[364] SP/NK1R's action upon BBB includes significant changes in ZO-1 and claudin-5 expression.[365] SP's activity may lead to transcellular transport by increasing caveolae-mediated transcytosis,[12] dependent on NK1R localization in endothelial caveolae.[366] SP's activation of NK1R also increases leukocyte migration via chemotaxis while upregulating adhesion molecules expression.[367] Neurogenic inflammation is capable of directly interfering with BBB's integrity while enhancing/perpetuating classical inflammation.[368]

1.4.3 Structural injury

Besides obvious macroscopic brain injuries, TBI can result in distinct microstructural findings.

Structural disruption

Moderate and severe TBI induces overall chronic impairments and structural disruption, a consequence of both primary and secondary injury, leading to disturbances in intrinsic brain structuring connectivity. Quantitative MRI studies display noticeable brain volume loss years following initial injury,[369] namely with white matter volume loss up to 4 years post-injury.[370] Most studies show that even mild TBI might result in diffuse axonal degeneration and neurodegenerative changes in the injured brain.[167]

Most post-traumatic impairments and deficits arise, in part, from disturbances in sensory input processing, based on persistent inhibition-related neuronal hyperexcitation in upper cortical layers.[163, 371] Other authors have described intracortical connectivity compensatory mechanisms, specifically inter-regional and through direct corticospinal projection pathways.[372] These mechanisms might explain motor improvement following TBI along with compensatory behaviours, despite more deeply integrated cognitive deficits.[373]

Several studies demonstrate, in Diffusion Tensor Imaging (DTI) MRI scans, post-TBI microstructural changes in white matter tracts,[374] with significant repercussion in cognitive performance (attention, memory, executive function). Animal models also confirm white matter disruption and loss of integrity, including a substantial decrease in *corpus callosum* thickness and other changes in its functional anatomy (anisotropy, lower diffusivity), still present up to 12 months following TBI.[375] C*orpus callosum*-specific myelinated axon conduction deficits and degenerative mechanisms (with demyelination) lead to white matter atrophy.[376] Damage from impact-acceleration forces is obvious, with corresponding neural circuit deficits following myelinated pathways impairment.[41, 171] Frequently, concomitant loss of neuronal cell bodies (grey matter regions) precludes white matter structural recovery.[377, 378]

Traumatic Axonal Injury (TAI) [or Diffuse Axonal Injury (DAI), in its clinical context)] is the main microstructural event concerning axonal projections running in white matter tracts, after sustaining distinct forces involved in TBI (torsion, tension, compression) (see Neurons section).[171] These shear forces and consequent deformation stress are mainly due to a speed differential concerning cortex and subcortical/deep white matter dislocation.

Experimental models consistently confirm specific notions as follows[379]:

- TAI frequently occurs in axons surrounded by non-affected axons;
- Axons are particularly vulnerable to rotational-acceleration injury due to their significant anisotropic arrangement and specific structural design[380];

- Breaking of axonal microtubules is an important feature[381];
- Primary axotomy is a relatively rare event[380];
- Death of corresponding neuron cell body is not a necessary condition for TAI[382];
- TAI may co-occur in several neuroanatomical regions.

Myelin disruption is another major factor in white matter damage, although unmyelinated fibers are also very susceptible to TAI.[171, 383] Damaged axons overcoming the disconnection phase undergo Wallerian degeneration and myelin sheaths collapse while the axon fragments and degenerates.[171] Myelin debris slow clearance arguably invokes additional detrimental effects, inhibiting axon regeneration[384] and activating microglia.[385]

Hippocampal injury

The hippocampus is a major component in human and other vertebrates´ brain, located in the medial temporal lobe in primates **(Figure 1.12)**. The hippocampus is included in the limbic system, playing crucial roles in acquiring and processing information and consolidating long-term, semantic, social and spatial memory.[386, 387]

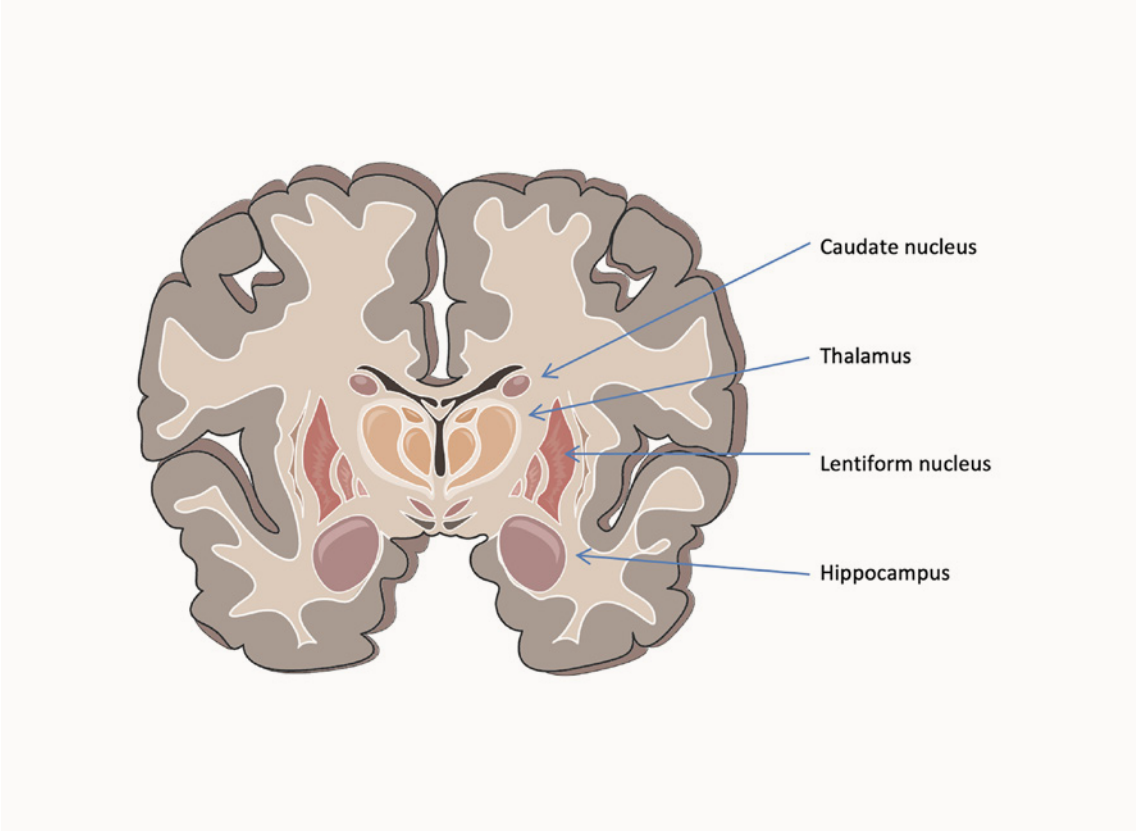


Figure 1.12 - Hippocampus, representation of a coronal section of the human brain.

Hippocampus is classically divided into two parts: *Cornu Ammonis* (CA) and Dentate Gyrus (DG), separated by hippocampal sulcus and curved into each other.[388, 389] Other specific areas, *subiculum* (inferior to hippocampal fissure) and entorhinal area (providing primary input to the hippocampus), are also mentioned in varying anatomical classifications. *Cornu ammonis,* consisting of pyramidal cells, is further divided into CA1, CA2, CA3 and CA4.[389, 390] These highly interconnected areas **(Figure 1.13)** communicate with different cortical regions.

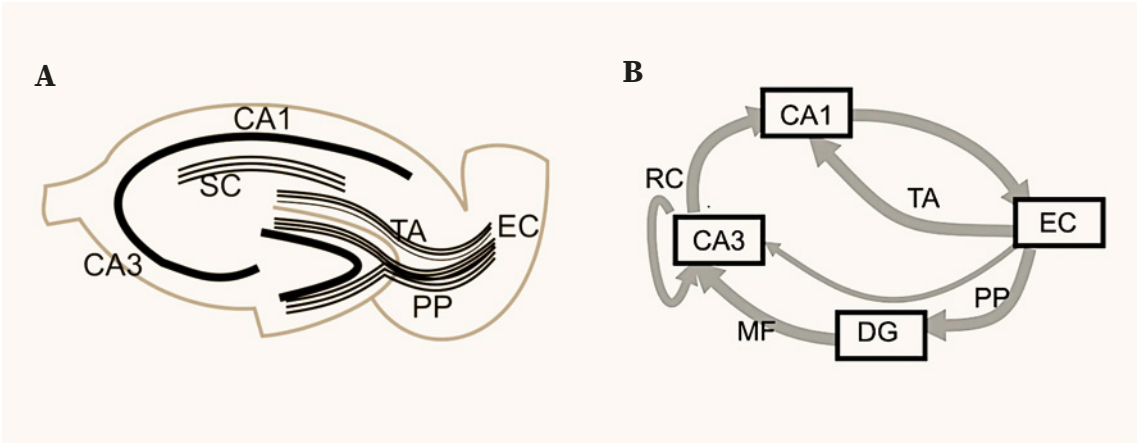


Figure 1.13 - A - Schematic illustration of human hippocampus structure (adapted from Takano et al.,[391] with permission). **B - Schematic illustration of human hippocampus major excitatory pathways** (adapted from Takano et al.,[391] with permission). **Legend**: CA, *cornu ammonis*; DG, dentate gyrus; EC, entorhinal cortex; MF, mossy fibers; PP, perforant path; RC, recurrent network; SC, Schaffer collaterals; TA, temporoammonic pathway.

In animal models of repetitive minor TBI, although not showing significative hippocampal neuronal damage, disperse gliosis and minimal changes in deeper layers are still present.[392] Post-traumatic hippocampal damage is characterized by neuronal disruption in the CA1 and CA3 layers and deafferentation in the CA1 layer.[393, 394] Neuronal loss is present bilaterally, even at later stages (following 30 days post-TBI), despite being more pronounced in the ipsilateral hippocampus.[395] Pyramidal hippocampal neurons in the CA3 layer and granule cells in the DG appear to be most vulnerable to this bilateral phenomenon.[396] Pathological changes specifically in contralateral hippocampus include neuronal loss in the CA3 layer (up to 60% in the first 48h) and CA1 layer deafferentation.[394, 397] Immunohistochemical studies with GFAP and Fluor-Jade staining documented significant neuronal and astrocytic damage, namely in the previously mentioned CA1 and CA3 hippocampal layers, as soon as 24h following TBI.[398, 399] Hippocampal damage is also reflected in cells that, although not sustaining apoptosis/necrosis, display

significant structural damage, including dendritic and synaptic degeneration, with diminishing local synaptic density.[400] A main location for hippocampal cell death is the DG,[401] where many dying cells are not NeuN-expressing mature neurons but immature NCAM (neural cell adhesion molecule)-expressing granular neurons, significantly compromising future neurogenesis.[402, 403]

Once Schaffer's collateral pathways are affected, significant post-traumatic disruption of the pyramidal CA3 layer will result in deafferentation of superior CA1 dendritic component.[404] A substantial decrease in the number of dendritic spines has been reported in the ipsilateral CA1 and DG,[33, 405] following repeated TBI, with an expected impact in synaptic plasticity, acquisition and retention of spatial memories.

Imaging data analysis shows increased mean hippocampal diffusivity in the CA1 and *stratum radiatum/lacunosum-moleculare* (SLRM) regions, in relation to necrosis and edema.[406] White-matter tractography detected smaller tract volumes seeded from the right-hemispheric hippocampus (CA1, SRLM, CA4) (again with hemispheric asymmetry).[406] Other studies demonstrate obvious volume loss in the hippocampus of victims of repetitive concussions.[407]

1.4.4 Substance P

SP is an 11-aminoacid peptide derived from the preprotachykinin-A gene by alternative splicing, widely distributed in CNS (cortex, hippocampus, basal ganglia, hypothalamus, amygdala), peripheral nerves (dorsal root ganglion neurons) and enteric nervous structures.[408, 409] It is more abundant in grey than in white matter.[410] Preferred NK1R are expressed on astrocytes and microglia, diverse endothelial cells and circulating inflammation-activated immune system cells.[411] Signal transduction through NK1R (and its truncated isoform, with less affinity for SP and decreased induced inflammatory response)[412, 413] takes place via G protein signalling and cAMP (secondary messenger), leading to changes in gene expression and enzymatic activity patterns, and regulation of ion channels activation.[411, 414] SP, via NK1R coupling to phospholipase C, facilitates AMPA and NMDA receptors function,[415, 416] namely in the dorsal horn and granular layer of hippocampal DG.[411, 417] Most intrinsic fibres containing SP develop symmetrical synapses in GABAergic interneurons in mouse hippocampus .[418]

SP promotes inflammatory mediators´ production (cytokines, histamine), endothelial cell adhesion molecule expression, leukocyte activation and migration.[353, 358] It is responsible for increased BBB permeability, with studies demonstrating di-

rect interference with TJs proteins, decreasing ZO-1 and claudin 5 levels upon direct application to cerebral capillary endothelial cells.[419] SP also upregulates adhesion molecules and MHC class II antigens, implying recruitment/migration of inflammatory cells across BBB.[419]

A regulatory role for SP in inhibitory GABAergic circuits is mediated preferably by NK1R.[420] Concerning SP's role in self-sustained seizures in animal models (self-sustaining *status epilepticus*), early reports have shown increased extracellular glutamate concentration and SP's promotion of *status epilepticus* and consequent hippocampal damage.[421] SP also acts as an element of mediation in memory and behavioural components (anxiety, stress, fear).[422]

Extensive literature reports an immediate post-traumatic increase in SP's serum levels and perivascular immunoreactivity.[423, 424] Increased SP's immunoreactivity following TBI has been described in animal models (from 5h up to 3 days post-TBI, perivascular location)[425] and human post-mortem studies.[426] Significantly, SP's perivascular location and increased immunoreactivity are co-localised with regions of significant BBB disruption, with an apparent linear relation between SP's immunoreactivity, the severity of injury and mortality.[297, 424] This leads to experimental research and clinical trials focusing on SP as a possible biomarker and, above all, as a therapeutic target.[130, 362] Targeting SP's expression with NK1 antagonists has shown beneficial effects in TBI rat models, decreasing BBB impairment and improving outcome.[423, 427] Some studies have shown a possible mechanism for post-traumatic brain release of SP (from sensory neurons) via mechanical activation of TRPV1 and similar receptors (e.g., TRPA1),[133, 353] with the influx of cations (sodium, Ca^{2+}) triggering neuropeptides release.[428] External mechanical insults to the brain, and eventual brief spikes in blood pressure, will activate TRPV1 and others and lead to SP's release.

SP is therefore a potent initiator of neurogenic inflammation **(Figure 1.14)** while also playing a role in classical inflammation pathways.

CGRP, included in the calcitonin family of peptides, has also been implicated in post-traumatic neurogenic inflammation **(Figure 1.14)**, displaying overlapping pathological pathways with SP.[429] Increased serum levels of this peptide were obvious 2 days post-TBI,[429] with similar findings in experimental studies.[430] Even so, CGRP's behaviour is less well studied and somewaht unpredictable when compared to SP.[12]

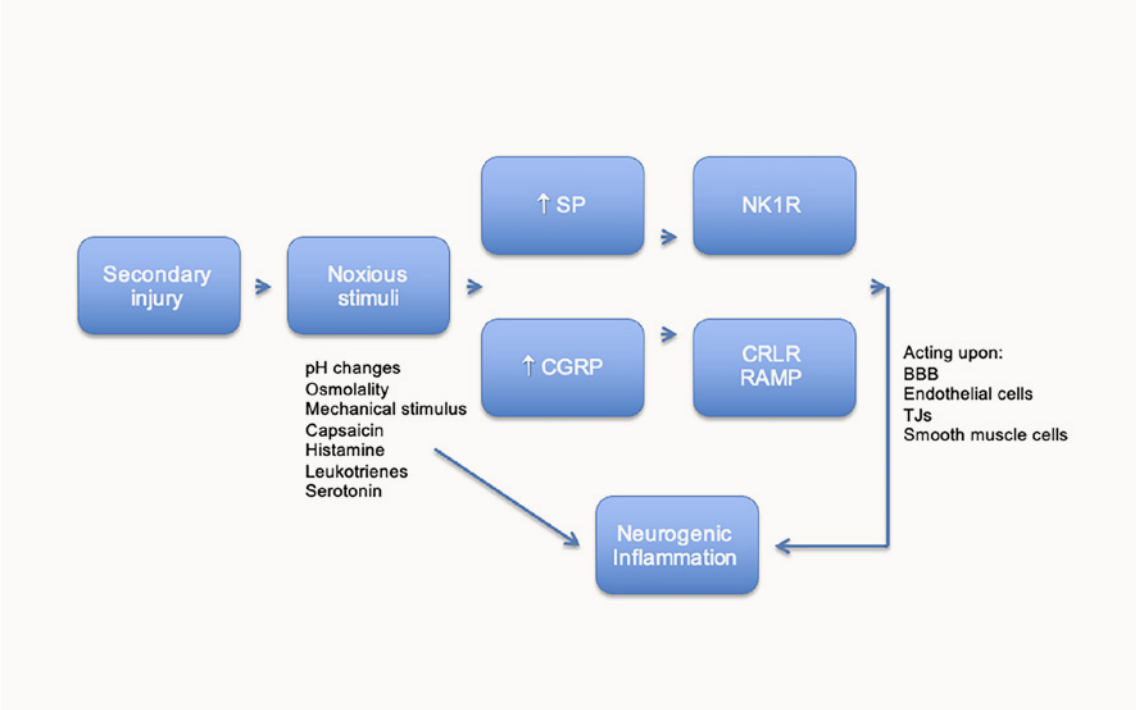


Figure 1.14 - SP and CGRP in neurogenic inflammation. Both neuropeptides are clearly and precociously increased in the context of TBI's secondary injury, playing a role in post-traumatic neurogenic inflammation. **Legend**: BBB, blood-brain barrier; CGRP, calcitonin gene-related peptide; CRLR, calcitonin receptor-like receptor; NK1R, neurokinin 1 receptor; RAMP, receptor activity-modifying protein; SP, substance P; TJs, tight junctions.

1.4.5 Neuropeptide Y

NPY, one of the most abundant brain neuropeptides, is a 36-aminoacid peptide highly expressed in the Central and Peripheral Nervous Systems, including prosencephalon, diencephalon and brain stem.[431, 432] NPY is abundantly expressed in the hippocampus and cortical interneurons, Sub-Ventricular Zone (SVZ) and thalamic reticular nucleus.[433] Astrocytes, namely cortical populations, display an abundance of NPY receptors,[434] and NPY is co-localized with SP in GABAergic interneurons, where it is mainly produced and released.[434] NPY is involved in the primary response to different events (stroke, epilepsy), modulation of post-aggression cytotoxic environment and neuronal regeneration.[432, 435] NPY is also included in nociceptive pathways, namely via activation of NPY Y1 receptor subtype.[432, 436]

NPY is included in the same family of peptide YY (PYY) and pancreatic polypeptide (PP).[437] The members of this family act via protein G coupled receptors, with

six G Protein-Coupled Receptors sub-types identified (NPY1R to y6R).[438] NPY1R, Y2R, and Y5R receptors are the most prominent in the brain, while Y4R is mainly expressed in the gastrointestinal tract and y6R is present in mice and rabbits, but not in primates or rats.[439, 440]

NPY regulates several biological functions, such as blood pressure, neuroendocrine system, feeding behaviour, circadian rhythms, neuroplasticity and memory.[441, 442] Increased levels of NPY expression in distinct brain regions correspond to different effects (both stimulating or inhibiting), according to different types of receptors.[442, 443] In fact, NPY is involved in the regulation of neuronal activity and hyperexcitability states.[444, 445] This peptide has a well-described anticonvulsant effect via its different receptor subtypes with a hippocampal and cortical location.[435, 446] Seizures increase NPY expression in specific hippocampus regions and cell populations, including gamma-aminobutyric acid (GABA)-ergic interneurons and mossy fibers and granule cells.[447, 448] NPY receptors coupling to Gi/o proteins leads to decreased cAMP accumulation (upon inhibition of adenylate cyclase), Ca^{2+} and K^{+} channels modulation and intracellular Ca^{2+} mobilization.[442] NPY's role as an endogenous anticonvulsant is partially based on hippocampal glutamatergic excitotoxicity modulation.[445, 449] Studies in post-ischemic retina models have also shown an inhibitory action of NPY over cytotoxic edema upon glutamatergic regulation.[450]

Other regulatory roles are attributed to NPY, including cellular proliferation (including neuronal) in hippocampal DG and SVZ,[451] vascular tonus regulation[452, 453] and angiogenesis.[454] Its role as a pro-neurogenic agent has been shown, on animal models of brain ischemia and epilepsy, acting on the SVZ stem cell population as a stimulus for new neurons and glial cell proliferation (via Y1 receptors) and promoting its migration in order to repopulate damaged areas.[451, 455]

In sum, NPY is clearly involved in neuroprotective pathways modulating glutamatergic hippocampal excitability (via receptors NPY2R), pro-neurogenic and pro-migratory activity,[437, 455] as demonstrated in animal models of ischemic stroke or induced degeneration.[456, 457] Its pro-neurogenic action is also exerted via Y1 receptors.[431, 432]

Given its role as a potent neuromodulator and its ubiquity in CNS, the therapeutic potential of NPY's pathways modulation has been addressed. One example is the intranasal administration of NPY or NPY 13-36 (a selective agonistic ligand for NPY2R) in animal models of Huntington's disease.[458] Other studies with recombinant adeno-associated viral vectors carrying human NPY gene have shown sup-

pression of acute and chronic seizures and reduced excitability upon increased endogenous NPY expression.[459, 460] NPY's potential as a neuroprotective agent in kainate models of hippocampal neurotoxicity has been shown.[461]

NPY is also mentioned as a response regulator in stress and depressive states, with different studies displaying suboptimal values of NPY in animal models and pre-clinical studies of post-traumatic stress and depression.[433, 462] NPY is upregulated (namely in the pre-frontal cortex) as a response to therapeutic protocols with antidepressants.[463] Anxiolytic effects, with amygdala interference, are also well characterized.[437, 464] In fact, chronic stress increments NPY RNAm levels in the amygdala, leading to increased NPY levels in CSF and plasma in major depression patients.[433] Amygdala's NPY1 receptors play a fundamental role not only as an adaptative response to stress but also in epileptogenic phenomena of temporal origin.[465, 466] Morgan and colleagues[467] report pre-clinical studies in which injection of NPY in the amygdala induces an anxiolytic effect at a central level, an effect later reproduced with artificial CSF solutions.[468] A likely NPY's anxiolytic endogenous effect, namely by down-regulating other neuropeptides (e.g., Corticotropin-Releasing Hormone), is therefore well documented both in human and animal studies.[469, 470]

An obstacle to more sustained pre-clinical studies involving NPY, which might explain in part the discrepancies among different teams, is the uncertain correlation between CSF and plasma levels of NPY.[433, 471] However, in various research protocols and independently of its questionable direct relationship, plasmatic and CSF levels of NPY indeed reflect an objective neurobiological response, able to modulate stress and coping strategies.[433] Concerning the possibility of NPY directly inhibiting SP's peripheral action via Y1 receptors, NPY decreases capsaicin-induced SP's immunoreactivity in dorsal horn's microdialysate and stimulus-evoked NK1R internalization.[431]

Regarding NPY's role in TBI, there are no significant studies on its role. Early reports show increased NPY levels in CSF and plasma of TBI victims and in animal models of TBI,[452, 472, 473] with special focus on cortical contusions and with an obvious peak at 48-72h. Its potential role as a promoter for focal neurogenesis following TBI was also shown.[474, 475] Some authors have previously theorized about its potential vaso-regulatory role in a post-TBI context.[452] Animal models of mild TBI and PTE display an apparent upregulation of hippocampal NPY associated with neuroprotective mechanisms in response to TBI.[476]

Several reports also mention NPY's involvement in post-TBI intestinal dysfunction in a synchronic way and complemented with AQP4 action.[477, 478] Intestinal ischemia, consequent hypoxia and edema are speculated to result from ongoing AQP4 activation.[479] The severity of structural changes in villi is proportional to

initial trauma severity, and the same happens with plasma levels of NPY and AQP4.[479] The authors correlate increased levels of NPY with intestinal ischemia and hypoxia, while AQP4 is supposedly related to intestinal edema.

Several reports show an embracing immunomodulatory role for NPY (both in a autocrine and paracrine fashion).[480, 481] Microglia migration and mobility, as a response to inflammatory events and dependent on lipopolysaccharide (LPS) stimulation, is inhibited by higher levels of NPY, downregulating p38 mitogen-activated kinase protein phosphorylation.[480] Moreover, NPY inhibits microglia phagocytosis stimulated by LPS via modulation of IL-1β levels.[482]

1.4.6 Magnesium

Mg^{2+} is mentioned in the literature as an important element in neuropathologic pathways, as a likely neuroprotective agent (memory, cognition, learning) with direct intervention on excitotoxicity phenomena.[483] Mg^{2+} functions as a physiological modulator for Ca^{2+} signalling and a direct Ca^{2+} antagonist, regulating NMDA receptors and attenuating smooth muscle contraction while diminishing neurotoxicity.[484, 485] It plays a role in inhibiting Endothelin-1 production, inflammatory mediators and free radicals,[486] while operating as a cofactor for innumerous enzymatic mechanisms.[485]

Several studies report its sustained decrease (intracellular and serum) following TBI[487, 488] and in trauma/shock patients in general.[489] Post-TBI hypomagnesemia is associated with exacerbation of secondary deleterious phenomena (apoptosis, oxidative stress, excitotoxicity).[490]

Mg^{2+} depletion, and concomitant SP's increase, have been tested as possible biological markers[491] or therapeutic targets,[492, 493] through specific antagonists (N-acetyltryptophan, cannabinoid agonistic receptors), with promising albeit insufficient results concerning functional outcome. Administration of magnesium sulphate appears to positively influence the degree of neuronal damage following TBI and ensuing functional recovery (cognitive and motor).[493, 494]

A direct relationship was outlined between the initial and final volume of intraparenchymal haematoma, its expected expansion and hypomagnesemia levels upon admission.[495] Mg^{2+} also displays significant repercussion in functional outcome after 3 months, suggesting an important role for Mg^{2+} in hemostasis and platelet aggregation (through activated Factor VII and Factor IX).[495, 496] Its true impact in TBI management, stroke or non-traumatic subarachnoid haemorrhage is still to be confirmed.[497]

1.5 Biomarkers for brain trauma

The field of biomarkers in Neurotrauma is rapidly evolving, and new molecules and approaches are constantly being added to the current body of knowledge. The choosing of a preferred biomarker should be based on rigorous assessment and comparison of specific characteristics, being minimally invasive and cost-effective while allowing proper identification of patients benefitting the most from a close and desirably tailored management.[498] The perceived clinical applicability of a biomarker is dependent on its biological and pathological grounding, including a possible relation to a threshold of disfunction of BBB and eventual mechanisms for post-TBI adaptation.[498] In respect to biomarkers characteristics, several questions must be addressed (based on Kawata et al.)[498]: What is the origin of the protein? Is the protein expression limited to CNS, or does it have a systemic repercussion? Are post-TBI abnormal values due to cellular damage and systemic spilling, or are they a consequence of up/down-regulation phenomena? What is the specificity of the phenomena in question? What is the role for molecular transporters in BBB?

As several reports have shown, panels of biomarkers outperform a single biomarker, namely in distinguishing CT-negative and CT-positive patients.[499, 500] Imaging is not entirely reliable as a single tool for a proper diagnosis/prognosis assessment.

When dealing with biomarkers of complex multifactorial phenomena, as in TBI, the danger of appreciation errors, with false positive/negative errors, is significant. An example of this is a likely interference of skull fractures when assessing brain trauma biomarkers, considering the increase in S100 serum levels when in the presence of injured osseous tissue.[501] GFAP and Ubiquitin C-terminal Hydrolase-L1 (UCH-L1) were both deemed unreliable as mild TBI biomarkers when in the presence of concomitant orthopedic injuries, as the rate of false positives would lead to unnecessary brain imaging.[500] Even so, several studies have shown the feasibility of employing recognized biomarkers for TBI, given its relevance in the short and long-term.[502, 503]

Promising biomarkers

It would be strenuous and ineffective to mention all biomarkers currently under scrutiny, namely in recent studies with Protein Network Analysis, employing multiple protein microarray detection and bioinformatics analysis (still in development and of uncertain utility at the moment).[504, 505] Thus, herein are described in detail some of the most promising biomarkers in neurotrauma to date.

1.5.1 S100B

S100B, an intracellular S100-group Ca^{2+}-binding protein, with a relatively small size (9-14 kDa), located primarily in astrocytes, is a widely accepted biomarker for TBI,[107, 506] despite its still rather limited use in clinical practice. S100B is expressed mainly in mature perivascular astrocytes in its two forms (homodimer S100BB and heterodimer S100AB).[3] It is present, to some extent, in other CNS cell types (oligodendrocytes, neural progenitor cells, specific neuronal populations) and peri-glial extracellular space.[507] S100B assists the regulation of cell Ca^{2+} influx/efflux, being linked to apoptotic environments.[508, 509] S100B is also involved in cell differentiation and cycle progression.[3]

Several studies display S100B's sensitivity in detecting brain lesions (namely focal contusions) and their progression **(Figure 1.15)**, as it is directly correlated to the amount of cerebral tissue affected.[3, 510] Upon trauma or metabolic stress, astrocytes will release previously stored S100B.[511] At the same time, S100B mRNA levels will also increase, confirming that increased S100B levels in TBI victims are simultaneously due to secretion and upregulated intracellular synthesis.[3, 512] Based on connections between glial cells and AQP4-dependent paravascular pathways, recent studies raise the possibility of the glymphatic system playing a role in S100B outflow from the brain and into the bloodstream.[513, 514] The degree of BBB disruption should also be influential in S100B clearance from CNS.[514]

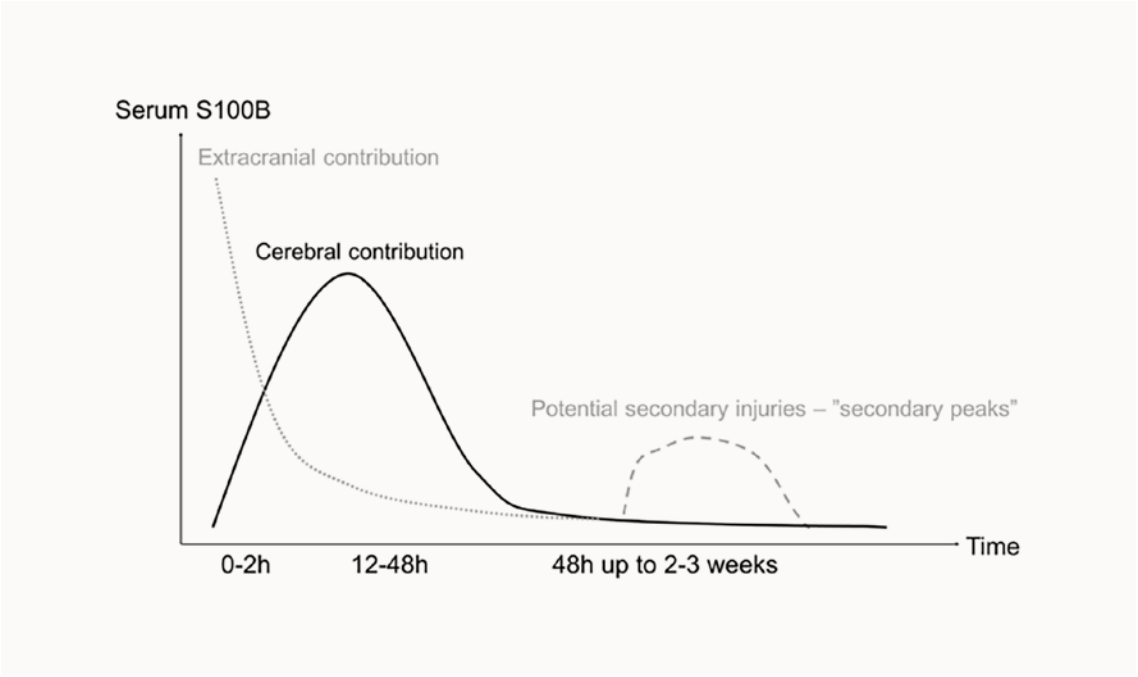


Figure 1.15 - Schematic overview of S100B levels in traumatic brain injury, displaying a typical temporal progression for a biomarker (adapted from Thelin et al.,[3] with permission).

S100B value as a biomarker for TBI has been clearly demonstrated, both in mild and moderate/severe TBI, with some authors emphasizing its negative predictive value.[3, 515] Specifically in the case of brain contusions, its volume is directly correlated to S100B serum levels.[516] S100B is also a reliable indicator of secondary injury,[517] although its significant concomitant elevation in extracranial injuries might represent a confounding factor.[518] S100B is already used in the clinical setting in some Institutions, strengthening diagnostic accuracy.[519, 520] For all its characteristics, S100B was included in the Scandinavian CT guidelines as the preferred biomarker for TBI.[521, 522] High levels of S100B are also associated with neuropsychological impairments and poorer work resumption.[515, 523]

1.5.2 Glial Fibrillary Acidic Protein

GFAP, an astrocyte intermediate monomeric filament cytoskeleton protein, is vastly studied in neurotrauma literature.[524, 525] Increased seric levels of GFAP have been detected following TBI (pre-clinical and clinical studies), with persistently increased levels up to 7 days post-TBI.[501, 526] Reports show an apparent increase in GFAP levels 1h following initial trauma, with a peak at 20h and progressive decline in the next 72h.[527, 528] Several studies display its sensitivity in detecting concussion and traumatic intracranial lesions.[528, 529] Its validity as a TBI marker was equally shown in pediatric patients.[530] Even so, the positive predictive value of GFAP is limited and its suitability for individual patient outcome assessment is questionable.[525] Importantly, biomarker expression and accuracy may decrease with age: GFAP's accuracy in detecting post-traumatic intracranial lesions decreases in older patients.[524]

Some studies mention the possibility of combining GFAP and S100B, or GFAP and p-tau, in more effective prediction models,[515, 524] although GFAP apparently displays superior detection capabilities for intracranial lesions, especially in the presence of skull fractures.[501]

1.5.3 Cytokines

Interleukin-1 (IL-1) is produced by activated microglia, astrocytes, endothelial cells and recruited leukocytes.[180] An early increase in IL-1**β** levels in CSF and brain (namely hippocampus) following TBI (3-8h) is noticeable.[180] IL-1**β** is known to upregulate other proinflammatory molecules such as IL-6, TNF, COX-2 and iNOS (inducible Nitric Oxide Synthase), following activation of protein kinases and nuclear factor kappa B (NF-κB).[531] IL-1**β** was tested as a therapeutic target in

TBI: following IL-1β attenuation or neutralization, distinct post-traumatic histological features (neuronal death, edema, inflammation) are improved.[532] Cognitive and behavioural improvements were also obvious following IL-1β neutralization, through relatively unknown mechanisms.[180, 533]

Tumor Necrosis Factor (TNF), expressed on the cell surface, plays several roles in physiological (immunity, body development) and pathological conditions, such as inflammation, cell death, septic shock, ischemia, tumour growth and others.[534, 535] It is known to modulate multiple signalling pathways, mediated by its two receptors, TNFR1 and TNFR2 (specifically expressed in endothelial cells, neurons and immune cells),[536-538] TNF is another potential biomarker for TBI, with consistently increased levels (both in CSF and plasma) up to 1 year following even mild TBI.[539, 540] This is in clear opposition to other studies, exhibiting transient or negligible post-TBI increases in TNF.[539, 541] Most often regarded as a potent proinflammatory cytokine, produced primarily by monocytes/macrophages,[534] TNF's action is thought to be mostly detrimental in a post-traumatic context, as part of the initial response to neuronal injury.[542]

Following an immediate increase in TNF and IL-1, a later increase in IL-6 and **IL-10** ensues,[543, 544] as a result of increased production by resident microglia and infiltrating monocytes/macrophages. IL-10 increased levels are detectable both in CSF and serum of TBI patients,[499] and clinical studies have shown its usefulness as an early predictor of intracranial lesions, injury severity and mortality.[499, 544]

1.5.4 Other possible biomarkers

Tau proteins, mainly expressed in neuronal axons, are considered to reflect neuronal injury.[545, 546] Increased CSF tau levels are a consequence of stroke or recent TBI (along with long-lasting findings in CTE),[547, 548] raising the possibility of using it as a biomarker for repetitive head trauma.[545] **UCH-L1**, a neuronal deubiquitinating enzyme acting on ubiquitin monomers,[549] is a potential early-stage biomarker.[550, 551] A simultaneous use of GFAP and UCH-L1 has been suggested.[551] **Neuron-Specific Enolase** was shown to be an independent biomarker for post-TBI mortality and functional outcome,[552, 553] with the advantage of displaying a longer half-life.[554, 555] **HMGB1** protein, a proinflammatory intracellular factor, is another potential TBI biomarker[148, 556] and an eventual therapeutic target.[336, 556] HMGB1 acts as a typical DAMPs molecule, stimulating proinflammatory pathways and specific cell secretion (monocytes, neutrophils, NKs). HMGB1 also promotes microglial activation, following its release from innate immune cells (macrophages, dendritic cells) and injured/necrotic cells.[336, 557] **BDNF** (Brain-Derived Neurotrophic Factor) levels are increased as part of the brain's response in

an initial neuroprotective effort.[558] Post-traumatic significant increase in BDNF levels are evident in cortex, hippocampus and CSF.[559]

Assessment of **ceramide** and **sphingomyelin** levels, as a reflection of overall myelin lipid status, and **Neurofilaments Light**, an integral part of axonal cytoskeleton, displays promising initial results.[3, 560] Another line of research stresses the feasibility of using specific microRNAs as diagnostic and prognostic tools.[561]

1.6 Neuromonitoring

Comprehensive intensive care-based neuromonitoring of patients with severe TBI should provide helpful information to guide treatment protocols and prevent secondary injury.

ICP monitoring is, by far, the most common modality, with widespread use among Neurosurgical units,[562] despite some contradicting outcome results concerning its use and cost-benefit in Randomized Controlled Trials.[563]

Multimodal monitoring is becoming standard-of-care in many NeuroIntensive Care units,[27, 564] based on continuous, simultaneous assessment of distinct parameters: brain tissue oximetry, brain temperature, electroencephalography, microdialysis (sampling brain metabolites and small molecules).[564] Availability of tested technologies (e.g., cerebral microdialysis catheters with a larger membrane cut-off)[565] and advanced microdialysis protocols [Multiplex Proximity Extension assay (PEA) technology][565, 566] allows for comprehensive pre-clinical and clinical trials, simultaneously testing distinct potential biomarkers.[253, 567] Cerebral microdialysis is unique in its ability to provide a direct, continuous perspective on parenchymal concentrations of different molecules.[565, 568]

Czosynka's pressure reactivity index is routinely used to assess and establish specific parameters concerning cerebral perfusion pressure, allowing a dynamic adjustment aiming at optimal brain perfusion.[569, 570] Future clinical protocols should routinely include the assessment of different parameters: pressure reactivity indices, pulse amplitude index, optimal-cerebral perfusion pressure.[31, 315]

Continuous electrocorticography enables detection of ictal discharges with potential for cortical spreading, another contributing factor for secondary damage.[27, 571]

Other specific techniques, as sensory evoked potentials and bispectral index monitoring, are also available.[572] Non-invasive monitoring of cerebral blood flow resides mostly in transcranial Doppler (first choice on assessing vasospasm), despite other available techniques (near-infrared spectroscopy, rhoencephalography).[573]

1.7 Imaging

Imaging is a crucial component in trauma care,[574] assessing injury in an acute setting, allowing critical decisions concerning intensive care and surgical procedures, and, in the long-term, characterizing and quantifying structural and functional damage.

In the context of TBI, a non-contrast CT scan is the gold-standard initial imaging study.[575, 576] CT scans can detect most traumatic pathologies, namely those requiring prompt surgical intervention (fractures, subdural or epidural haematomas, large contusions). Unfortunately, non-contrast CT has several shortcomings on a broader perspective of neurotrauma care: failure in detecting DAI, underestimation of parenchymal contusions, limitations in detecting signs of intracranial hypertension.[575] Because of intrinsic limitations, specific MRI sequences with short acquisition time are being tested.[577]

Following an initial evaluation, a thorough and specific assessment of intracranial injuries may take place. Susceptibility-weighted imaging (SWI) allows detection of subacute microhaemorrhages in DAI.[575, 578] Other advanced MRI modalities, yet with no proved clinical usefulness and dubious cost-benefit analysis,[575, 576] are undoubtedly useful for research purposes: Perfusion imaging identifies areas of hypo and hyperperfusion;[579] Diffusion Tensor Imaging evaluates diffusivity concerning axonal tracts integrity;[198, 575] functional MRI assesses local brain activation,[576, 580] detecting patterns of recovery in network connections and uncovering higher cortical functions.[581, 582]

Magnetic resonance spectroscopy quantifies molecular compounds in brain tissue microenvironment.[583, 584] Typical features of TBI include early reduction in N-acetylaspartate (NAA) levels and decreased NAA/choline and NAA/creatine ratios, well correlated with long-term functional outcome.[585, 586]

1.8 Therapeutic modalities

The therapeutic approach to TBI is based on complementary aspects of treatment, based on the surgical management of traumatic lesions and medical/neurointensive care protocols, attempting to attenuate the expected increase in ICP and, therefore, indirectly optimize brain perfusion pressure and function. Unfortunately, these therapeutic protocols and procedures are so far unable to significantly interfere with crucial secondary mechanisms of brain damage.[587]

1.8.1 Medical therapies

Innumerous molecular compounds have been tested in animal models and clinical trials as potential therapeutic agents,[588, 589] with diverse properties, mechanisms of action, delivery methods and preferential targets. However, they have in common the fact of not being used in daily clinical practice, as their therapeutic effect and relevance for vital prognosis is dubious, unremarkable or still to be shown. Some of the most relevant are::

Corticosteroids, diuretics[590]
Superoxid dismutase[591]
Cyclosporin[592]
Selfotel (competitive NMDA antagonist)[593]
N-Acetylcysteine (antioxidant)[594]
Insulin[595]
Deltibant (bradykinin antagonist)[596]
Denaxabinol (non-competitive NMDA antagonist)[597]
Nerve growth factor[598]
Progesterone[599, 600]
Cerium oxide nanoparticles (targeting free radicals)[601]
4-amino-TEMPO antioxidant particles[602]
Amantadine[603]
Statins[604]
Magnesium sulphate[605]
Erythropoietin[606]
Candesartan (Angiotensin II Receptor 1 blocker)[607]
Retigabine (reducing neuronal excitability)[608]
Quetiapine[609]
Dietary supplementation with phospholid precursors[223]
Lithium Chloride[610]
IL-1 1R Knock-out[611]
TNF/Fas receptor Knockout[612]
Topiramate (glutamate release inhibitor)[613]
Doxycicline[614]

Several of these agents and protocols specifically target neuroinflammation.[138] Medical therapies directly targeting cytotoxic edema (bumetanide, aquaporumab, amiloride)[615, 616] or vasogenic edema (rosiglitazone, pioglitazone, bevacizumab, N-acetyltryptophan)[114, 425] were or are being tested in pre-clinical trials.

Magnesium sulphate is an obvious candidate for therapeutic protocols in TBI. Despite its use in several pathological contexts (arrhythmias, asthma, obstetric complications),[486, 494] recent clinical trials in neurological and neurosurgical disorders failed to display unequivocal therapeutic benefits.[488, 494] In theory, considering Mg^{2+}'s ubiquitous role, the therapeutic effects of magnesium sulphate should be objective and easy to demonstrate, as they are in animal models.[605, 617]

SP antagonists display promising results concerning functional outcomes in animal models of disease.[130, 618] N-acetyltryptophan seems to promote Mg^{2+} renovation and attenuate vasogenic edema.[130, 425] However, no successful phase III clinical trials have taken place, and its implementation in routine clinical practice seems unlikely at the moment.

Tissular Oxygen Partial Pressure is another monitoring parameter for therapeutic adjustments,[619] assessing ischemic damage and mitochondrial use of oxygen.[620] **Therapeutic use of oxygen** has also been tested, with conflicting results.[621, 622]

Hypothermia appears to reduce metabolic rate and overall mortality.[623, 624] Theoretical beneficial effects include decreased apoptosis and formation of free radicals, attenuation of post-TBI excitotoxicity[625, 626] and improved BBB function.[627, 628] However, standard hypothermia protocols fail to display efficacy beyond Grade III evidence and are yet to be fully integrated into standard clinical practice.[629]

1.8.2 Management of Intracranial Pressure

Several distinct modalities of treatment for raised ICP are currently used in clinical practice: head of bed elevation, transient hyperventilation, osmolar therapies (mannitol, hypertonic saline), barbiturates, decompressive surgery.

Osmolar therapies are believed to create an osmolar gradient, drawing water into intravascular space,[320, 630] while reducing cytokine-mediated oxidative stress and inflammation.[631] Unlike mannitol, hypertonic saline has the advantages of not being a diuretic agent and not accumulating within the brain parenchyma (which can lead to paradoxical intraparenchymal accumulation of fluid and refractory edema).[632]

In respect to **transient hyperventilation**, as induced hypocapnia will reduce arterial carbon dioxide´s partial pressure (promoting vasoconstriction), cerebral blood flow and ultimately ICP will be decreased.[633] Despite obvious risks (tissue hypoxia, cerebral ischemia), hyperventilation is one valid therapeutic option, only to be used for brief periods of time.[633, 634]

Barbiturates display beneficial features concerning TBI and brain edema: increase in vascular tone, anti-seizure activity, reduction of brain metabolic rate and excitotoxicity, inhibition of free radical-mediated lipid peroxidation.[113, 635] Its general profile and potentially severe side effects (pulmonary failure, arterial hypotension) preclude its wider use, being used only as a last resort.[635]

1.8.3 Surgical procedures in Neurotrauma

The role of surgical procedures in brain trauma is of extreme relevance, despite being somewhat limited in its possibilities. Its main aspects are drainage of intracranial bleeds (haematomas or contusions), monitoring and management of ICP and correction of structural abnormalities (fractures, lacerations, CSF fistulas).[636] Concerning management of ICP, besides treatment of hydrocephalus, placement of intracranial ICP sensors is crucial by allowing medical treatment optimization. Decompressive craniectomy is considered a last resort procedure, only when in the presence of intracranial hypertension refractory to medical treatments. It is, however, a controversial procedure, raising significant doubts concerning its actual usefulness and cost-benefit relationship.[637, 638]

Hypothesis and study design

Our research project was based on the intent of exploring the possibility of modulating the neuropeptide response to TBI. Considering the multitude of neuropeptides, we focused on the most abundant - NPY - and its potential as a therapeutic agent, a subject scarcely studied so far. As discussed in previous sections, extensive literature demonstrates NPY's neuroprotective role in other pathologies.

We hypothesized that:

- NPY plays a multifaceted neuroprotective role in TBI, and this physiological response can be potentiated;
- NPY is encompassed in a broader, multistage neuropeptide brain response to TBI.

This translational research project is divided into two main components:

- **Animal studies**, in which the possibilities of a rigorous animal model of head trauma were explored, and several post-traumatic pathological phenomena were assessed, as well as a possible action by NPY against TBI-induced changes;
- **Human clinical studies,** in which post-traumatic neuropeptide response (focusing on SP and NPY) was evaluated in its different timings and correlated to biomarkers of TBI.

CHAPTER II

Animal studies

2.1 Introduction

In order to determine the role of NPY in TBI and confirm our hypothesis of a potential role for NPY as a neuroprotective agent (with multiple aspects in its mechanisms of action), an animal model of trauma was developed and implemented in our laboratory. Following controlled trauma, several known aspects in deleterious response to TBI, from cellular activation and degeneration to BBB's disruption, were assessed and the influence of exogenous NPY in these same phenomena was evaluated.

2.2 Materials and Methods

All experimental procedures with animals were performed by certified researchers, in consonance with European Community Council Directives (2010/63/EU) and portuguese law for care and use of experimental animals (DL no. 113/2013). The present study was approved by the Institutional Animal Care and Use Committee (ORBEA) from FMUC, University of Coimbra (Coimbra, Portugal) and by the Portuguese National Authority for Animal Health (DGAV; Ref. 004015). Moreover, studies were conducted in accordance with the principles and procedures outlined as "3Rs" in EU guidelines (86/609/EEC), FELASA, and the National Centre for the 3Rs (the ARRIVE). All efforts were made to minimize the animal suffering and to reduce the number of animals used.

2.2.1 Animal model of trauma

Sprague-Dawley rats were subjected to sham injury or head trauma upon a TBI weight-drop model, as described below.

Sprague-Dawley male rats, aged between 10 and 14 weeks, weight 250-400g (Charles River Laboratories), were housed in standard plastic cages (with wood shavings) and kept in an accredited Animal Facility room at the Faculty of Medicine, University of Coimbra. The room temperature was 24 ± 1°C and humidity preserved at 50 ± 5%. Animals were fed *ad libitum* with standard chow and fresh water. Animals were kept under controlled light conditions with a 12 h/ 12 h light/ dark cycle. As a standard protocol, all animals were acclimated to the facilities 1 week before the experimental procedures. All procedures took place between 11:00 and 17:00 to minimize circadian rhythm influence.

General monoanesthesia (4% isoflurane in air) was induced in a designated plastic chamber (**Figure 2.1**). Anesthesia was thereupon maintained through a tubular delivering device, using 2% isoflurane (**Figure 2.1**), assuring correct positioning and immobility through the planned procedure.

Animals included in this study were divided into specific groups as follows:

- Controls (sham injury), sacrificed at 48h post-injury.
- Controls (sham injury), euthanised at 7 days post-injury.
- TBI group, euthanised at 48h post-TBI.
- TBI group, euthanised at 7 days post-TBI.
- TBI group + NPY administration, euthanised at 48h post-TBI.
- TBI group + NPY administration, euthanised at 7 days post-TBI.

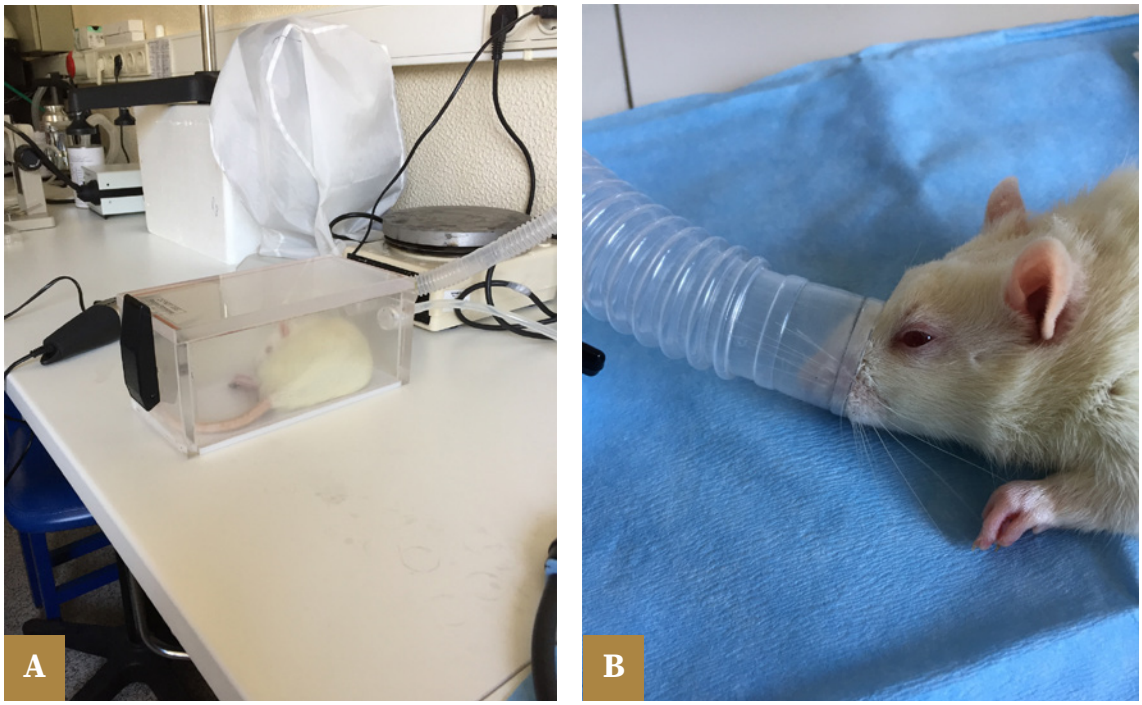


Figure 2.1 - Induction (A) and maintenance (B) of anesthesia.

Following loss of righting and toe-pinch reflexes, the scalp was shaved with an electric razor and wiped with a gauze soaked in 70% ethanol followed by iodopovidone solution. A skin mini-incision was undertaken (left frontal location, 1.5 mm lateral to the midline) and the skull exposed following skin retraction **(Figure 2.2-A)**, defining the impact area for a *weight-drop injury* – para-median left location, 2 mm lateral to the midline and 2mm posterior to the coronal suture **(Figure 2.2-B)**. The periosteum was gently separated and moved away from the impact area.

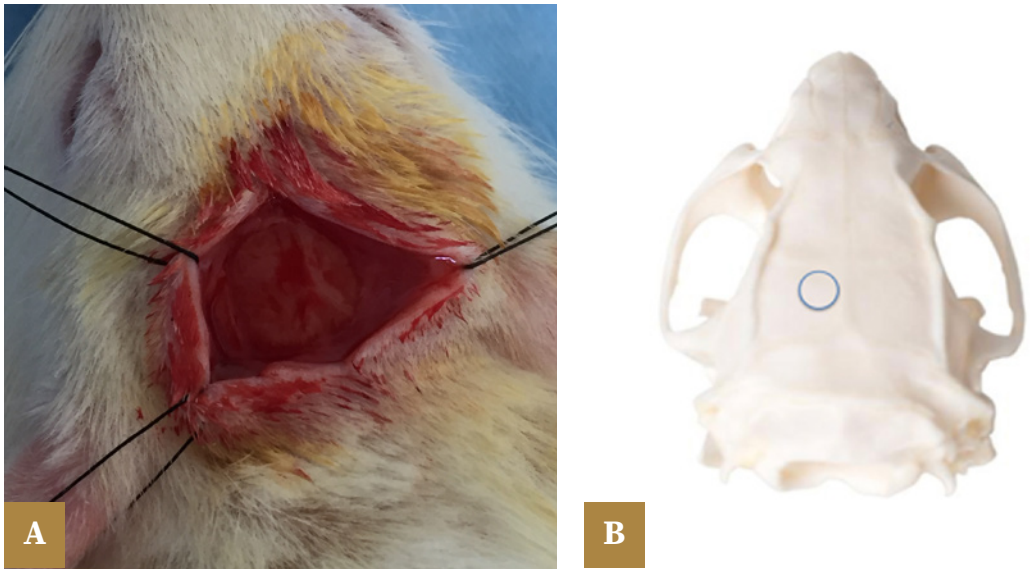


Figure 2.2 - Exposure and impact point. A - Skull exposure in rat's experimental groups for planned impact. B - Planned impact area (blue) in rat skull.

Sedated animals were then transferred to an adapted stereotaxic instrument for rat **(Figure 2.3)**, with the head fixed with two pins, preventing lateral movements as the impact was delivered, and the whole animal's body placed over a semi-rigid structure in a neutral position **(Figure 2.4-A)**. The whole device was placed on a rigid surface to avoid overall energy dissipation.

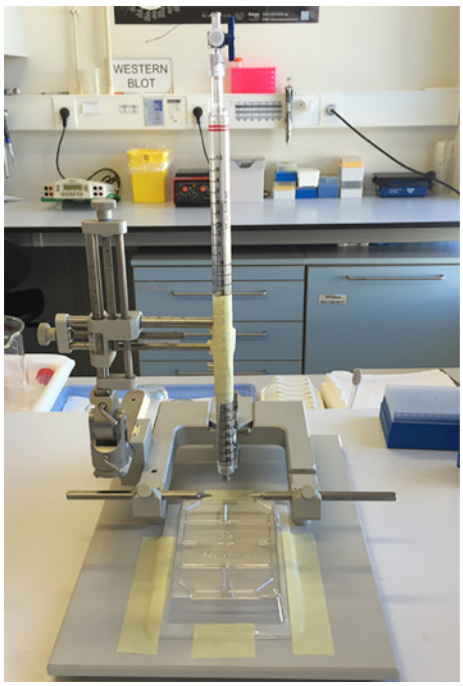


Figure 2.3 - Adapted stereotaxic instrument for rat head injury.

The animals were randomly assigned to be submitted to head trauma or sham injury (no cranial impact). After confirming the head's neutral position and correct exposure of desired impact area, the impact device - plastic tubular structure containing the impact object (weight - 55g), supported by a metal frame - was adjusted in its location accordingly. The impact weight (with the tip enclosed by a rubber covering for a more uniform impact) was then dropped over the skull (height of free-fall - 24cm). After inducing the lesion, the skin incision was closed (2-0 silk sutures, Medline®) **(Figure 2.4-B),** and the animal was then withdrawn from anesthesia. Physiological saline solution was applied on animal's eyes and no further protection measures for the eyes were deemed necessary, given the context of a short-duration procedure. This trauma model was established based on the work of Shohami and team[639-641] and following an observership with the Neurotrauma research team in Heidelberg University Hospital, Germany (Managing Director: Prof. Dr. med Andreas Unterberg).

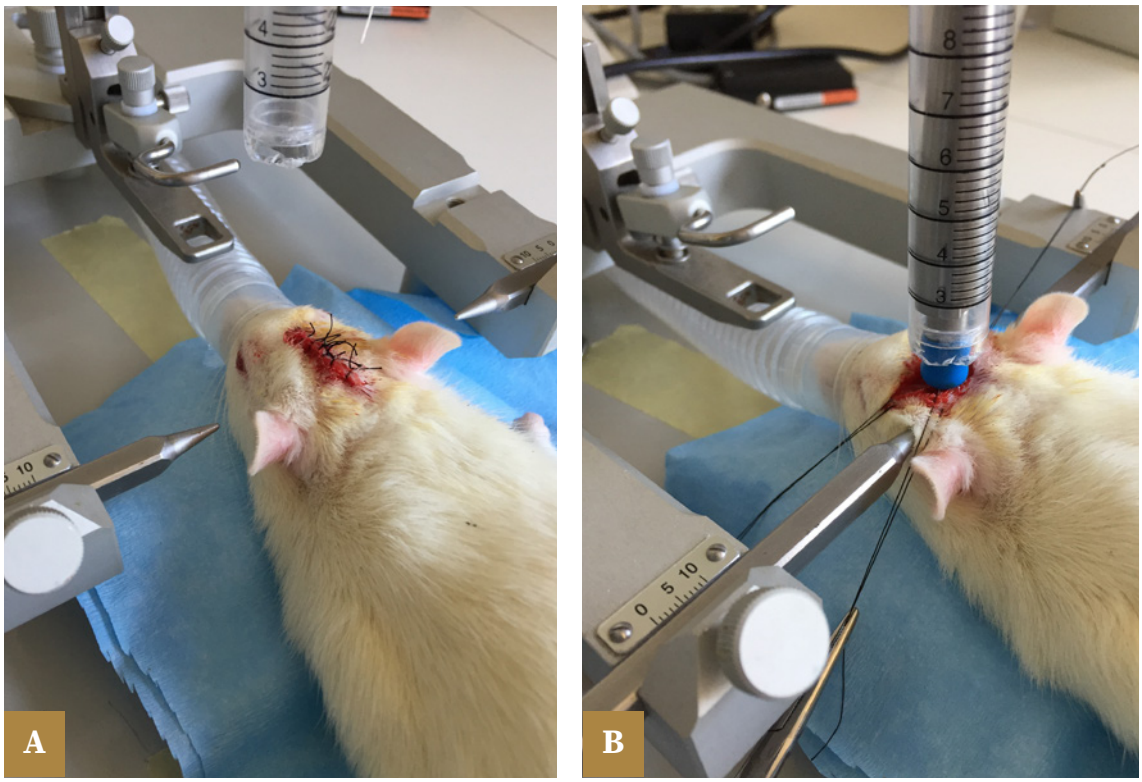


Figure 2.4 - A - Animal sedated and placed in position for impact. B - Skin closure after impact.

Following the procedure, the animal was placed in a designated separate cage until fully recovered from anesthesia, and its general and neurological status were assessed. Animal's body temperature was maintained throughout the procedure.

Sham-injured animals were subjected to identical procedures as described above, with the exception of not being submitted to cranial impact. The corresponding duration of anesthesia was intentionally prolonged to match the timings in the head-impact group.

Animal weight was daily recorded after the injury. If weight loss of more than 10% was noted, the animal would be excluded from the study. After 48h or 7d following TBI, rats were sacrificed for western blot and immunohistochemistry studies, as described below in the respective sections.

2.2.2 Intranasal administration of Neuropeptide Y

The group of rats submitted to head trauma was randomly divided into 2 subgroups, with the rats in one group being administered with NPY, by gently injecting NPY solution (500 µg - 100 µL PBS 1x; Bachem®, 4012616.0500), 100 µg /20 µL per animal, 10 µL per nostril **(Figure 2.5)**, 10 minutes after induced trauma. The head of the animal was held in a tilted back position for NPY administration, followed by approximately 10 seconds in the same positioning to prevent unintended drainage of the solution from the nares.[642-644] Extreme care was taken to avoid contact with intranasal mucosa.

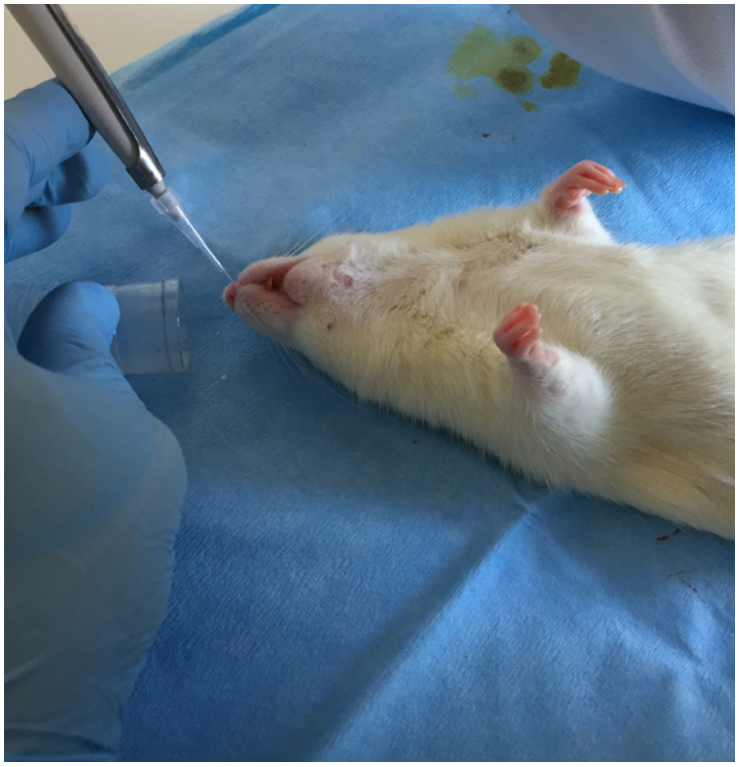


Figure 2.5 - Intranasal administration of NPY.

For immunohistochemistry studies, a minimum of 2 animals per group was defined, comprising a minimum of 12 animals in total.

For Western blot studies, a minimum of 3 animals per group was defined, comprising a minimum of 18 animals in total.

2.2.3 Animal sacrifice

All 3 groups - controls (sham injury), head trauma and head trauma with NPY administration - were further randomly subdivided into two groups, 48 hours and 7 days, corresponding to two specific time points in which the animals were euthanized.

The animal was placed on his back and the limbs pinned. An initial intraperitoneal injection with 100 mg/kg ketamine and 10 mg/kg xylazine was performed. Upon lifting the skin, a subdiaphragmatic incision was extended laterally and then up through the rib cage. The diaphragm was cut and the sternum lifted - the loose flap was pinned. Next, a needle was inserted through the left ventricle and 0.01M PBS (for western blot analysis) or 4% paraformaldehyde (PFA; for immunohistochemistry studies) were transcardially infused, followed by an incision in the right atrium, until all blood was perfused out from the right atrium. The animal was then decapitated, the skull open and the brain was rapidly removed.

2.2.4 Western blot analysis

Following hippocampi isolation in ice and lysis in RIPA buffer (0.15 M sodium chloride, 0.05 M Tris-base, 0.005 M ethylene glycol tetraacetic acid, 0.5% sodium deoxycholate, 0.1% SDS and 1% X-Triton, pH 7.5) supplemented with protease inhibitor cocktail tablets (Roche Applied Science, Basel, Switzerland) and anti-phosphatases (PhosSTOPTM, Roche Applied Science, Mannheim, Germany), protein content was quantified using bicinchoninic acid method (BCA), and stored at -20 °C until further use. Samples of total protein were separated by electrophoresis, transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore) and blocked with 5% non-fat milk or 4% BSA, as previously described by Leitão and colleagues.[645] Primary antibodies were as follows: goat anti-albumin (1:20000; Bethyl Laboratories Inc.®, USA); rabbit anti-GFAP (1:1000; Sigma-Aldrich®, USA); rabbit anti-occludin (1:100, Invitrogen, Inchinnan Business Park®, UK); rabbit anti-iNOS (1:500, Novus, BioTechne®, UK); rabbit anti-caspase 3 cleaved protein (1:500, Cell Signalling®, USA). Secondary antibodies were as follows: alkaline phosphatase-conjugated secondary antibody anti-rabbit (1:20000;

GE Healthcare Biosciences®, USA), and anti-goat (1:10000; Invitrogen®, USA). Immunoblots were reprobed with an antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1000; Thermo Scientific®, USA) to ensure equal sample loading. Quantification of band density was performed using Image Studio (LI-COR Biosciences®, USA).

2.2.5 Immunohistochemistry

After brain removal, it was placed in a 4% PFA solution for 24 h, and afterwards in a 30% sucrose solution for 72 h. Then, the brains were wrapped in parafilm (Bemis ®) and aluminium foil and then stored at -80°C until sectioned as desired. Coronal sections of 12 µm (for immunostaining quantification) or 50 µm (for cell reconstruction) were cut on a cryostat (Leica CM3050S, Leica®, Germany) and stored until further use. Immunostaining studies were performed as previously published.[645, 646] Briefly, brain slices were incubated with anti-GFAP-Cy3 conjugated (1:1000; Sigma-Aldrich®, USA) or goat anti-albumin (1:2000, Bethyl Laboratories Inc.®, USA) primary antibodies followed by donkey anti-rabbit Alexa Fluor 488 or donkey anti-goat Alexa Fluor 594 secondary antibodies (both 1:200; Invitrogen®, USA), and 5 µg/mL Hoechst 33342 (Sigma-Aldrich®, USA). Finally, slices were mounted with Dako fluorescence medium (Dako®, Denmark), and images were recorded using the LSM 710 Meta Confocal microscope (Carl Zeiss®, Germany).

2.2.6 Immunostaining analysis for albumin

Quantification of albumin (a marker of BBB disruption) immunoreactivity was accomplished using NIH ImageJ 1.47 analysis software.[645, 647] All photograph area was considered as well as three different areas without staining (black) to be used for background subtraction. We used the following formula to determine the corrected total fluorescence: correct total fluorescence = (integrated intensity) − (area of picture × mean background). The results were obtained from at least five brain slices obtained from two different animals for each experimental group.

2.2.7 Morphological analysis of astrocytes and microglia

GFAP-labeled astrocytes and Iba-1-labeled microglia cells were analyzed, as previously described,[648] using ImageJ-based Fiji software (Simple Neurite Tracer plugin), in order to assess the total length [expressed in micrometers (µm)] and number of astrocytic and microglial processes in each experimental group.[645, 647]

In order to evaluate the arbor complexity of astrocytes and microglia cells, Sholl analysis was performed, which counts the number of intersections at concentric spheres originated from the cell soma.

A minimum of 40 cells (20 cells/animal from two different animals) for each experimental group were analyzed.

2.2.8 Statistical Analysis

In western blot studies, results are expressed as mean + Standard Error of the Mean (SEM). Morphological data were analysed using the Kruskal-Wallis test followed by post hoc Dunn's multiple comparison test. For the Sholl analysis, a two-way ANOVA followed by Bonferroni's post hoc test was used. Regarding immunohistochemistry studies, results are expressed as the mean of fluorescence intensity (arbitrary units) of at least five brain slices obtained from two different animals for each experimental group. The level of significance was set at $p < 0.05$, and "n" represents the total number of animals. Statistical analysis was performed using Prism 6.0 (GraphPad® Software, USA).

2.3 Results and specific discussions

Upon being submitted to either sham injury or TBI (with and without ensuing NPY administration), all rats recovered from anesthesia to their previous general and neurological status. No rats were excluded from the study due to significant changes in general status, weight loss or nutritional abnormalities. No abnormal findings were present in routine neurological assessment in the following days and until euthanasia was performed. No noticeable skull fractures (linear, compound or depressed) were visible at the time of induced trauma or upon euthanasia, despite minor skull indentations at the site of impact in 3 rats. In addition, no obvious intracranial bleed (epidural or subdural haematoma, brain contusion or haematoma) was found at the time of sacrifice.

All brains from rats sustaining TBI presented some degree of traumatic subarachnoid haemorrhage upon direct inspection **(Figure 2.6)**. One rat presented with significant and immediate sub-periosteal bleed close to the impact site, with spontaneous cessation.

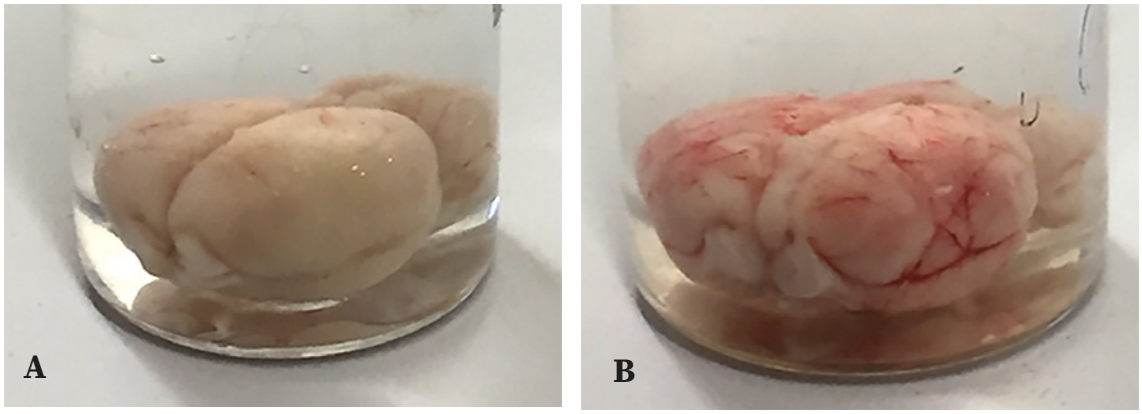


Figure 2.6 - Brains following removal from the skull, from animals included in Control (A) and TBI group (B).

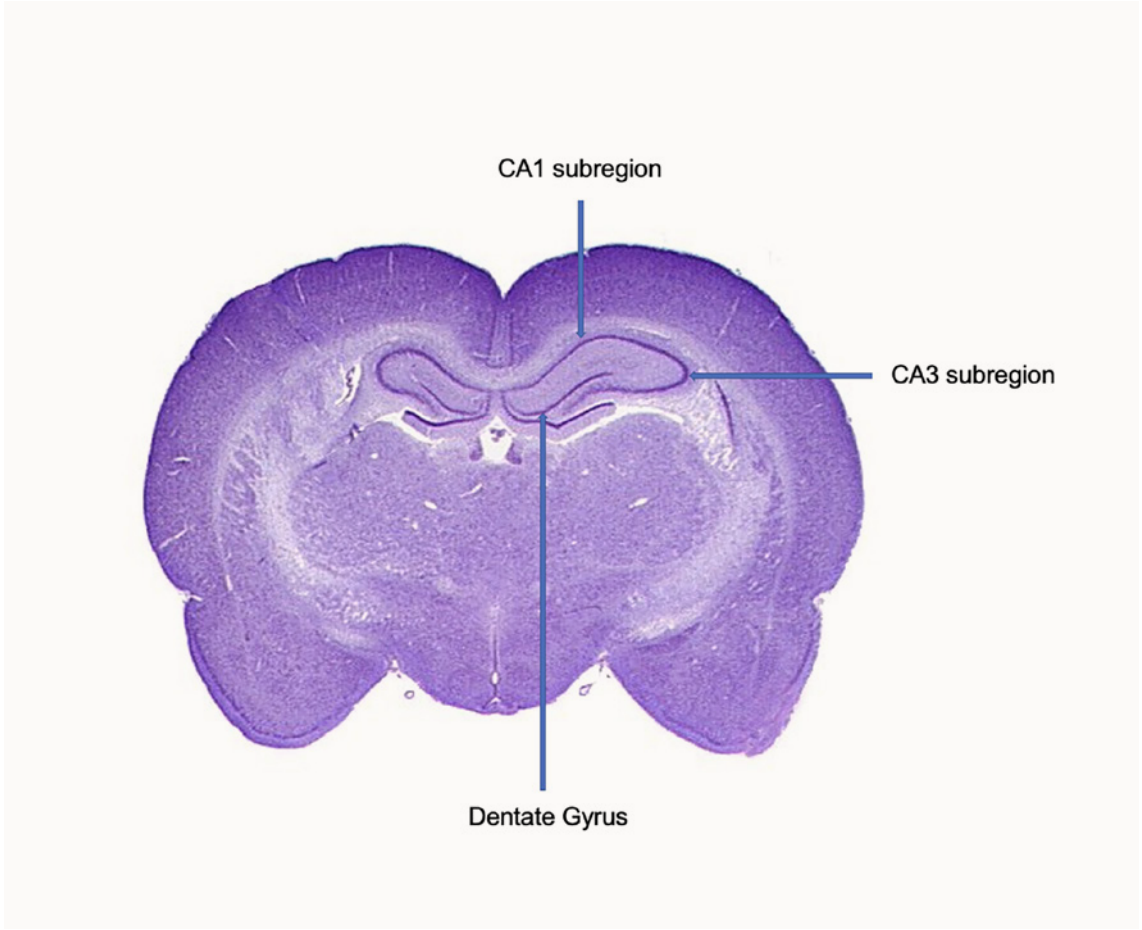


Figure 2.7 - Example of a Nissl-stained coronal section obtained from a rat brain displaying hippocampal subregions (adapted from SynapseWeb ®).

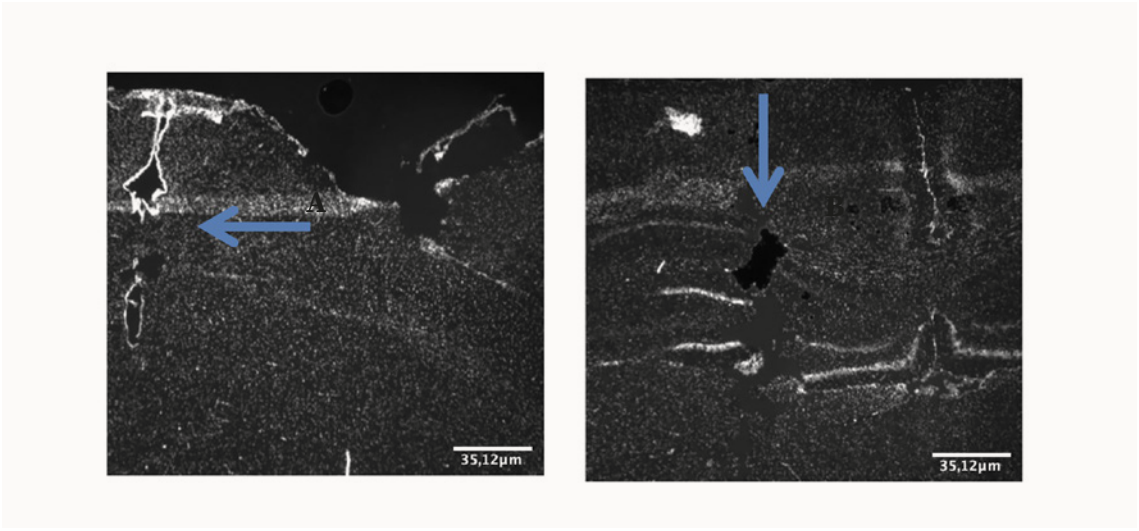


Figure 2.8 - Injury caused in the cortex and subcortical region (A) and hippocampus (B) following induced parasagittal trauma. Immunofluorescence microscopy, 15 µm coronal sections of rat brains. Scale bar = 35.12 **µm** .

2.3.1 Post-traumatic cellular degeneration

In order to characterize post-traumatic hippocampal cellular degeneration and potential interference by exogenous NPY administration, cleaved caspase-3 protein levels were assessed **(Figure 2.9)**.

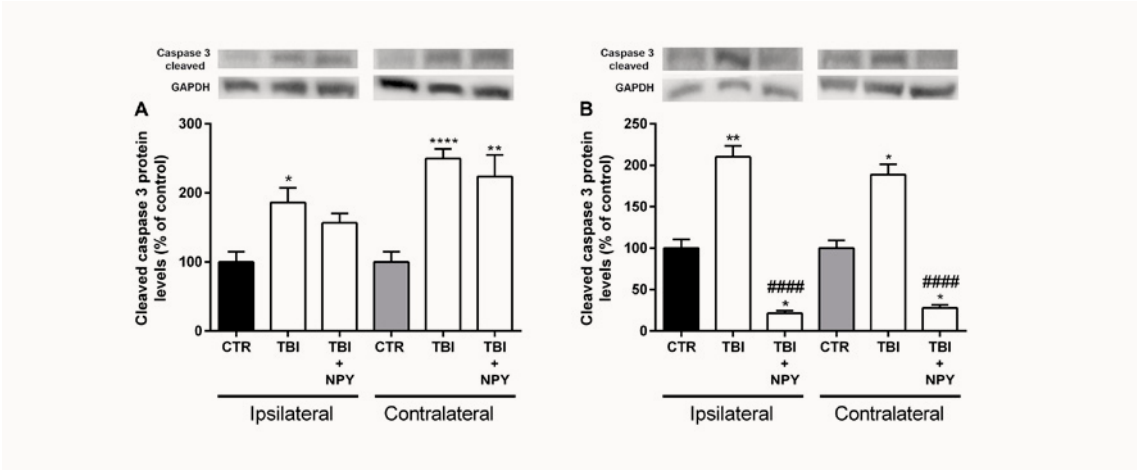


Figure 2.9 - Cleaved caspase-3 protein levels in ipsi- and contralateral hippocampus, 48h (A) and 7 days (B) post-TBI. Above the bars, representative western blot images of cleaved caspase-3 protein (17 kDa) and GAPDH (37 kDa) are shown. **A)** TBI increases cleaved caspase-3 protein levels in ipsi- and contralateral sides at 48h. **B)** Cleaved caspase-3 protein levels are increased in ipsi- and contralateral hippocampus. NPY is able to prevent TBI-induced cellular degeneration. Results are expressed as mean + SEM. *p<0.05; **p<0.01; ****p<0.0001 significantly different from CTR. ####p<0.0001 significantly different from TBI group. **Legend:** CTR, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NPY, Neuropeptide Y; TBI, traumatic brain injury.

At both 48h and 7 days post-TBI and concerning cleaved caspase-3 **(Figure 2.9)**, there was a significant increase in its levels in the ipsi- and contralateral hippocampus of TBI animals when compared to controls. NPY's beneficial effect was only obvious at 7 days post-TBI **(Figure 2.9-B)**, with no beneficial effect at 48h **(Figure 2.9-A)**. Curiously, at 7 days post-TBI, NPY was able to revert the activation of caspase 3 to levels even lower than the ones observed in controls. This is not surprising considering that basal cell death is always present in control conditions and our results suggest that NPY can also further prevent such basal events.

Discussion

The intention of these experiments was to identify post-traumatic cellular degeneration, namely in the hippocampus, and to evaluate a possible influence of NPY in attenuating it. Despite cortical lesion and cellular death being immediate, our working model and extrapolations are based on early sequelae hippocampal damage, 48h and 1 week following TBI.

Our findings document a significant attenuation of post-traumatic cell death by NPY, only at 7 days and not perceptible at 48h, as shown by cleaved caspase-3 protein levels **(Figure 2.9)**, a known marker for apoptosis.[649] These results suggest that attenuated cell death by NPY, as found in our model, is due to delayed secondary injury phenomena and not from direct primary injury.

Post-traumatic cell death is significantly dependent on excitotoxicity, via necrosis or apoptosis, depending on the initial stimulus, local conditions and cell populations,[401] previously shown to be related to NMDA receptors activation or NO/superoxide upregulation.[650] All cell types (neurons and different glia cells) display post-TBI apoptotic phenomena, following pro/anti-apoptotic protein factors imbalance and secondary injury mechanisms. Importantly, animal TBI studies show that the majority of dying hippocampal cells following TBI are newborn immature granular neurons,[401, 651] even upon a moderate level of impact and lasting for weeks, as shown by studies with caspase-3 activation.[652] Caspases (cysteine-dependent aspartate specific proteases) are a crucial element for the initiation and execution of apoptosis.[653] External factors and cellular signals will trigger proteolytic caspases activation and subsequent cell death cascade.[654] Specifically, caspase 3, a consistently cleaved and activated protein upon an insult, is a widely used biomarker for apoptosis[649] and is frequently used in animal studies of TBI as a marker for apoptosis and cell death.[655]

Equally relevant to our findings, Ou and colleagues[656] showed that NPY confers a neuroprotective effect against NMDA-induced apoptosis (both in *in vitro* and *in*

vivo models), in line with other studies reporting a neuroprotective role for NPY by inhibiting glutamate release.[657] This NPY-mediated attenuation of excitotoxicity and neuroinflammation is indeed based, at least partially, on counteracting proinflammatory mediators released by glial cells.[456]

The prospect of attenuating neuronal loss in the hippocampus and other areas, as observed in our experimental studies, opens the possibility of realistically minimizing hippocampal impairment and eventually potentiating overall recovery. Recent studies demonstrate persisting neurogenesis and astrocytogenesis throughout aging, namely in DG, despite decreased quiescent stem cell pools, neuroplasticity and angiogenesis.[658] As mentioned in the literature, a pro-neurogenic role for NPY (via Y1R) acting on specific neurogenic niches (including subventricular and hippocampal subgranular zone) is evident, directly interfering with neural progenitors.[456] Importantly, exogenous administration of NPY has also resulted in incremented integration of functional newly generated neurons in local circuits.[659]

Considering all this, one can speculate on a dual role for exogenous NPY, concerning neuronal populations, upon TBI: while preserving existing neurons and attenuating neuronal death, as strongly suggested by our research data, NPY can also actively stimulate neurogenesis and promote circuits renewal. This possibility is even more relevant if we consider, as mentioned, the more vulnerable but over-represented population of older patients. Importantly, granular neurons, glia and DG volume are relatively unchanged with aging,[658] unlike anterior and total hippocampal volume,[660] reinforcing the prospect of medically enhancing post-traumatic cognitive-emotional resilience.

2.3.2 Post-traumatic microglia alterations

Post-traumatic changes in hippocampal microglia cells and a possible interference by NPY were assessed.

48h post-TBI

At 48h post-TBI, it was possible to observe trauma-induced microglia activation, as reflected by a decrease in the number of processes and total length of cell ramifications **(Figure 2.10-C and D)**, particularly in the contralateral hippocampus when compared to controls (both hippocampus were analysed). Additionally, intranasal administration of NPY opposes such effects induced by TBI and also promoted an increase in the number of processes and total length both in the ipsi- and contralateral hippocampus compared to control condition. Interestingly, NPY's effect was more significant in the contralateral hippocampus in both parameters.

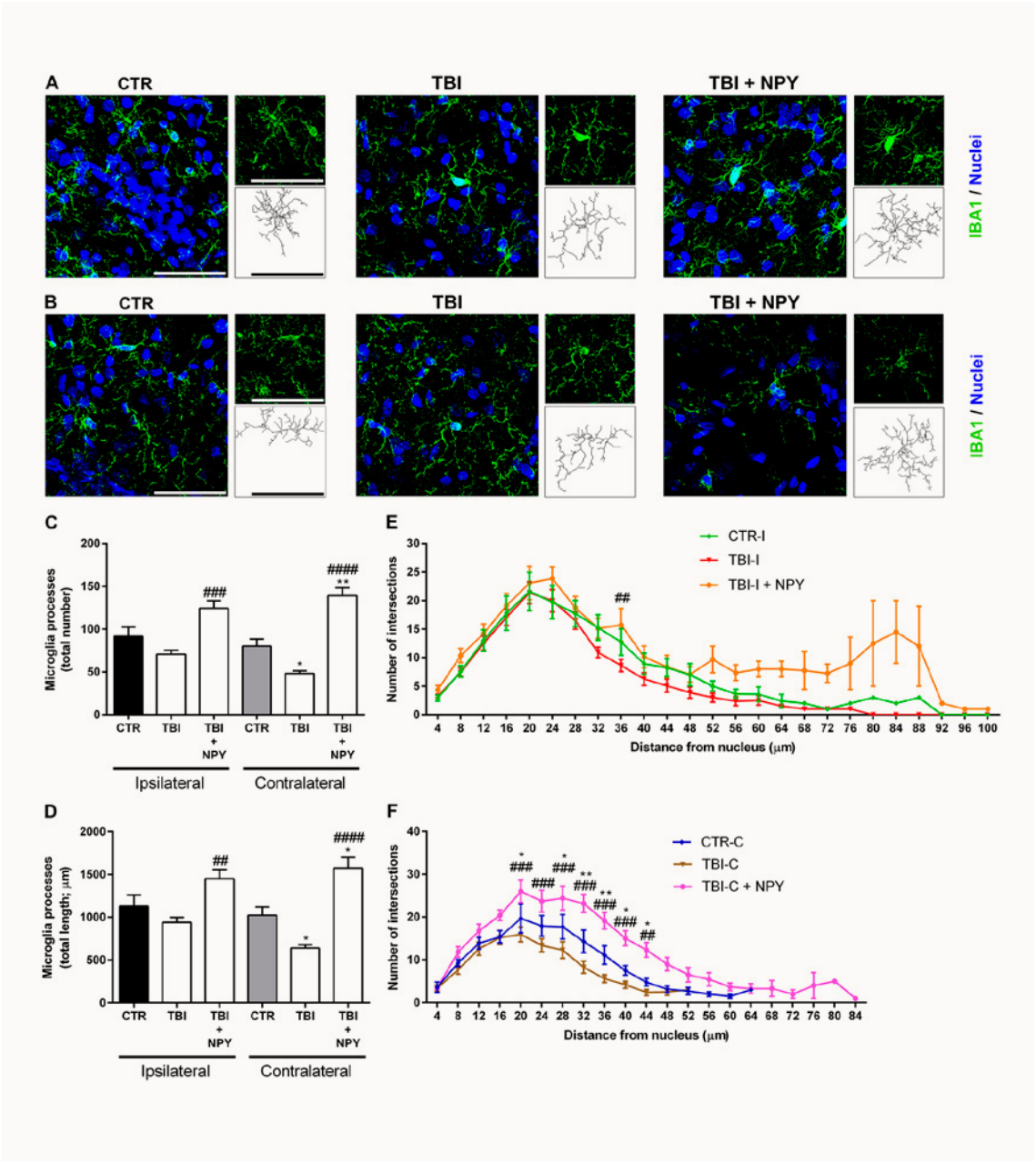


Figure 2.10 - Representative images of Iba-1 (green, microglia cells) and Hoechst (blue, nuclei) in different experimental groups, obtained in the A) ipsilateral and B) contralateral hippocampus, 48h post-TBI. Representative morphological reconstructions of microglia cells from each experimental group are shown. Scale bar = 50 μm. Quantification of (C) total number and (D) total length of microglial cell processes and number of interceptions in the (E) ipsilateral and (F) contralateral hippocampus - morphological characterization by Sholl analysis. TBI significantly decreases the total number and length of processes and the number of intersections in the contralateral hippocampus, with the same tendency in the ipsilateral side. NPY significantly blocks this effect. C, D) Results are expressed as mean + SEM. *p<0.05; **p<0.01 significantly different from CTR. ##p<0.01; ###p<0.001; ####p<0.0001 significantly different from TBI group. **Legend**: CTR, control; NPY, Neuropeptide Y; TBI, traumatic brain injury; -C, contralateral; -I, ipsilateral.

Moreover, microglial activation by TBI is supported by quantitative data showing a decrease in maximum branch length and distance from the soma where microglia process interaction occurred. Noteworthy, this activation is counterbalanced by NPY and microglial cells are highly ramified with long processes.

As demonstrated in **Figure 2.10**, TBI induces a clear state of microglia activation, with a decrease in the total number and length of processes (in comparison to controls). Importantly, NPY is able to reinstate microglia's basal status and counteract its activation.

7 days post-TBI

After 7 days following initial trauma, there was a significant decrease in the number and total length of processes of microglial cells in the contralateral hippocampus **(Figure 2.11-C and D)**. In the ipsilateral hippocampus, the observed number of microglia processes are not statistically distinct from controls. Interestingly, an increase in the total length in the ipsilateral hippocampus was observed. Overall, intranasal administration of NPY significantly attenuated post-traumatic morphological alterations induced by TBI and was even able to decrease the basal total number of processes in the ipsilateral hippocampus.

Concerning increased total length, NPY still displays a significant effect in the contralateral hippocampus after 7 days. These last findings represent a late consequence of early administration of NPY, as a reflection of NPY's interference with post-TBI microglia, still perceptible after 7 days.

Another overall trend is clear: contralateral hippocampus still displays, at 7 days post-TBI, more obvious traits of microglial activation when in comparison, in both parameters, to the ipsilateral hippocampus, as the decrease in number and total length of processes is still present only in the contralateral hippocampus when comparing to controls **(Figure 2.11)**.

Graphical representation of Sholl analysis displays a curious curve conformation in respect to ipsilateral hippocampus following TBI, with an obvious and significant bimodal curve suggesting that post-TBI microglia presented with a hyper-ramified conformation **(Figure 2.11-E)**, and NPY was able to prevent this effect, influencing global morphological rearrangements.

In summary, following TBI, microglia of contralateral hippocampus presented with fewer and shorter processes, features of activated microglia. This is more evident at 48h, but is still present after 7 days, and is significantly counterbalanced by exogenous NPY administration.

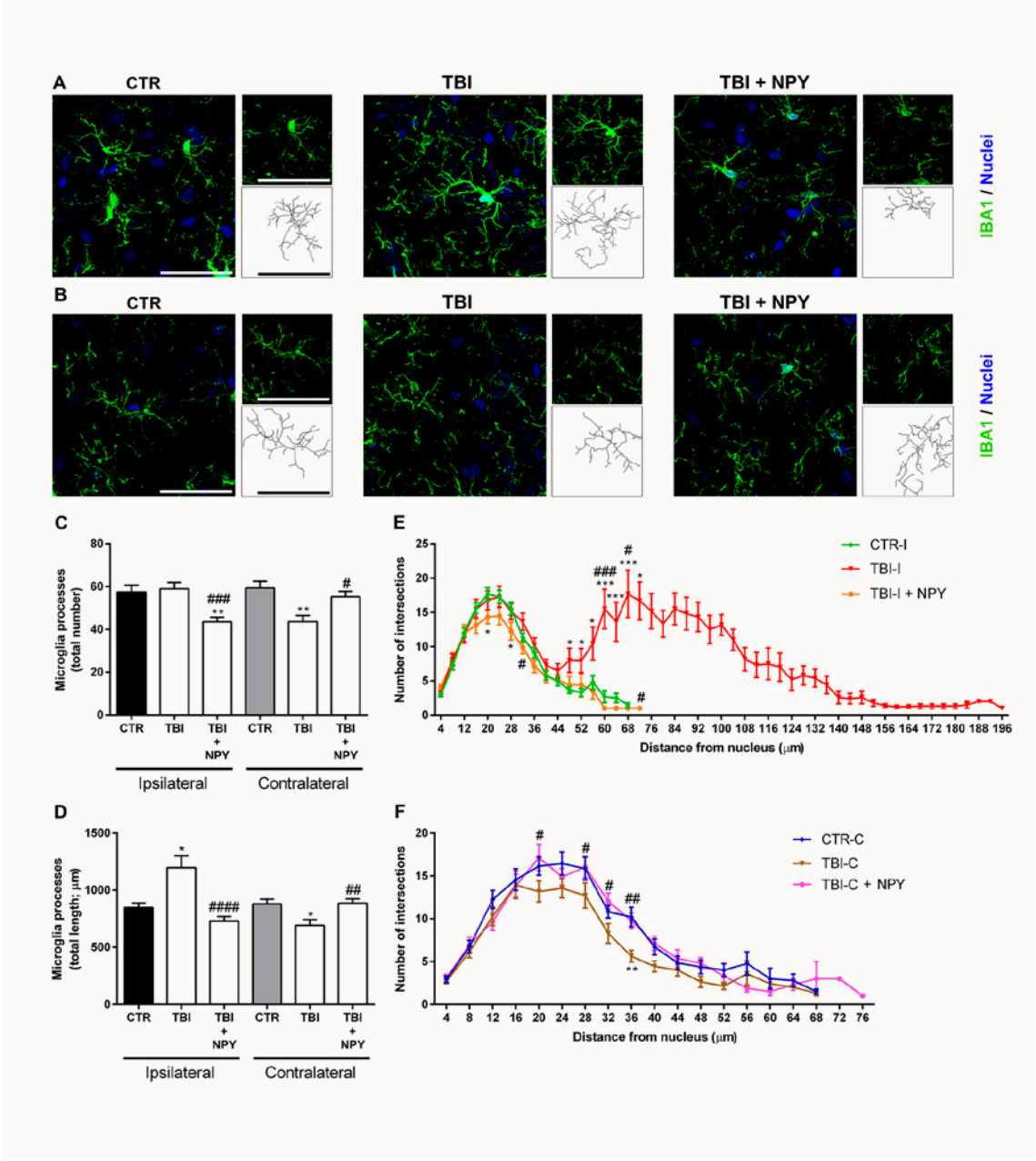


Figure 2.11 - Representative images of Iba-1 (green, microglia cells) and Hoechst (blue, nuclei) in different experimental groups, obtained in the A) ipsilateral and B) contralateral hippocampus, 7 days post-TBI. Representative morphological reconstructions of microglia cells from each experimental group are shown. Scale bar = 50 µm. Quantification of the (C) total number and (D) total length of microglial cells processes and the number of interceptions in the (E) ipsilateral and (F) contralateral hippocampus - morphological characterization by Sholl analysis. TBI induces a significant increase in total length of processes in the ipsilateral hippocampus and a decrease in the number and total length of processes in the contralateral hippocampus. NPY opposes these phenomena. C, D) Results are expressed as mean + SEM. *p<0.05; **p<0.01; ***p<0.001 significantly different from CTR. #p<0.05; ##p<0.01; ###p<0.001; ####p<0.0001 significantly different from TBI group. **Legend**: CTR, control; NPY, Neuropeptide Y; TBI, traumatic brain injury; -C, contralateral; -I, ipsilateral.

Microglia alterations in CA1/CA3 hippocampal subregions

Hippocampal regional disparities were assessed **(Figure 2.12)**, as this is a topic never reported before in the literature.

48h post-TBI, CA1/CA3 hippocampal subregions

At 48h post-trauma, TBI induced a significant decrease in the number and total length of processes in the ipsi- and contralateral hippocampus, both in the CA1 and CA3 subregions **(Figure 2.12)**, which indicates that microglia is globally activated. Findings concerning contralateral CA1 subregion did not reach statistical significance.

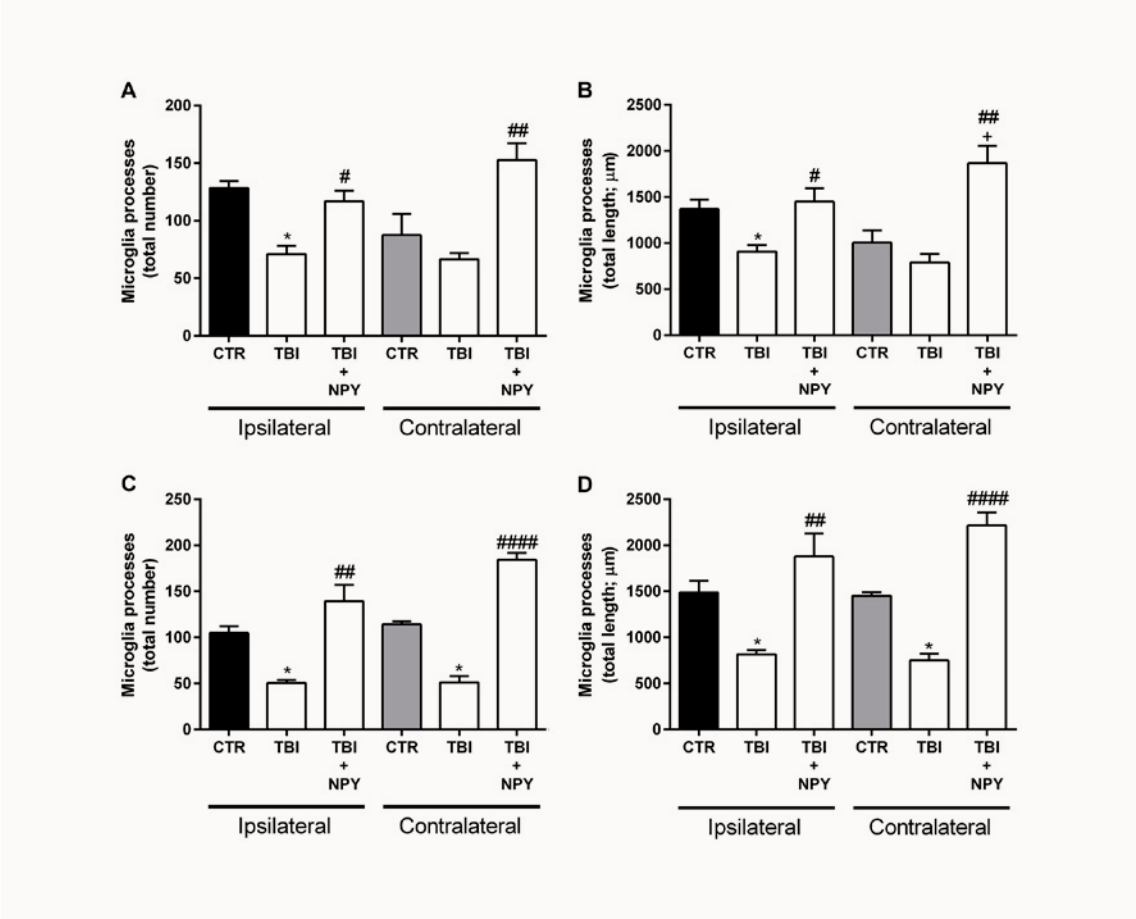


Figure 2.12 - Morphological analysis of hippocampal microglia, 48h post-TBI, CA1 (A, B) and CA3 (C, D) subregions. TBI induces a decrease in the number and total length of processes in the CA1 and CA3 subregions, namely in the ipsilateral subregions. NPY clearly opposes this decrease. Results are expressed as mean + SEM. *p<0.05 significantly different from CTR. #p<0.05; ##p<0.01; ####p<0.0001 significantly different from TBI group. **Legend**: CTR, control; NPY, Neuropeptide Y; TBI, traumatic brain injury.

Moreover, intranasal administration of NPY attenuates previously mentioned post-traumatic changes, inducing a higher number and total length of processes (compared to the TBI group, with statistical significance) both in ipsi- and contralateral CA1 and CA3 subregions. These findings document a relevant effect of NPY, by attenuating microglial activation, in both hippocampal subregions.

The effects of intranasal administration of NPY in CA1 and CA3 subregions, at 48h post-TBI, are similar to the ones described concerning the whole hippocampus. Again, these findings are in line with an obvious interference of NPY with microglial activation.

7 days post-TBI, CA1/CA3 hippocampal subregions

As shown in **Figure 2.13** and regarding intranasal administration of NPY, the only statistically significant finding in line with our previous results is in contralateral CA3 subregion, with a recovery to control values (**Figure 2.13-C and D)**.

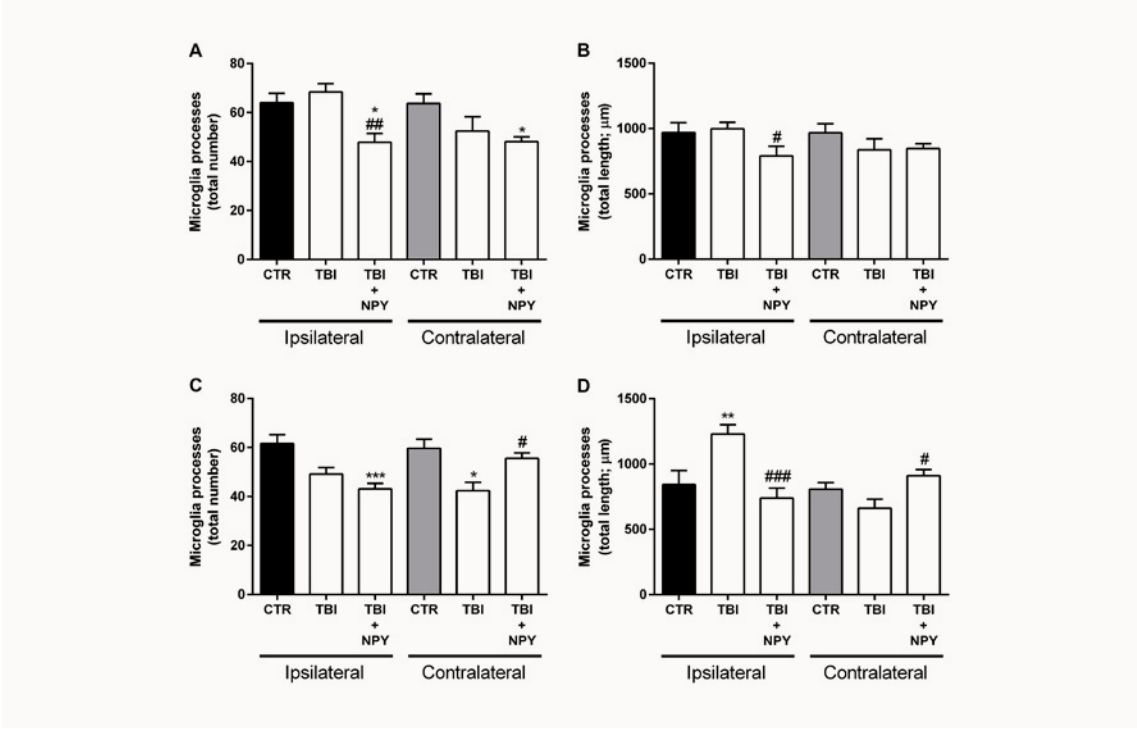


Figure 2.13 - Morphological analysis of hippocampal microglia, 7 days post-TBI, CA1 (A, B) and CA3 (C, D) subregions. NPY decreases the number and length of processes (compared to TBI group) in the ipsilateral CA1 subregion and, concerning total length, in the ipsilateral CA3 subregion. NPY displays an opposite effect concerning the contralateral CA3 subregion. Results are expressed as mean + SEM. *p<0.05; **p<0.001; ***p<0.001 significantly different from CTR. #p<0.05; ##p<0.01; ###p<0.001 significantly different from TBI group. **Legend**: CTR, control; NPY, Neuropeptide Y; TBI, traumatic brain injury.

Concerning the total length of microglia processes at 7 days post-TBI and regional discrepancies, there was a significant increase in the ipsilateral CA3 subregion (unlike CA1 and contralateral CA3 subregions) **(Figure 2.13-D)**. Intranasal administration of NPY significantly impacted both ipsi- and contralateral CA3 subregions concerning the total length of processes, although with opposite effects (compared to TBI groups) and returning to control levels.

In summary, in our animal model of TBI, trauma injury consistently induces microglial activation with known morphological changes. Early administration of NPY clearly prevents this deleterious activation **(Figure 2.14)**, namely in the acute phase (48h post-TBI).

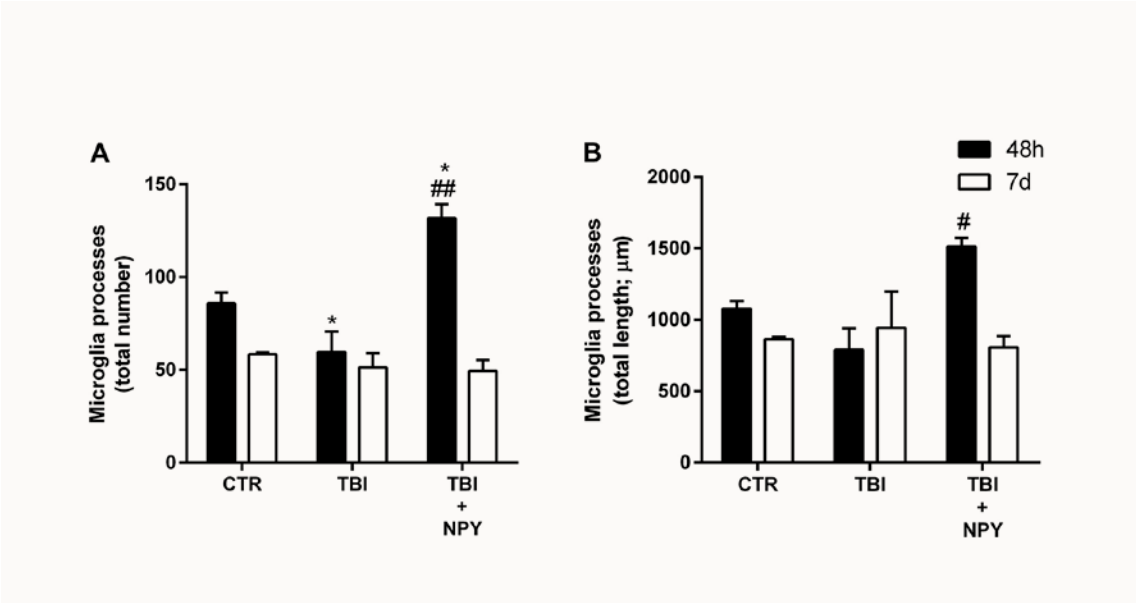


Figure 2.14 - Overall picture of post-TBI microglia morphology varying with time and NPY's influence, 48h and 7 days post-TBI. A) Number of processes. B) Total length of processes (mean value per group, both hemispheres considered). TBI induces microglial activation, as reflected in the number and length of processes at 48h post-TBI. NPY administration blocks post-traumatic activation. Results are expressed as mean + SEM. *$p<0.05$ significantly different from CTR. $^{\#}p<0.05$; $^{\#\#}p<0.01$ significantly different from TBI group. **Legend**: CTR, control; NPY, Neuropeptide Y; TBI, traumatic brain injury; 7d, 7 days.

Discussion

Concerning post-TBI microglial activation over time, this is a long-lasting encompassing phenomenon, with post-mortem studies showing signs of mild activation at 3 months post-TBI but lasting for years.[661] Thus, given its central role in most events concerning secondary injury and its surprisingly extended time frame, microglia is a potential therapeutic target to be considered in the short term, reason why it received so much attention in our study.

The role of microglia in TBI is based on both neuroprotective and neurodegenerative effects. Regardless of direct or indirect TBI-triggered microglia response, these cells play an important role in post-aggression acute stages, namely by clearing cellular debris, but prolonged activation is detrimental, by producing ROS and proinflammatory cytokines.[595, 662] As such, our demonstrated ability to attenuate microglial activation at 48h and 7 days following injury is significant and promising in its downstream possibilities.

In regard to our results at 48h, a prominent role of NPY as a deterrent to microglial activation is demonstrated. In all assessed variables (number of processes, total length), both in ipsi- and contralateral hippocampus, NPY clearly impedes expected transitioning into a more activated state. Even when considering CA1 and CA3 subregions separately, the same trends are present. In our work, in some instances, NPY's role in attenuating microglial activation is even more obvious in the contralateral hippocampus, considering overall secondary injury. This fact might reflect a more significant and direct injury to ipsilateral structures, preventing them from fully participating in specific global secondary injury mechanisms, unlike more spatially distant contralateral structures.

As thoroughly described in the literature, microglial activation is characterized by a progressive transformation from a ramified to an amoeboid-like morphology, by enlarging cell bodies, retracting and thickening microglia processes and posteriorly extending dynamic protusions.[218, 655] Nevertheless, this phenotypic plasticity includes a range of intermediate states: "bipolar", "rod-like", "hypertrophied". Concerning our findings in microglia after 7 days, an unexpected "double peak" is shown on Sholl analysis, which might represent a change in morphology, eventually related to formation of a glial scar, a comprehensive response of glial cells to external damage, involving proliferation and hypertrophy of glial cells. Moreover, migration of macrophages and microglia takes place within hours of initial injury. Gliosis, with the contribution of peripheral cells,[663] is part of standard post-injury microglial activation, with phagocytic activity, antigen presentation and cytokines production. It should be difficult to effectively and comprehensively approach and modulate such a complex system from a therapeutic perspective, with inputs both

from the extracellular environment and intracellular mechanisms. Microglia's role in TBI requires further elucidation, considering the danger of having far-reaching anti-inflammatory therapies for TBI not translating into effective clinical therapies as their effects influence both noxious and beneficial steps in the pathological continuum.[664] As mentioned, it should be noted that post-TBI microglial activation arguably displays a deleterious effect, namely in relation to neuroinflammation and white matter disruption,[665] along with a possible influence in β-amyloid accumulation, leading to axonal damage.[666]

The M1-M2 paradigm is, beyond doubt, an oversimplified model that only represents two extreme states within an activation continuum.[201] A rigid classification into M0, M1 and M2 microglial phenotype states is arguably not applicable in complex biological systems, and it is probably wiser to consider it to be a spectrum of phenotypes with some degree of functional overlap.[184, 667] It is also more accurate to use expressions as "M1-like" and "M2-like" to describe observed states, accounting for biases arising from targeted experimental studies and techniques.[216, 668] Jassam and colleagues[209] propose to go beyond the rather simplistic M1 vs. M2 classification and set a new TBI-specific profile classification, defining specific transcriptional microglial networks,[209] allowing for tailored immunomodulatory therapies. Shifting post-traumatic microglia phenotype into a more permanent and supposedly neuroprotective M2-like phenotype is, in theory, a valid and promising approach (e.g., via a positive allosteric modulator).[143] But, as M1-like phenotype actions play an important role in early repair processes and possibly in the clearance of cellular debris, it can be a mistake to upset a pre-determined, however insufficient, sequence of inflammatory, proliferative and repair mechanisms.[669]

On a closer look, our findings in the population of microglia 7 days post-TBI in ipsilateral hippocampus, displaying a "double-peak" conformation in Sholl analysis **(Figure 2.11-E)**, are in line with a "hyperactive" conformation (as one possible intermediate state of activation). It is usually perceptible around 5 days following initial aggression[181] and peaks at 7 days post-injury,[670] aligning along the injury site during the initial recovery phase and potentially displaying a higher proliferative capacity when compared to amoeboid conformation.[217] Considering obtained results in our morphological studies, one can presuppose that we are in the presence of a well-described hypertrophied morphology,[182, 671] associated with microglia pathological activation, characterized by cell bodies hypertrophy, more intense Iba1 immunoreactivity and asymmetrical processes distribution.[191, 672] Besides neurodegenerative diseases and overall brain insults,[673] TBI is also mentioned to induce hypertrophied microglia.[674] Decreased levels of cell-polarity protein Par1b/MARK2 are apparently associated with some of these morphological changes, activation and increased phagocytosis.[673]

Other intermediate morphological states, namely the rod-like type, can also assume a bipolar conformation and have also been described in TBI.[675] Animal models of both focal[676] and diffuse TBI[677] specifically display microglial activation with bipolar/rod-shaped microglia as well (in *corpus callosum*, hippocampus and cortex).[675]

Another important discussion concerns the true meaning of attenuating microglial activation, given considerable doubts regarding whether this chronic activation has a fundamental role in chronic neuronal degeneration or, contrarily, constitutes a response to this same neuronal damage.[201] Distinct groups have shown that, by reducing classical microglial activation (via reduced NADPH oxidase activation), even 1 month after initial TBI, progressive neuronal degeneration is decreased and functional recovery is improved.[201, 678] These data are important as they show that microglial activation is indeed a suitable therapeutic target, as shown in our study, and, equally important, the therapeutic window might be significantly longer than usually assumed. Concerning our intent of manipulating and improving cell survival following TBI, it should be stressed that neuronal survival is not the only factor directly influencing cognitive outcome. Microglia is now known to play a crucial role in brain development and synaptic plasticity by regulating synapse elimination, cell turn-over and neuronal surveillance.[679]

Our study clearly shows an objective action of NPY in attenuating microglial activation in different timings, reinforcing the notion of this being a beneficial effect by inhibiting a mostly deleterious phenomenon. As indicated previously, NPY may exert its role via different mediators and pathways that warrant future investigations.

2.3.3 Post-traumatic astrocytic modifications

Post-traumatic changes in hippocampal astrocytes and a possible interference by NPY were also assessed **(Figure 2.15)**.

48h post-TBI

As shown in **Figure 2.15**, TBI induces an obvious astrocyte activation in the ipsilateral hippocampus, with an increased overall number of elongated processes (compared to controls). Importantly, this reactive status is significantly opposed by NPY. In fact, NPY-administered groups display an average number and length of processes even lower than controls. Moreover, NPY administration also implies a lesser astrocytic arbor complexity displayed in the graphical representation of Sholl analysis (compared to the TBI group) **(Figure 2.15-E and F)**. A similar

tendency, although not statistically significant, is present when considering the number and total length of processes in contralateral hippocampal astrocytes.

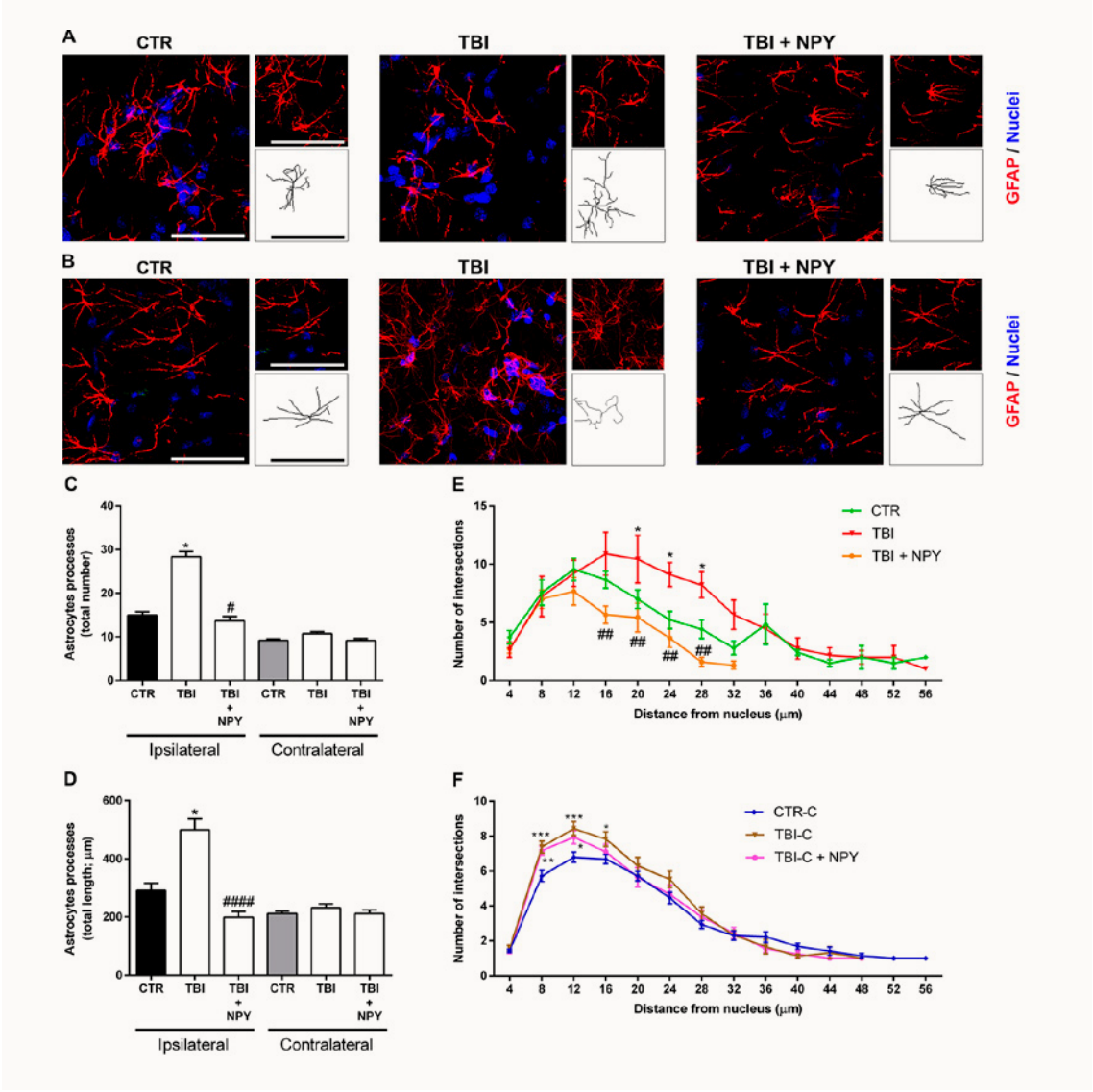


Figure 2.15 - Representative images of GFAP (red, astrocytes) and Hoechst (blue, nuclei) in different experimental groups, obtained in the A) ipsilateral and B) contralateral hippocampus, 48h post-TBI. Representative morphological reconstructions of astrocytes from each experimental group are shown. Scale bar = 50 μm. Quantification of (C) total number and (D) total length of astrocytic processes and number of interceptions in the (E) ipsilateral and (F) contralateral hippocampus - morphological characterization by Sholl analysis. TBI induces an obvious increase in the number and total length of astrocytic processes in the ipsilateral hippocampus. NPY significantly hinders this increase. C, D) Results are expressed as mean + SEM. *p<0.05; **p<0.01; ***p<0.001 significantly different from CTR. #p<0.05; ##p<0.01; ####p<0.0001 significantly different from TBI group. **Legend**: CTR, control; NPY, Neuropeptide Y; TBI, traumatic brain injury.

Astrocytes alterations in CA1/CA3 contralateral hippocampal subregions, 48h post-TBI

Data is displayed according to hippocampal subregion, due to disparate topographical findings, never reported before in the literature **(Figure 2.16)**.

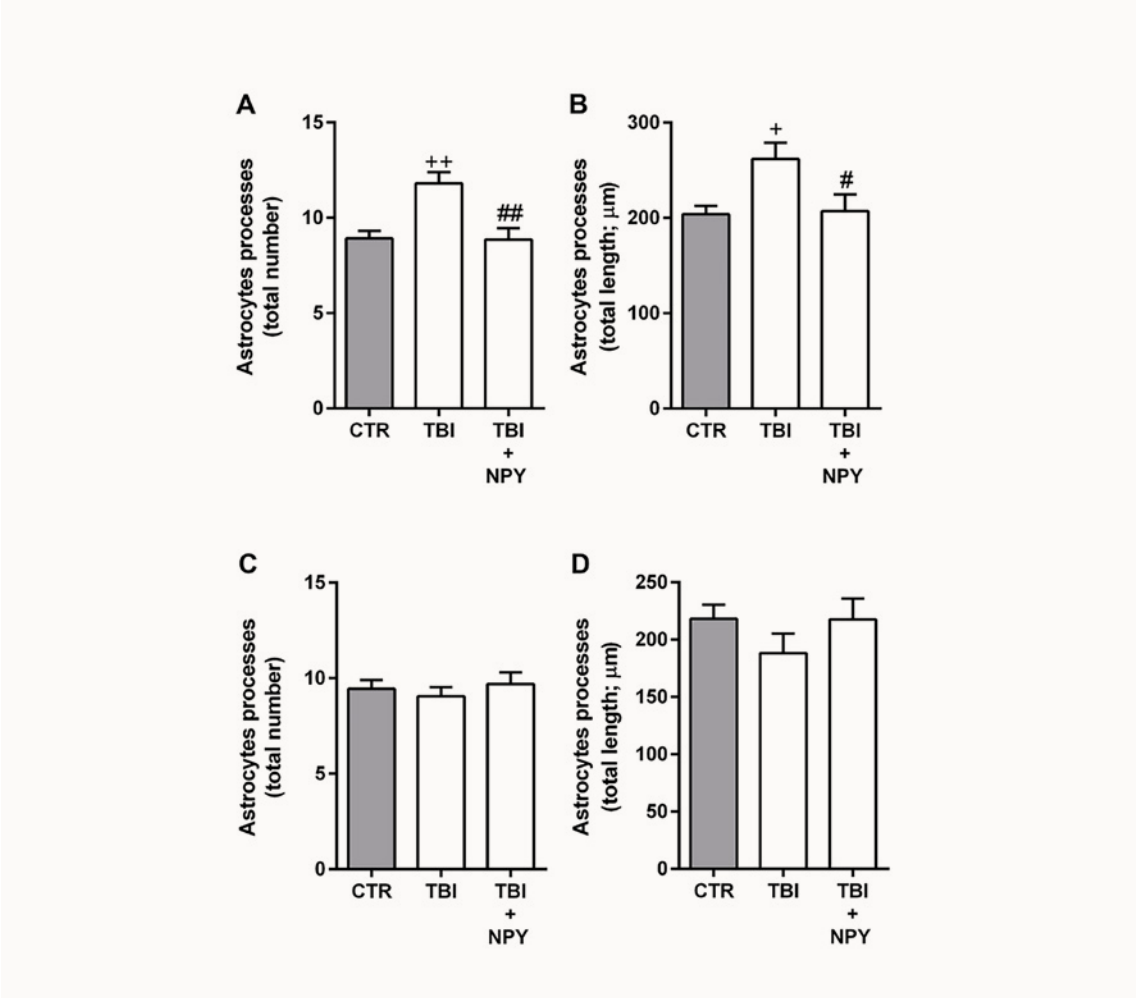


Figure 2.16 - Morphological analysis of contralateral hippocampal astrocytes, 48h post-TBI, CA1 (A, B) and CA3 (C, D) subregions by Sholl analysis. TBI induces an obvious increase in the number and total length of astrocytes processes in the CA1 subregion. NPY significantly hinders this increase. Results are expressed as mean + SEM. $^{+}p<0.05$; $^{++}p<0.01$ significantly different from CTR. $^{\#}p<0.05$; $^{\#\#}p<0.01$ significantly different from TBI group. **Legend**: CTR, control; NPY, Neuropeptide Y; TBI, traumatic brain injury.

Traumatic brain injury displays an increase both in the number and total length of astrocytic processes in the contralateral CA1 subregion **(Figure 2.16-A and B)**. Importantly, NPY attenuates these abnormal findings induced by brain injury and also displays a lower astrocytic arbor complexity, as depicted in the graphical representation of Sholl analysis (compared to the TBI group) **(Figure 2.17)**.

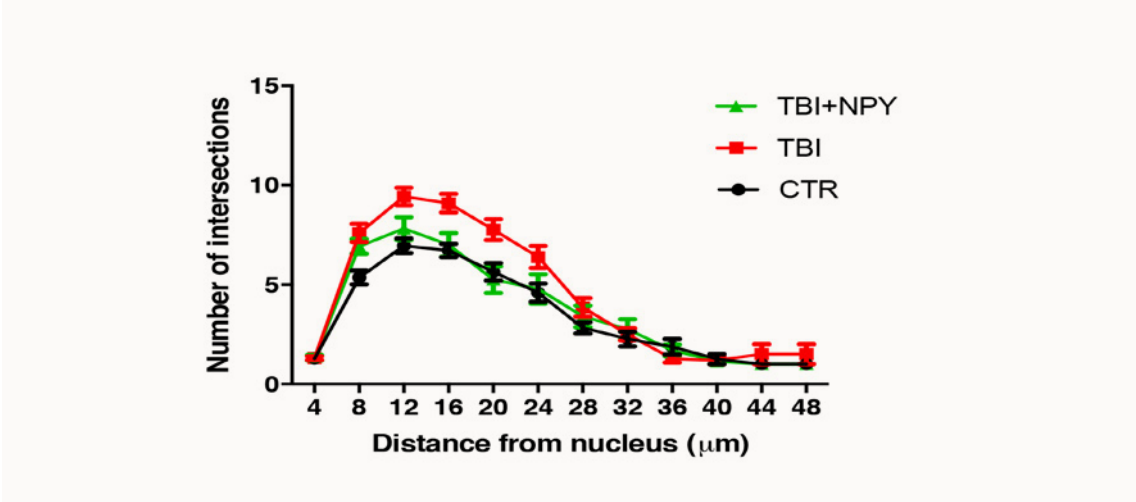


Figure 2.17 - Astrocytes hippocampal response, 48h post-TBI, in the contralateral CA1 subregion, Sholl analysis. Legend: CTR, control; NPY, Neuropeptide Y; TBI, traumatic brain injury.

Regarding the contralateral CA3 hippocampal subregion, TBI did not cause a significant effect on the number or total length of astrocytic processes **(Figure 2.16-C and D)**. Moreover, NPY had no overall significant effect in the contralateral CA3 subregion.

Therefore, an obvious discrepancy is described, with most of the post-traumatic astrocytic changes and corresponding response to NPY occurring in the CA1 subregion.

7 days post-TBI

As shown in **Figure 2.18**, at 7 days, animals submitted to TBI present a statistically significant decrease in the number of processes, in the ipsi- and contralateral hippocampus, and in the total length of processes in contralateral hippocampus. Importantly, NPY clearly attenuates these findings in injured brains in both hemispheres, with an obvious trend both in number and total length of astrocytic processes.

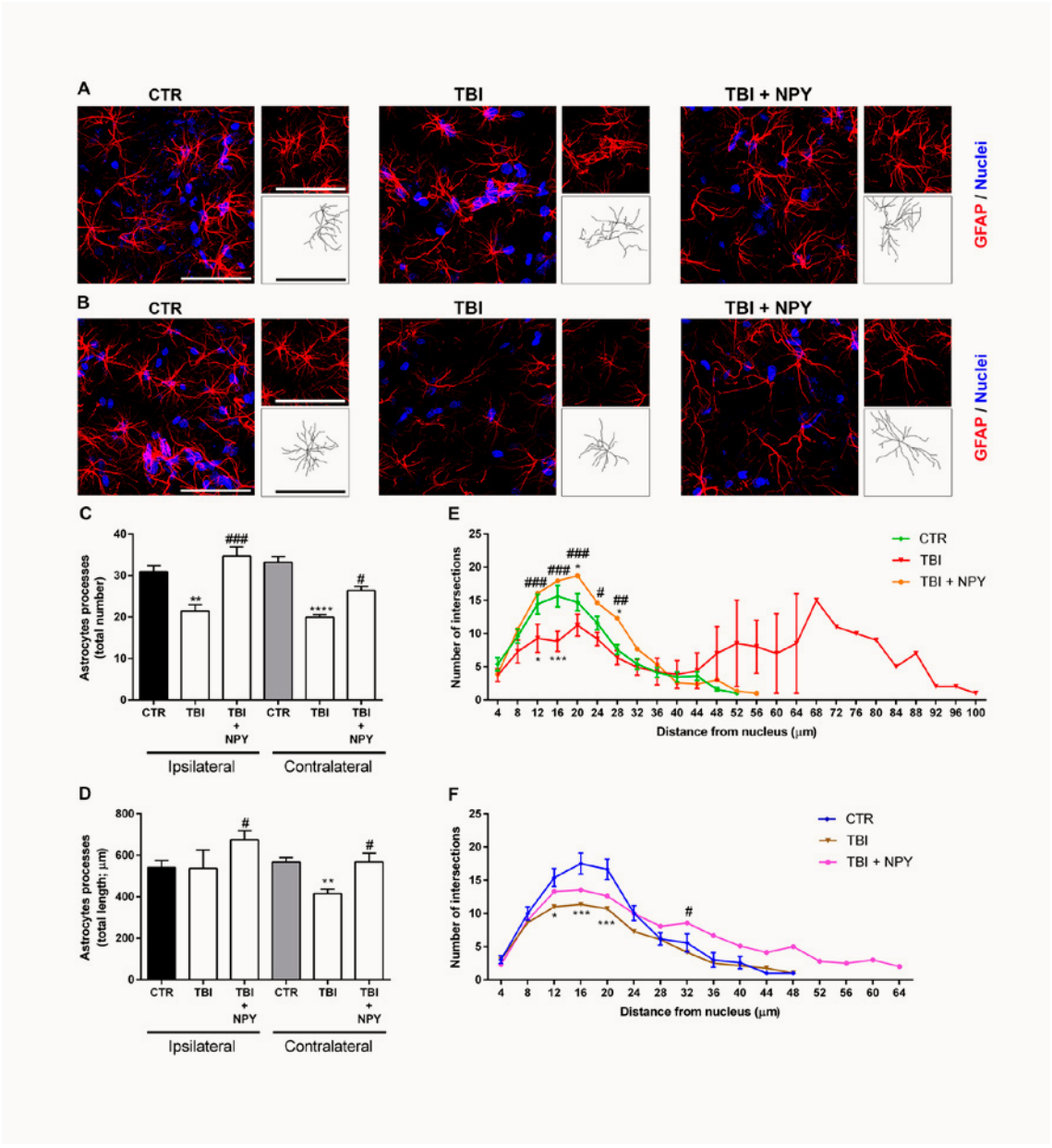


Figure 2.18 - Representative images of GFAP (red, astrocytes) and Hoechst (blue, nuclei) in different experimental groups, obtained in the A) ipsilateral and B) contralateral hippocampus, 7 days post-TBI. Representative morphological reconstructions of astrocytes from each experimental group are shown. Scale bar = 50 µm. Quantification of (C) total number and (D) total length of astrocytic processes and number of interceptions in the (E) ipsilateral and (F) contralateral hippocampus - morphological characterization by Sholl analysis. TBI induces a reduction in the number and total length of astrocytic processes (with the exception of total length in ipsilateral hippocampus). NPY stops this reduction in all groups, namely regarding the number of processes in the ipsilateral hippocampus. C, D) Results are expressed as mean + SEM. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 significantly different from CTR. #p<0.05; ##p<0.01; ###p<0.001 significantly different from TBI group. **Legend**: CTR, control; NPY, Neuropeptide Y; TBI, traumatic brain injury.

Sholl analysis graphical representation displays a curious curve conformation in respect to ipsilateral hippocampus following TBI **(Figure 2.18-E)**, with an apparent bimodal display (not present at 48h post-TBI) and statistically significant differences in TBI groups upon NPY administration.

In summary, in our animal model, trauma injury consistently induces astrocytic activation with known morphological changes. Early administration of NPY prevents this deleterious activation **(Fig. 2.19)**, with obvious effects in the acute phase (48h post-TBI), reducing the hyper-ramification induced by trauma. At 7 days post-TBI, NPY attenuates an otherwise clear reduction in number and total length of processes following trauma, apparently preventing later astrocyte atrophy.

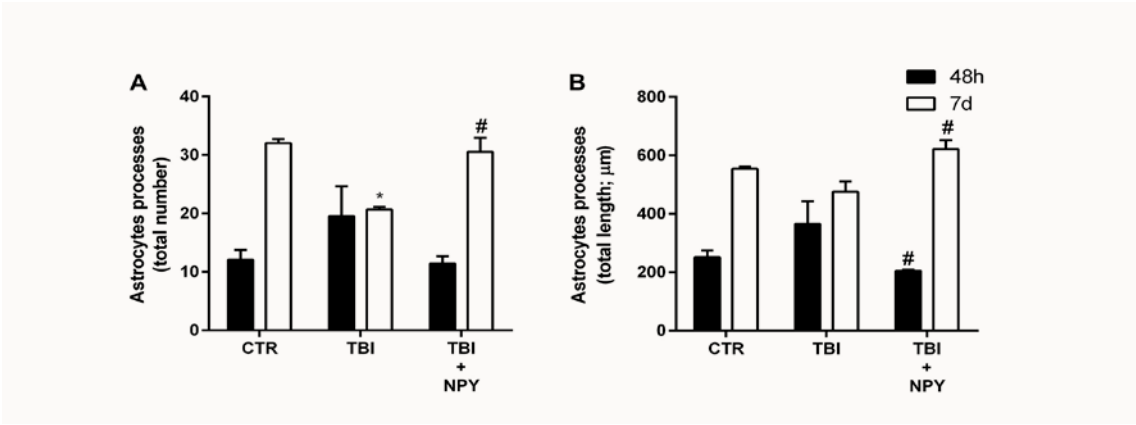


Figure 2.19 - Overall picture of post-TBI astrocytic morphology varying with time and NPY's influence, 48h and 7 days post-TBI. A) Number of processes. B) Total length of processes (mean value per group, both hemispheres considered). NPY administration blocks post-traumatic astrocytic activation at 48h post-TBI and promotes an increase in the number and length of processes at 7 days post-TBI (compared to TBI group). Results are expressed as mean + SEM. *p<0.05 significantly different from CTR. #p<0.05 significantly different from TBI group. **Legend**: CTR, control; NPY, Neuropeptide Y; TBI, traumatic brain injury; 7d, 7 days.

Glial fibrillary acidic protein

Besides astrocytic morphological evaluation, we also investigated possible changes in GFAP protein levels **(Figure 2.20)**, a protein expressed by astrocytes and which respective increased levels indicate astrogliosis.

At 48h **(Figure 2.20-A)** and 7 days post-TBI **(Figure 2.20-B)**, there was a notable increase of GFAP protein levels in the contralateral hippocampus in the TBI group, as an indirect sign of expected post-traumatic reactive astrogliosis. No other significant changes were discernible. Most importantly, this effect was thwarted by NPY administration.

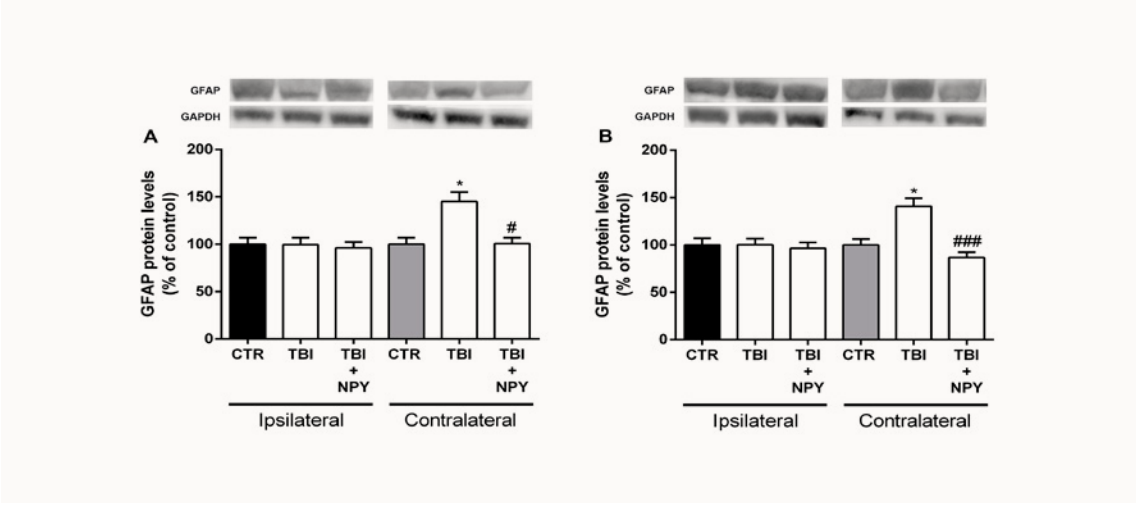


Figure 2.20 - GFAP protein levels in ipsi- and contralateral hippocampus, 48h (A) and 7 days (B) post-TBI. Above the bars, representative western blot images of GFAP protein (50 kDa) and GAPDH (37 kDa) are shown. At both timings, post-TBI GFAP expression is significantly higher in the contralateral hippocampus. NPY attenuates this increase. Results are expressed as mean + SEM. *p<0.05 significantly different from CTR. #p<0.05; ###p<0.001 significantly different from TBI group. **Legend:** CTR, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NPY, Neuropeptide Y; TBI, traumatic brain injury.

Discussion

Our study clearly shows a role for NPY in attenuating astrocyte activation upon TBI, both in ipsi- and contralateral hippocampus. These findings are present at 48h and are still notorious at 7 days following injury.

Humans' astrocytes are larger than rodent's astrocytes and display faster Ca^{2+} wave transmission,[680] showing a more complex structure and functioning. Even so, it is still valid to presume significant parallelism between human and rats' astrocytic response to TBI, as shown in our study and in the literature.[238, 681]

Our study shows that, 7 days after the initial injury, reactive astrocytes display decreased number and total length of processes when compared to controls (both in ipsi- and contralateral hippocampus), most likely contributing to long-term post-traumatic hippocampal atrophy and cell loss.[87, 682] These abnormal changes in hippocampal volume, following TBI and other brain insults (e.g., aneurysmatic subarachnoid haemorrhage), are in part explained by well-described retraction of GFAP-positive astrocyte processes.[398, 682] Recent studies correlate bilateral changes in post-traumatic hippocampal volume with astrocyte morphological abnormalities.[683] Importantly, our experimental protocol objectively shows an effect of NPY in this overall retraction, predominantly in the ipsilateral

hippocampus, as a consequence of an expected (although mild) lateralization of inflicted injury following a unilateral physical injury.

Being able to reverse or attenuate overall organ retraction and spatial reorganization, by acting upon specific cell populations as shown in our experimental data, is undoubtedly a promising finding concerning long-term hippocampal atrophy and hypofunction. There was no obvious post-traumatic decrease in GFAP protein levels, reinforcing the previously discussed notion that post-TBI cellular degeneration and death primarily concern the neuronal population (see Cellular Degeneration).

As previously mentioned, post-traumatic AQP4 polarization and expression are dependent on reactive astrogliosis, which peaks at 7 days post-injury but may be present even 28 days post-injury (as well as AQP4 abnormal expression).[243] Studies on micro-injuries in the brain (e.g., diffuse multiple microinfarctions) confirm that the mentioned loss of perivascular AQP4 polarization is, in the end, a feature of reactive astrocytosis.[684] Considering the possibility that physiologic AQP4 paravascular polarization enables clearance of soluble β amyloid, as suggested by Iliff and colleagues,[685] the prospect of targeting reactive gliosis in therapeutic protocols is theoretically beneficial by indirectly normalizing AQP4 expression and recovering the expected interstitial clearance of wastes, avoiding some of the chronic consequences of brain trauma.

Considering the previously discussed dual role for astrocytes regarding the global response to TBI, one may argue about the true benefit of this inhibition of astrocyte activation, as shown in our Results. First, it is important to mention that this work did not specifically address histopathological and functional outcomes, possibly influenced by astrocyte modulation. Future studies should fully characterize the relevance of this potential therapeutic intervention.

Second, astrocytes can play a key role in responding to and producing proinflammatory mediators (cytokines, chemokines, DAMPs).[148, 150] Pattern recognition receptors, as TLRs, are present in astrocytes and microglia, and its activation results in NFκB signalling pathways and cytokines (namely TNF), chemokines and inflammatory mediators (COX2, MMPs).[148, 151] It is evident that astrocytes play a crucial role in secondary injury, following prior astrocytic involvement in primary injury, susceptibility to membrane distortions[686] and the presence of activated astrocytic mechanotransduction ion channels leading to a rapid influx of extracellular Ca^{2+} and sodium.[687] Therefore, DAMPs signalling and astrocyte involvement can be considered a major promoter of inflammation and edema formation, with increased DAMPs' CSF levels being associated with worst post-TBI functional outcome.[688]

Upon primary injury, astrocyte connexin-mediated ATP signalling and release induce increased cytoplasmic Ca^{2+},[245, 689] microglial activation and further astrocytic activation, namely in spinal cord injury models.[148] Taking this into consideration, inhibition of post-traumatic astrocyte activation should be beneficial. In line with this notion, NFκB signalling inhibition (in astrocytes) was reported to down-regulate post-traumatic inflammation.[690] However, NFκB signalling is also known to stimulate production and secretion of neuronal and gliioprotective growth factor,[148] namely BDNF and Nerve Growth Factor (NGF), while DAMPs were shown to promote, on a more extended period of time, phagocytic debris clearance, vascular remodelling and BBB repair.[148] Even previously mentioned connexin-dependent ATP release can be indirectly beneficial in the long-term, as innate immune cell recruitment depends on it.[148, 245] Considering the hypothesis of a dual-role for astrocytes,[148] one should be careful when interpreting our results. Demonstrating an interference with astrocyte activation at 48h, our findings are aligned with peak inflammatory response and would be suited for early intervention in the acute stage. Even so, the role for post-traumatic astrocyte activation mandates further research and specific multi-factorial assesments.[237]

There is significant uncertainty and less-than-optimal knowledge concerning astrocyte phenotypic polarization (A1/A2) in TBI.[235] Although relatively straightforward in experimental models involving LPS challenge or middle cerebral artery occlusion,[228] A1/A2 dichotomy of reactive astrogliosis molecular profile is relatively absent from recent TBI studies.[238] Ideally, by shifting/directing cell populations to an intended phenotype, therapeutic protocols would potentiate protective/regenerative mechanisms and dampen deleterious effects.[216] Of relevance is the fact that microglial activation is needed in order to A1 astrocytes phenotype fully develop,[228, 235] as supported by previous *in vitro* work.[691] This delicate balance and close cell interconnection, delineating downstream inflammatory response, again shows the difficulty in correctly embracing all aspects of TBI.

To add further complexity, studies clearly show distinct astrocytic molecular profiles upon activation depending on anatomical region,[692] suggesting specific regional functional roles. Hippocampus seems to be more vulnerable than the neocortex,[238] highlighting another contributing factor to cognitive disturbance in all types of TBI. This fact also confirms, concerning TBI research, the relevance of specifically focusing on the hippocampus, as in our study.

Specific astrocyte populations express NPY and NPY receptors[456, 693] and are described as being capable of NPY secretion via dense-core granules.[694] Most im-

portantly, and in a similar fashion to what was mentioned concerning microglia, administration of exogenous NPY significantly increased astrocyte proliferation in the subventricular zone, via Y1R activation.[455] These reports, when combined with our findings, raise the possibility of a dual role for NPY from a therapeutic point of view, by attenuating deleterious effects of secondary injury concerning astrocytes (as shown in our work) while simultaneously promoting the renewal of affected cell populations.

As previously mentioned, older patients are at the highest risk for worse outcomes following TBI. Animal studies have shown a pronounced tendency for proinflammatory phenotype and loss of function in astrocyte populations following TBI in aged brains,[238] in another parallelism to microglia post-traumatic reactivity.[695, 696] Curiously, recent works show that the process of normal aging by itself tilts astrocytes towards a so-called A1 proinflammatory bias, being more responsive to inflammatory challenge.[692, 697] Previously mentioned studies outline a difference between young and aged brains and peak timing in astrocyte reactivity and inflammatory phenomena (at 3rd and 7th day, respectively).[238, 692] Again, this fact reinforces the notion that our NPY protocol, with an early intervention, should have a transitory but significant protective role in the acute phase, namely in an aged brain.

Although these experimental protocols and consequent Discussion are focused on hippocampal astrocytes, one should not forget, when assessing the overall impact of our findings, that BBB's integrity and NVU's homeostasis function also rely on astrocytic function.

2.3.4 Inflammatory pathways

In order to characterize NPY's interference with post-traumatic inflammatory status, IL-1**β** and iNOS protein levels were assessed **(Figures 2.21 and 2.22)**.

IL-1β, 48h post-TBI

As depicted above in **Figure 2.21**, TBI induces a significant increase in IL-1**β** protein levels in the contralateral hippocampus at 48h pos-TBI, an effect largely attenuated by NPY administration. Regarding IL-1**β** expression in the ipsilateral hippocampus, there were no significant effects and the overall trend is opposite to what was described in the contralateral hippocampus. No relevant findings were present at 7 days post-TBI.

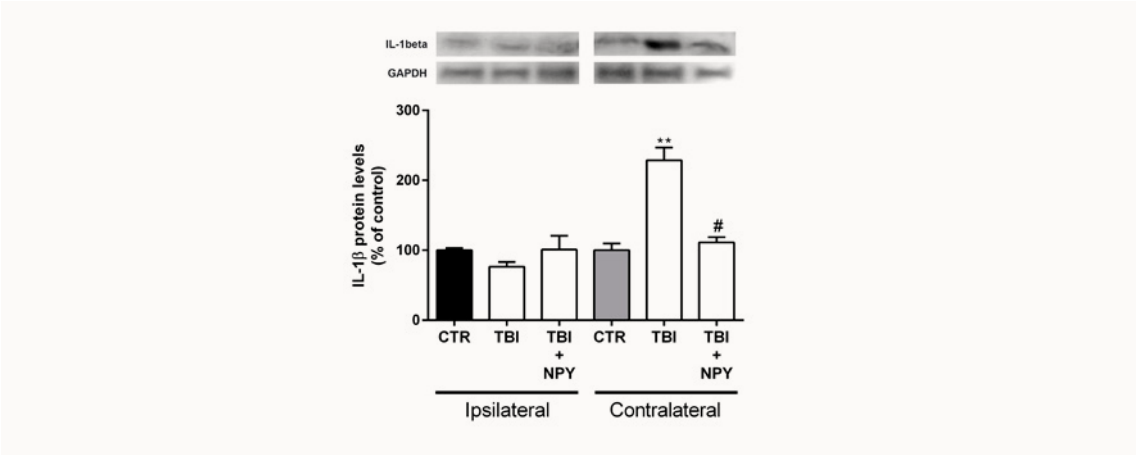


Figure 2.21 - IL-1β protein levels, ipsi- and contralateral hippocampus, 48h post-TBI. Above the bars, representative western blot images of IL-1β protein (17 kDa) and GAPDH (37 kDa) are shown. TBI induces an obvious increase in IL-1**β** protein levels in the contralateral hippocampus. NPY opposes this phenomenon. Results are expressed as mean + SEM. **p<0.01 significantly different from CTR. #p<0.05 significantly different from TBI group. **Legend:** CTR, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL-1, interleukin-1; NPY, Neuropeptide Y; TBI, traumatic brain injury.

iNOS

At 48h, no relevant findings were present concerning iNOS expression in distinct groups, with no significant disparities among them **(Figure 2.22-A)**. Concern-

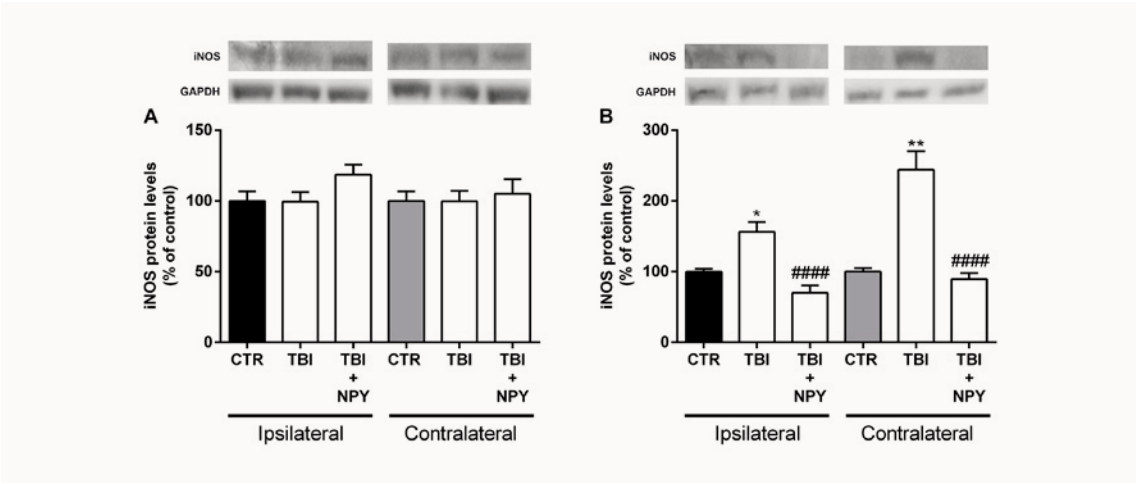


Figure 2.22 - iNOS protein levels in ipsi- and contralateral hippocampus, 48h (A) and 7 days (B) post-TBI. Above the bars, representative western blot images of iNOS protein (131 kDa) and GAPDH (37 kDa) are shown. TBI induces an increase in iNOS protein levels in the ipsi- and contralateral hippocampus at 7 days post-TBI. NPY counterbalances this increase. Results are expressed as mean + SEM. *p<0.05, **p<0.01 significantly different from CTR. ####p<0.0001 significantly different from TBI group. **Legend:** CTR, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase; NPY, Neuropeptide Y; TBI, traumatic brain injury.

ing iNOS expression at 7 days post-TBI **(Figure 2.22-B)**, the occurrence of trauma implies significantly higher protein levels of iNOS in both hemispheres (TBI vs. controls). Importantly, NPY-administered groups display significantly lower iNOS levels compared to TBI groups, both in ipsi- and contralateral hippocampus, with iNOS levels even lower than those shown in controls. This proves a relevant late effect of early NPY administration in attenuating post-traumatic inflammatory and stress response.

Discussion

In our study, TBI interfered with the expression of proinflammatory cytokine IL-1**β**. Animals submitted to TBI showed a significant increase in IL1-**β** protein levels in the contralateral hippocampus, although no effect was obvious in the ipsilateral hippocampus. Moreover, the administration of NPY interfered with its contralateral increase. In order to assess and describe an overall picture regarding inflammatory status at a later stage, iNOS was also assessed at 48h and 7 days. iNOS expression is significantly increased with TBI at 7 days post-TBI and is significantly decreased in the group receiving NPY. In brief, it seems clear that TBI triggered, in our model, an inflammatory response with an initial increase in proinflammatory cytokines followed by a nitrosative stress response.[698] This overall response appears to be significantly reduced by NPY.

Studies regarding human astrocyte cultures response to specific cytokines stimuli, with pro- and anti-inflammatory profiles, display a stronger effect in downstream cytokines production, such as IL-1β, TNF and IL-6.[235] This suggests that IL-1**β** is indeed an essential component of astrocytic inflammatory response. These findings highlight the relevance of our data concerning both astrocytes and IL-1**β** and shows that our intervention acted upon main inflammatory drivers, at least for the astrocyte population.

These findings must be contextualized in a probable broader action of NPY on different cell populations, hampering upstream microglial and astrocyte activation and indirectly decreasing proinflammatory cytokine-based profile. In line with this context of post-traumatic diffuse brain inflammatory profile is the fact that, again and as in previous experimental tasks in our work, distinct responses in ipsi- and contralateral hippocampus take place. As it is not structurally disrupted by trauma, the contralateral hippocampus appears to preserve more of its ability to functionally respond both in terms of inflammation and susceptibility to NPY's influence.

Recent studies have shown, by determining plasma cytokines levels in TBI patients, that systemic inflammation persists up to 1 year following even mild TBI.[540, 699] Importantly, by targeting post-TBI cytokines increased expression, namely IL-1**β**, a significant improvement was shown not only concerning cognitive outcome but also in respect to underlying phenomena as microglia/macrophage activation.[700] In fact, Flygt and colleagues[180] have shown a specific IL-1**β**-related attenuation of microglia/macrophage immunoreactivity following antibody neutralization, as well as a promising reduction in caspase-3 expression (indicator of post-traumatic apoptosis).[180]

Brain inflammation is in some degree proportional to injury severity and, above all, involves several phenomenons subject to considerable inter-individual variability.[138] This variability is another factor to be considered in experimental studies, namely in its correlation to clinical outcome assessment, undoubtedly more challenging and prone to under or overestimations.[701]

Neuronal death is not reversible and excitotoxicity can be attenuated only to some extent. On the other hand, inflammation is clearly a more transitory state and, eventually, more prone to be temporarily influenced by outer elements. Specific protocols targeting cytokines pathways and (somehow neglected) chemokines are therefore a promising line of research.[138, 676] Even so, considering data provided by basic science and clinical trials, it becomes evident that blindly and massively suppressing neuroinflammation, namely by using high doses of corticosteroids, will not only be unuseful but also potentially deleterious.[702] The aim should be at pinpointing which agents/steps on which pathways should be suppressed and for how long.[335]

Several intersections and common elements to both classical pathways and neurogenic inflammation have been discussed throughout this work. To assess multiple elements in innumerous inflammatory pathways, blood and CSF cytokine profiling, in distinct timelines, may prove useful in the clinical setting. The goal would be not only to select new biomarkers but also to delineate a threshold for proinflammatory status that is potentially therapeutic while not invoking a deleterious neuroinflammatory response.[703]

Attenuating post-traumatic CNS classical pathways and neuroinflammation, especially in the context of BBB breakdown, might also prove helpful by preventing a systemic proinflammatory status and subsequent promotion of multi-organ dysfunction (from respiratory failure to systolic dysfunction).[704, 705]

In sum, studies focusing on post-traumatic inflammation should ideally contemplate all the complexity involving its upstream elements and downstream cascade of events.

2.3.5 Post-traumatic blood-brain barrier dysfunction

In order to study TBI's impact on BBB and a possible influence of NPY in BBB's post-traumatic disruption, post-TBI albumin immunostaining **(Figure 2.23)**, as well as albumin and occludin protein levels **(Figures 2.25 and 2.26)**, were assessed.

Albumin

Concerning the impact of TBI in BBB properties, TBI causes a statistically significant increase in hippocampal albumin immunoreactivity, as a reflection of albumin extravasation, at 48h post-TBI **(Figure 2.23-E)**, when compared to con-

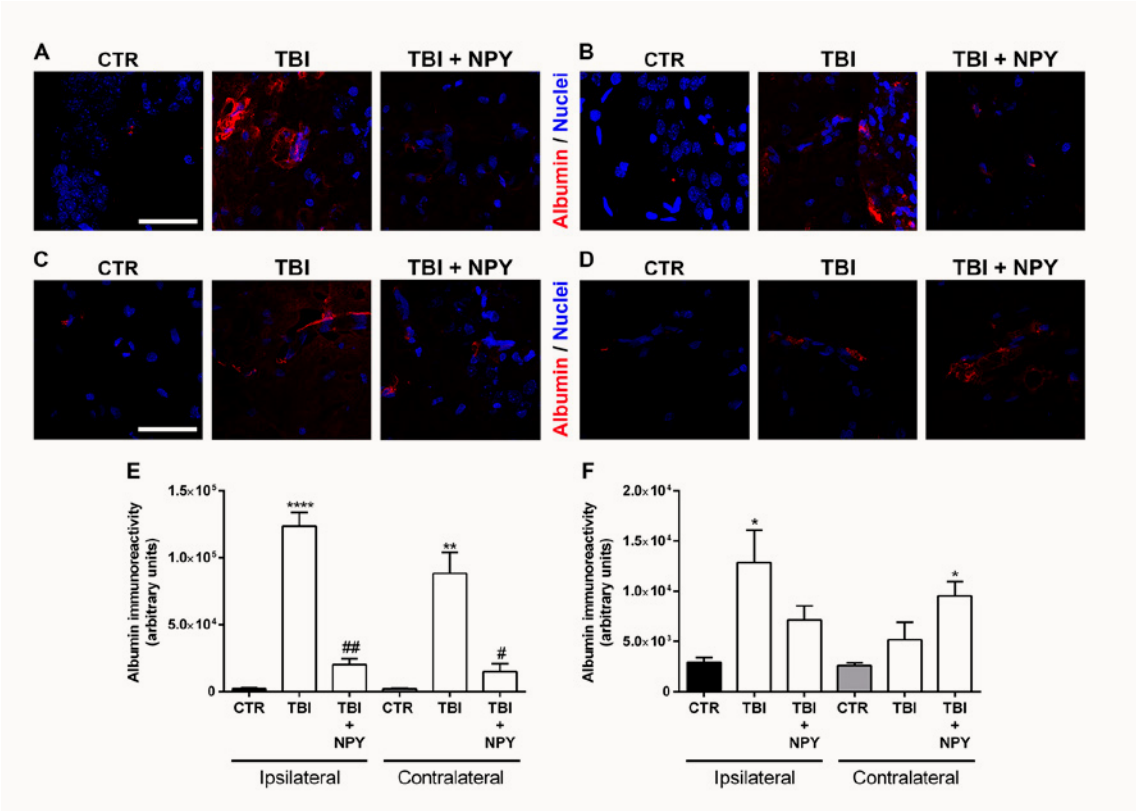


Figure 2.23 - Representative images of albumin (marker for BBB disruption) (albumin, red) and Hoechst (blue, nuclei) in different experimental groups, at 48h [obtained in the A) ipsilateral and C) contralateral hippocampus] and 7 days post-TBI [obtained in the B) ipsilateral and D) contralateral hippocampus]. Scale bar = 50 µm. Quantification of ipsi- and contralateral hippocampal albumin immunostaining (arbitrary units), at (E) 48h and (F) 7 days post-TBI. TBI induces a significant increase in albumin immunostaining, namely at 48h post-TBI. NPY clearly diminishes this phenomenon but apparently reinforces the increase in albumin immunostaining in the contralateral hippocampus at 7 days post-TBI. E, F) Results are expressed as mean + SEM. *p<0.05; **p<0.01; ****p<0.0001 significantly different from CTR. #p<0.05; ##p<0.01 significantly different from TBI group. **Legend**: CTR, control; NPY, Neuropeptide Y; TBI, traumatic brain injury.

trols. Post-TBI administration of NPY implies a significantly decreased albumin immunoreactivity when comparing to the TBI group. This fact shows a probable role for NPY in significantly attenuating post-traumatic BBB´s disruption.

In regard to albumin immunoreactivity at 7 days post-TBI **(Figure 2.23-F)**, our preliminary data suggest an increase of albumin immunoreactivity in both ipsi- and contralateral hippocampus. In the ipsilateral hemisphere, the trend is similar to our findings at 48h: a post-TBI increase attenuated with concomitant NPY administration. Unexpectedly and unlike ipsilateral findings, NPY significantly increases albumin immunostaining in the contralateral hemisphere.

In summary, in our animal model of TBI, trauma injury induces an increase in albumin immunoreactivity, due to post-traumatic albumin extravasation in the context of BBB's impairment. Early administration of NPY clearly prevents this deleterious phenomenon **(Figure 2.24)**.

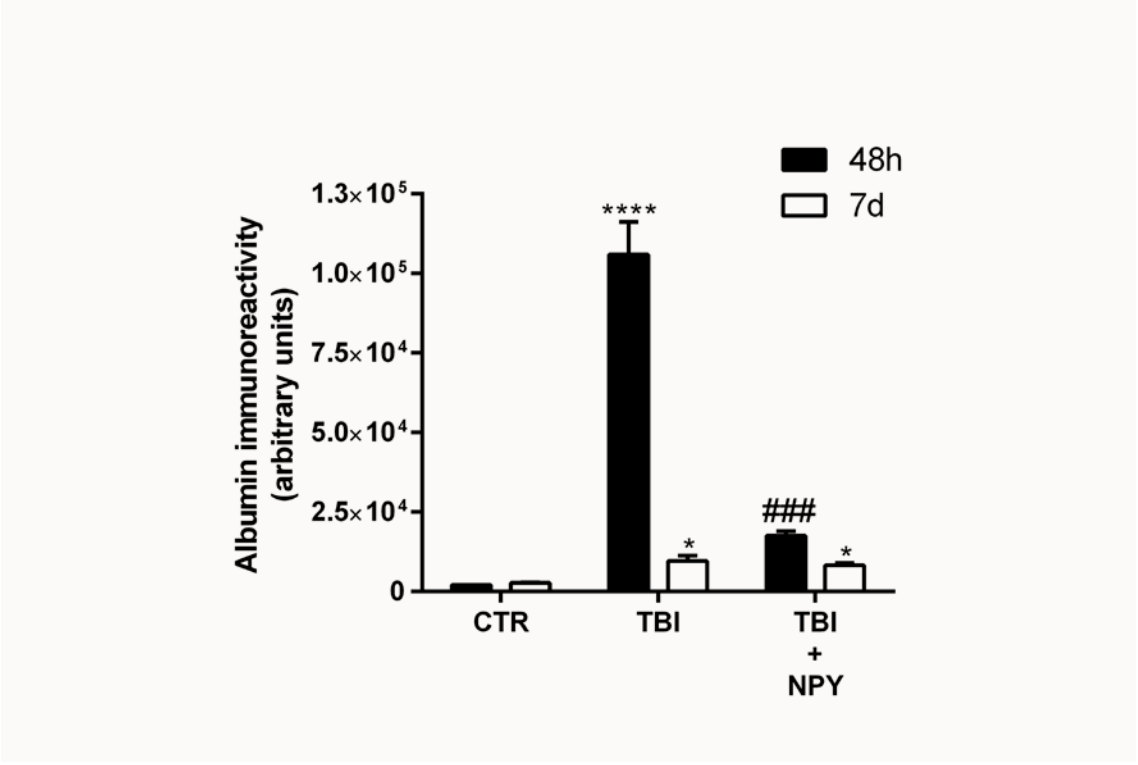


Figure 2.24 - Overall picture of post-TBI albumin immunoreactivity varying with time and NPY's influence, 48h and 7 days post-TBI (mean value per group, both hemispheres considered). NPY administration blocks post-traumatic albumin extravasation, as displayed at 48h. Results are expressed as mean + SEM. *p<0.05; ****p<0.0001 significantly different from CTR. ###p<0.001 significantly different from TBI group. **Legend**: CTR, control; NPY, Neuropeptide Y; TBI, traumatic brain injury; 7d, 7 days.

As shown in **Figure 2.25**, western blot studies confirm the impact of TBI in brain's vasculature and the role of NPY administration, by countering BBB impairment, as assessed by albumin protein levels.

Therefore, these findings are similar, both at 48h and 7 days, to our results in immunostaining studies. A significant increase in albumin protein levels upon TBI, namely in the ipsilateral hippocampus, is counteracted by NPY's action (not statistically significant in the contralateral hippocampus at 7 days post-TBI).

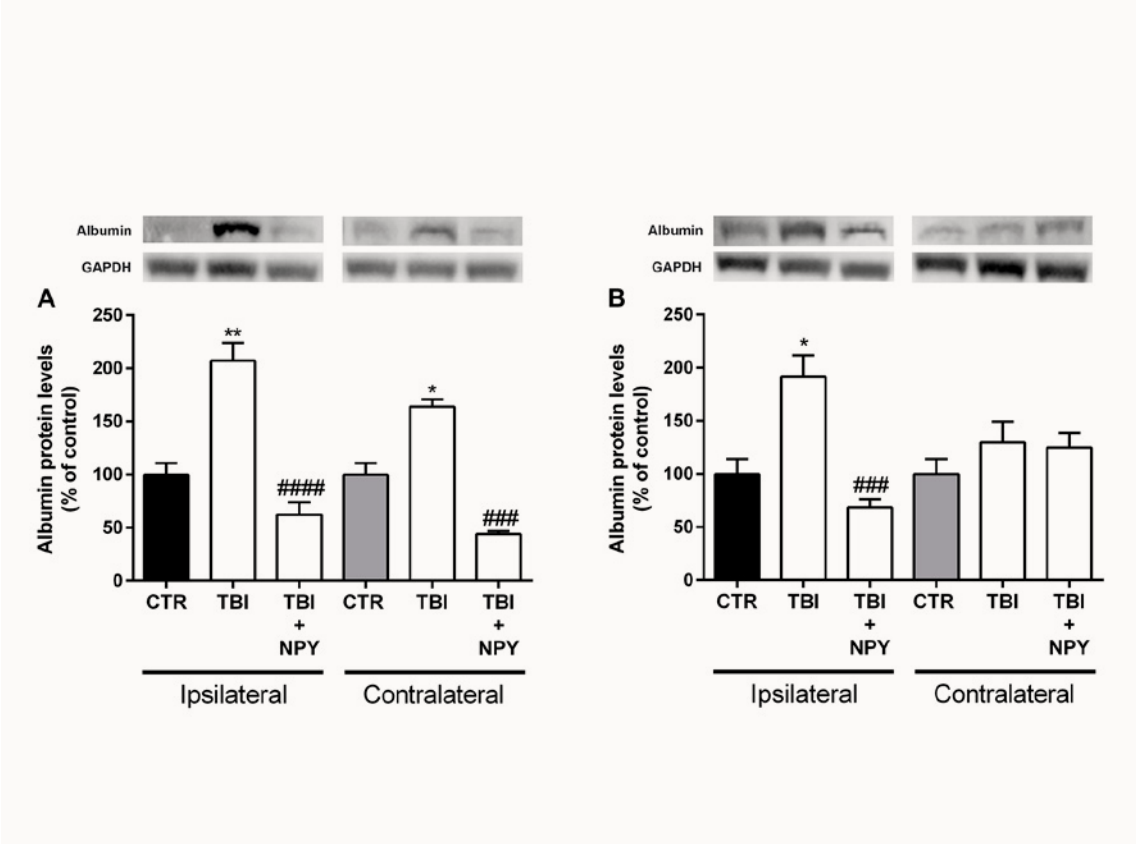


Figure 2.25 - Albumin protein levels in ipsi- and contralateral hippocampus, 48h (A) and 7 days (B) post-TBI. Above the bars, representative western blot images of albumin protein (66,5 kDa) and GAPDH (37 kDa) are shown. **A)** TBI induces a significant increase in albumin protein levels at 48h. NPY significantly counters this phenomenon. **B)** TBI induces a significant increase in albumin protein levels in the ipsilateral hippocampus at 7 days post-TBI. NPY counters this phenomenon. Results are expressed as mean + SEM. *$p<0.05$; **$p<0.01$ significantly different from CTR. ###$p<0.001$; ####$p<0.0001$ significantly different from TBI group. **Legend:** CTR, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NPY, Neuropeptide Y; TBI, traumatic brain injury.

Occludin

In order to elucidate the mechanisms involved in BBB disturbance, possible fluctuations in a tight junction protein levels (occludin) were analysed **(Figure 2.26)**.

Concerning occludin protein levels at 48h post-TBI **(Figure 2.26-A)**, no statistically significant findings were present upon the TBI group both in ipsi or contralateral hippocampus. A trend to its post-traumatic increase in both hemispheres is discernible, and it appears to be reinforced by NPY. At 7 days post-TBI **(Figure 2.26-B)**, its levels again tend to be increased in the TBI group, yet with no statistical significance, and NPY clearly prevented this tendency in the ipsilateral hippocampus.

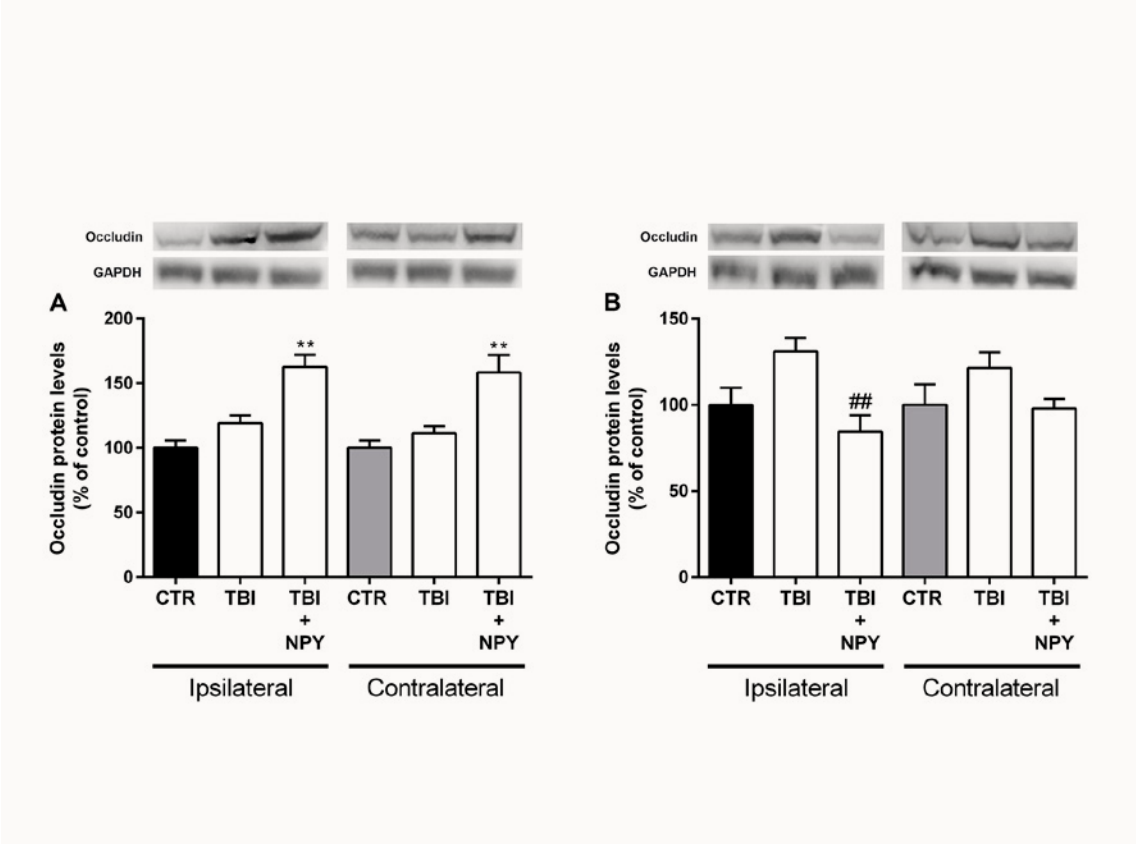


Figure 2.26 - Occludin protein levels in ipsi- and contralateral hippocampus, 48h (A) and 7 days (B) post-TBI. Above the bars, representative western blot images of occludin protein (59 kDa) and GAPDH (37 kDa) are shown. **A)** TBI apparently induces an increase in occludin protein levels at 48h (trend with no statistical significance). NPY reinforces this increase. **B)** TBI apparently induces an increase in occludin protein levels at 7 days post-TBI (trend with no statistical significance). NPY counters this tendency, namely in the ipsilateral hippocampus. Results are expressed as mean + SEM. **p<0.01 significantly different from CTR. ##p<0.01 significantly different from the TBI group. **Legend**: CTR, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NPY, Neuropeptide Y; TBI, traumatic brain injury.

Discussion

Our experimental protocol displays a significant attenuation of acute post-traumatic BBB disruption by NPY, as shown by albumin immunoreactivity studies at 48h, with a significant decrease in albumin extravasation. No meaningful effect at 7 days was shown. These findings were mostly confirmed, in regard to albumin, in western blotting studies.

This task focused on BBB disruption, an early and acute consequence of TBI, peaking at 2-3 days after initial injury. For this reason, as this is a phenomenon of early progression and is significantly settled or attenuated after 1 week, the fact that our most relevant findings take place at 48h (and not 7 days) is somewhat expected and in accordance with previous knowledge (see Introduction, Blood-Brain Barrier section). Another contributing factor to this time-dependent response is that exogenous NPY reinforcement took place a few minutes after trauma, and an absent long-term effect is plausible.

When considering ipsi- and contralateral hippocampus separately in regard to albumin immunoreactivity, the same trend is present at 48h: a significant increase upon TBI and decrease with post-TBI NPY administration. However, a more pronounced effect was identified in the ipsilateral hippocampus.

At 7 days, there was a tendency to increased ipsilateral albumin immunoreactivity but with no effect of NPY, probably as NPY's effect vanishes, as previously discussed. Once again, the present variability lead to no statistical significance and more experiments are needed to complete this study. Interestingly, specifically in contralateral hippocampus at 7 days post-TBI, but clearly influencing the overall picture, a paradoxical effect is present since post-TBI NPY administration seems to increase the amount of late albumin extravasation (no statistical significance). The possible reasons for this unexpected effect are diverse. First, a low "n" in our subgroups might explain this unexpected result in a specific subset of animals. Second, a "rebound effect" on the contralateral hippocampus, theoretically and functionally closer to its baseline status, in which one single NPY administration would preclude an initial response while leading to a more robust, delayed compensating response. Third, a possible late manifestation of microglia's dual role in neuroinflammation.[346] As mentioned before, upon induced inflammation in experimental conditions, microglia initially reinforces BBB integrity before its protective effect is reversed and BBB permeability is further impaired, and neuroinflammation ensues. One can speculate on a late manifestation of this dual role in the contralateral hippocampus, somewhat spared to overall damage in TBI, namely physical disruption. Another interesting hypothesis is based on the fact that post-traumatic angiogenesis is well documented, with evident up-

regulation of angiogenic factors and corresponding neovascularization as early as 48h post-TBI.[131] These immature and unstructured neovessels, with leaky BBB, might somehow contribute to overall increased albumin extravasation. Additionally, increased albumin synthesis and secretion by activated microglia is another potential confounding factor.[706, 707]

Nonetheless, the variability between the two hemispheres at 7 days post-TBI was significant, with conflicting results concerning NPY influence in albumin immunoreactivity (as a reflection of BBB impairment) and it is mandatory to increase the number of experiments in the future. At this point, it is only possible to speculate that, in general, NPY's protective action by attenuating BBB impairment is more pronounced after 48h, somehow losing its effect after 7 days.

Therefore, inhibiting post-TBI exacerbation of BBB's impairment is a promising therapeutic strategy to be implemented in several pathologic contexts.[346, 708] Decreased albumin extravasation, following NPY's reinforcement, is in itself a significant finding as, in abnormal conditions, albumin will also activate microglia and astrocytes, with all the harmful consequences previously addressed, including proinflammatory cytokines release.[311]

Concerning occludin and its levels upon TBI and NPY administration, disparate findings can be mentioned in relation to different timings (48h and 7 days post-TBI). Protein complexes that regulate BBB paracellular transport are composed of several proteins, able to compensate for each other, which can at least in part explain, in our findings, the increase in albumin levels without significant changes in occludin at 48h post-TBI. Nevertheless, in the presence of NPY, there was an increase in occludin levels at 48h post-TBI, suggesting its possible involvement in a NPY-mediated protective effect. When assessed at 7 days following initial trauma, there was a tendency to lower occludin levels in the animals initially administered with NPY. Such results were unexpected, since NPY was supposed to interfere with post-traumatic BBB disruption, usually associated with decreased levels or abnormal organization of TJs and adherens junctions. Further studies are necessary to confirm these results and, if so, these post-traumatic findings could probably be associated with abnormal rearrangements in brain endothelial cells leading to leaky junctions, as mentioned previously.

Some reports show NPY's action in apparently diminishing endothelial monolayer permeability.[709] These findings were contested by other studies, again with coronary endothelial monolayers,[710] and reports demonstrating BBB´s increased permeability in brain gliomas upon the use of highly selective Y1R ligands.[711] In relation to our results in reestablishing BBB's function with NPY, Ou et al.[656] have shown a significant action by NPY in stabilizing TJs in the retinal vascular

barrier (*in vitro* models), significantly increasing ZO-1 expression. Importantly, NPY-promoted preservation of cellular TJs integrity was associated with a 30% reduction in permeability (fluorescein-streptavidin studies).[656] Most importantly, the same action was observed in *in vivo* models, with considerable NPY-induced attenuation of vascular permeability in retinal vessels, again demonstrating NPY's ability to stabilize TJs complexes.[656] The previously discussed body of knowledge concerning NPY and BBB closure/reinforcing therefore provides solid ground for our findings, reinforcing our working hypothesis.

2.4 General discussion

2.4.1 Animal model of trauma

In this research project, an animal head trauma model was developed in conditions mimicking closed head injury, as there is no cranial penetration. In our opinion, and according to the literature, these conditions are ideal to best reproduce TBI's context and consequences in real life.

Representative open skull injury models, such as Fluid Percussion and Controlled Cortical Impact models, are partially artificial, as they induce lesions that, by mechanism and context, generally are not found in real life (an open skull before the induced trauma, a fluid column layed upon the *dura mater*, the opening of the *dura mater* before induced trauma). Nevertheless, these models indeed cause brain injury and predictable effects in cell populations and general pathophysiologic mechanisms of response. But, from a larger perspective, these models reproduce apparently similar results by employing much different means, with unknown consequences concerning true reproducibility and similarity to real TBI.

Animal models of TBI indeed replicate specific realities, namely biomechanical contexts (fluid percussion injury models, cortical impact injury, weight drop injury models, blast injury models) but will necessarily fail to apprehend all aspects of complex mechanisms of injury,[273, 712] with inevitable shortcomings. For example, Fluid Percussion injury models, a well-known and widely used model for diffuse TBI, predictably replicate brain edema, diffuse haemorrhages and grey matter damage.[273, 713] However, it is unable to replicate/induce neither skull fracture nor multiple gyri contusions, a common feature of moderate to severe human TBI.[273, 714]

Our trauma model was based on Shohami's group model[640]: weight-drop injury delivered to one side of the unprotected rat's skull, with the head placed on

a hard surface. A focal blunt injury is followed by BBB breakdown, microglia and astrocytes activation and neurodegeneration (all correlated to neurological impairment and neurobehavioural deficits). Considering this, this model is said by many to closely resemble the intrinsic conditions of human Closed Head Injury,[715] a condition almost universal to TBI in real life. It is an easy-to-assemble and predictable model,[716] quickly performed under gas-based anesthesia and allows, if indicated, immediate neurological assessment. Some authors mention a possible disadvantage of a higher variability in injury severity dependent on the intensity of impact, with mild impact inducing a diffuse injury pattern, whereas a severe impact will generate focal contusion.[717] This criticism does not seem relevant, as the severity of the impact can be directly adjusted and the intended effect calibrated. As mentioned by Johnson et al.,[718] this a valid and widespread model, replicating clinically-based brain injury with cranial vault deformation and indirect cortical compression/injury.

Research teams are supposed to balance the need to address a specific phenomenon with the essential requirement of some connection to reality, as translational research is understood in its intent ("from bench to bedside").[719] A simple gesture, e.g., performing a craniotomy in the desired impact zone before inducing direct trauma, might enhance post-traumatic findings but moves experimental models away from the clinical and pathological picture of an actual patient. A veiled artificiality is inevitable when each model depicts a specific injury, limited in its anatomical boundaries and pathological effects (e.g., unilateral or bilateral, penetrant or non-penetrant injury, DAI or focal injury), somehow failing to apprehend the intrinsic complex and all-encompassing nature of TBI.[720] Diffuse and focal TBI display some overlapping effects in post-TBI behavioural deficits,[721] but obviously differ in the type of induced lesions and consequences.

This research protocol did not consider gender differences for data analysis purposes. However, it is increasingly recognized that, despite mixed results (both in animal and human studies), gender accounts for non-irrelevant distinct responses to trauma.[722] Among other works, Doran and colleagues[723] have shown sexually specific neuroinflammatory responses in mice: male subjects displayed increased higher basal ROS in non-injured brains, increased post-TBI phagocytic activity and influx of peripheral myeloid cells; female subjects presented with increased production of TNF and IL-1**β** and significantly reduced microglial activation.[723] Even so and most importantly, a coherent and sustained response was shown in a uniform population in our study. Future studies might confirm further gender disparities, specifically regarding neuropeptides in trauma.

Bhatt and collaborators[724] have confirmed brain laterality in rats. Several functions and behaviours rely on an apparent left hemisphere dominance (learning,

depression, behaviour, appetite). This contradicts a report by Hum et al.,[725] denying any laterality facing similar stimuli, unlike what is predominant in humans: left hemisphere as dominant in most individuals, with preferential use of the right hand.[726] Goldstein et al.,[727] regarding cellular proliferation and immune response to external aggressions, also reported additional data that corroborates laterality in rats' brain function. However, as no behavioural or performance outcome tests were undertaken in the present work, the issue of functional lateralization does not seem relevant in this circumstance.

One frequently overlooked phenomenon in TBI is the differential effect of trauma in the two brain hemispheres. On this specific issue of differential injury exposure and course of recovery concerning ipsi- and contralateral hippocampus, as shown in our data, several reports mention an expected less severe morphological and functional disruption (including hyperexcitability) in the contralateral hippocampus.[395, 728] Regarding differential damage to distinct hippocampal subregions, no specific findings can allow straightforward extrapolations. Sporadic reports mention an apparent higher vulnerability of the CA3 subregion in an acute setting following TBI, with a long-lasting effect more evident in the CA1 subregion (at 6 months).[729] These findings in themselves are rather unspecific and with no apparent critical impact in the neurotrauma field, despite acknowledged differences in anatomical and functional connectivity among distinct hippocampal subregions.[730]

Herein, upon induced trauma and following animal sacrifice, no cranial fractures or epidural or subdural haemorrhages were obvious under visual inspection. If any of these lesions were present, our findings could be affected in their significance and our model's uniformity would be compromised. As mentioned in the Results section, one rat presented with immediate sub-periosteal bleed near the site of impact. However, it was an extracranial bleed of limited amount and spontaneous cessation, so this event was deemed non-relevant. Concerning neurological status, all controls and injured rats were assessed after waking up from anesthesia and in the following days. All rats were neurologically intact upon being euthanized.

A major difficulty in animal models of trauma is to calibrate the induced injury in order to uniformly obtain the desired consequences (at a structural and functional level) but with no overextended injury concerning space, time or sequelae. One scenario to avoid, specifically in head trauma models, would be to have such a significant initial injury that it would preclude a valid distinction between neuronal death due to primary or secondary injury. In our work, there were no animal deaths, cranial fractures or significant intracranial bleeds, and the rats presented with similar induced injuries and intact neurological status, leading

us to believe that our model is uniform and valid concerning our findings. These results were coherent among themselves and with similar studies in the field, as depicted previously. This illustrates the fulfilled objective (and initial premise of this project) of inducing an objective, predictable and reproducible traumatic lesion to the brain.

Our choice of animal strain (Sprague-Dawley rats) was based on the team's experience from past research projects and its intrinsic characteristics - a well-studied and extensively used strain in the trauma research field, with predictable behaviour and reproducible responses. Despite known strain-dependent disparities concerning response to CNS injuries,[731, 732] namely in the volume of induced ischemic lesion, the most striking variability in obtained responses is in relation to fundamental behavioural changes.[733] Even so, some structural differences pertaining to CNS are necessarily present. For example, Long-Evans strain rats display greater average cortical areas compared to Sprague-Dawley rats.[733] Fisher 344 strain also displays distinct features: increased basal caspase-3 protein levels, higher ICP, increased seizure activity, frequent motor deficits, better long-term cognitive performance.[732] Strain differences account for some variability among studies. Undoubtedly, the most important is to acknowledge confirmed differences between control and trauma groups and for them to be coherent, non-spurious and reproducible.

Another possible confounding factor might be the use, before experimental induced trauma, of isoflurane, the most used anaesthetic agent in experimental TBI, as it is easy to administrate and provides a rapid recovery. Further, isoflurane, and most anaesthetic agents, are known to be neuroprotective and supposedly might influence an animal's response to brain trauma.[734] As our study includes a well-defined control group, with animals being submitted to similar conditions and procedures except for trauma itself, one can reasonably argue that this possible bias is not a significant issue when assessing inter-group variability and distinct findings.

A relevant discussion concerns intrinsic validity, reproducibility and utility of animal models in general and this TBI animal model in particular. One should assume that the model used in this study is not a precise, faithful and complete reproduction of a TBI on a human victim, since human trauma takes many forms and contexts. This variability and brain's complexity most likely hinder developing a well-suited, comprehensive and valid trauma model that reproduces all human relevant phenomena. Besides intrinsic differences in cytoarchitecture (e.g., rat's lissencephalic brain displays lesser white matter percentage)[712] and pathophysiology in animals vs. humans, this is not a real TBI, an unexpected event in non-controlled circumstances and environment. Animal models of trauma pres-

ent two significant issues to be accounted for: they differ from humans concerning neurobiochemical pathways, cell population and pharmacodynamics, and the anatomical structures and corresponding biomechanics of trauma are different. A significant example of this is that human CSF post-traumatic cytokines increment lasts longer (several days) than in their rodents counterparts.[339]

2.4.2 Intranasal delivery of NPY

The intranasal route is a widely tested and practical drug-delivery way of gaining access to the brain's parenchyma and CSF.[735] It has the obvious advantage of acting promptly and being non-invasive. Several studies show that different peptides can reach high CSF concentration and brain parenchyma activity, including in the hippocampus, following intranasal administration, retaining their beneficial effects.[736, 737] A review by Lee and colleagues[738] mentions several reports on objective benefits following intranasal delivery of distinct drugs, aiming at either immune modulation,[595] neuronal protection[739] or regeneration.[737]

Intranasal delivery of therapeutic agents has for long been tested in different CNS pathologies,[740] from migraines[741] to PTSD[742] and AD,[743] as a way of enhancing therapeutic efficacy.[744] Saver and colleagues[497] have reviewed and confirmed intranasal delivery's utility in the context of stroke, concerning its adequacy in pre-hospital management and therapeutics with neuroprotective agents, even before performing any tests (imaging, blood tests).[497] Intranasal delivery of NPY and NPY_{13-36} attenuates microglial activation and IL-1**β** mRNA expression in Huntington's disease,[458] while depression and anxiety have also been addressed.[745] Interestingly, the anxiolytic effect of NPY is present both from a treatment perspective, upon a previous settled situation with persistent symptoms, and from a prophylactic perspective, preventing the upheaval of neurobiochemical findings and PTSD typical behaviour.[642]

The unique, straightforward relationship between the nasal cavity and cranial contents allows this route to effectively deliver medication by circumventing blood-brain and blood-cerebrospinal fluid barriers. Some compounds can reach the caudal brain via trigeminal nerve pathways, respiratory mucosa or lymphatic/perivascular spaces located on *lamina propria*.[744] Another major path is based on the olfactory structures,[746] with peripheral olfactory neuron pathways running along into olfactory bulbs and allowing drug distribution into the rostral brain (a somewhat slower process, dependent on pinocytosis and axonal flow).[747] In humans, neuropeptides administered through this pathway reach significant concentrations in the olfactory bulb within 10 minutes.[748] Intranasal brain delivery occurs mainly by the trigeminal and olfactory nerve pathways, following

paracellular and transcellular passage.[744] By overcoming BBB through the olfactory and trigeminal path, intranasal delivery is effective and does not imply systemic drug absorption, avoiding secondary effects, first-pass metabolism and gastrointestinal breakdown.[749, 750]

Intranasal drug delivery is, in theory, applicable to almost every TBI patient (including children) and subject to pre-hospital use.[751, 752] This simple approach, relatively cheaper and non-toxic, might become a valuable tool in future therapeutic protocols, attenuating secondary damage in the immediate post-trauma moment.[753] As an example, intranasal administration of NAD^+ decreased post-traumatic hippocampal neuronal death and anomalous microglia activation (CA1, CA3 and DG).[754] Lv et al.[755] studied the possibility of an anti-edematous effect by intranasal Nerve Growth Factor (NGF), interfering with AQP4 activity and inhibiting transcription and expression of proinflammatory cytokines, including IL-1β. Further, a study involving healthy human volunteers and intranasal delivery of exogenous NPY, through the nasal mucosa on the superior 1/3 of the nasal cavity, documented very low systemic absorption and no relevant side effects besides long-lasting nasal vasoconstriction.[756] Concerning central NPY injection in animal models, minor side effects are mentioned: hyperphagia and hypometabolism/hypothermia in the acute phase followed by a catabolic phase, if NPY infusion was to be continued for 7 days, with fever and interruption of weight gain.[757]

Distinct variables and factors can influence the success of intranasal delivery: drug's relative molecular weight (molecules exceeding 1000 Da display significantly poorer distribution)[758, 759]; lipophilicity[760] and degree of dissociation[761]; drug concentration and volume; nasal mucociliary clearance and mucoadhesive properties (with the possibility of enhancing it with designated polymers)[762]; the subject's position[763] and the depth of cannula's insertion. Several strategies for enhancing this route's efficacy are possible,[764] including formula modification[765] and transport mediation (nanoparticles, agglutinants).[766] In fact, studies in humans have shown a swift CNS delivery kinetics, with intranasally-delivered peptides reaching peak concentration within 30 minutes.[767]

Even so, the efficiency of this maneuver is diminished by the limited volume of the nasal cavity, small olfactory mucosa/nasal mucosa area ratio and the necessarily low drug dosage.[747] Besides that, not all drugs can be administered using this route, and its efficacy can be affected by the nasal anatomy (beyond the scope of this work) and condition, physical and chemical properties of the compound and its formulation.[747]

Another issue of concern is the timing of therapeutics administration, perhaps another significant factor hampering clinical success. Antagonists of

IL-1, for example, appear to be truly effective only if administered in the first few hours following TBI.[768] As expected, a later and more pronounced disturbance of neuronal and glial compartments may affect drugs pharmacokinetics and effectiveness.[738]

2.4.3 Pathological findings and NPY's influence

Our data shows a global brain response to TBI, involving different cellular elements and with obvious repercussion concerning overall inflammatory mechanisms and BBB's functional status. Importantly, our model convincingly demonstrates an effective and consistent neuroprotective action of NPY at different levels by attenuating cell death, reducing distinctive glial activation, downregulating inflammatory profile and reinstating BBB's function.

This neuroprotective role for NPY was somewhat foreseeable, considering existing literature and the current body of knowledge concerning NPY. Importantly, our results are in line with several findings in retinal studies (*in vitro* and *in vivo*), demonstrating NPY's protective action in diabetic retinopathy[656] by maintaining vascular integrity and tight junction protein expression, thus reducing induced leakage, and diminishing (excitotoxic-induced) neural apoptosis.

Even so, much remains to be elucidated about NPY's mechanisms of action, including possible modulation of multifactorial glutamate release, and overall true impact in Neurotrauma. For example, future studies might focus on comprehensively describing NPY's simultaneous influence in a multitude of inflammatory biomarkers, in order to obtain a more accurate and reliable picture. Notably, one cannot ignore the fact that inflammatory pathways and promoters act simultaneously upon different cells. For instance, SP is known to augment inflammatory mechanisms in human microglial and astrocytic populations directly.[769] Because of this, as we show an overall influence of NPY in different cell types and events, the notion of a global and staged response, prone to therapeutical intervention, is even more relevant.

2.4.4 Translating findings in animal studies into clinical practice

This type of study raises a crucial question: how can one counteract the complexity of multiple pathological mechanisms associated with TBI with a single therapeutic agent, focusing on a single target and with a relatively unknown ideal timing? A paradigm change combining therapeutic protocols and multiple targets is needed.[307, 770]

With respect to animal models, they are increasingly relevant in trauma research.[771] Rodents' brains, not to mention bigger and more complex mammalians, are known to share significant similarities, both functional and structural, with the human brain and cranial vault. Moreover, at a cellular level, neurons and other specialized cell types also display considerable morphological correspondence.[252] Considering this, animal models should provide valuable insights on a microscopic and macroscopic scale, despite the necessary limits to a direct extrapolation.

Human neocortex astrocytes are 2.6-fold larger, extending 10-fold more GFAP-positive primary processes than their rodent counterparts, while specific subclasses of human astrocytes are not even represented in rodents.[772] Thus, human cortical astrocytes, when compared to rodents, are larger, structurally more complex and more diverse.[773] Nevertheless, there are similarities among human astrocytes and murine models, not just morphological but also functional.[774]

Considerable differences between humans and other mammals are present concerning the CNS system, supportive cranial and spinal apparatus, craniospinal angle, brain's complexity and specifically gyrus architecture and white/grey ratio.[273, 775] The overall complexity of the human brain, compared to other animals, downplays the notion derived from a reductionist framework that, in order to understand a system, one should study its elements separately. Excessive research focus on neurons in artificial models of trauma (a neurocentric approach, as mentioned by Logsdon), although understandable, is perhaps one of the reasons for sub-optimal results in TBI therapeutics.[28]

The complexity of TBI and its symptoms makes it difficult to be replicated by a single animal model of trauma.[776] An important indirect consequence is that different models may display distinct (even opposite) responses to supposedly therapeutic interventions (e.g., IL1R1 knockout displaying beneficial or detrimental effects according to the model and sub-types of lesion).[611] Moreover, various timings for neuroimmunology mechanisms in TBI are also an issue.[209]

Importantly, most studies focus on biological and structural sequelae, despite widespread knowledge on the significant impact of TBI concerning neuropsychiatric symptoms,[777] arising from an impaired organ and the patient's own understanding of its limitations and challenges.[778] These symptoms are undoubtedly conditioned upon a biological basis of disease[779] but are, by definition, impossible to fully replicate in animals. Thus, emulating TBI-relevant neuropsychiatric symptoms in animal models is a challenging but necessary step to understand mechanistic relationships and develop realistic therapeutic approaches.[780] Given this, rigorous scientific judgement and further validation of the suitability of these models in assessing neuropsychological contexts are mandatory.

Other possible limitations, such as physiological and structural differences, variations in physiological parameters (blood pressure, ionic balance, brain temperature, partial pressures of carbon dioxide and oxygen) and others are ignored or undervalued contexts that can lead to distinct findings.[4, 273] Efforts should be taken to bring these models the closest possible to reality (by using primate models to the detriment of rodents, for example), considering distinct drug metabolism and reabsorption rates, and only then assess or manipulate individual variables of interest.[781] Within animal models, differences might be significant between rodents and bigger gyrencephalic animals (monkeys, pigs), as distinct bioenergetics response and anatomical differences might be relevant.[592] For example, rodents have a higher grey-to-white-matter ratio, posing another difficulty in extrapolating findings and outcome metrics concerning Diffuse Axonal Injury (DAI).[782]

Differences in patterns of gene expression when comparing rodents and humans[783] are another factor to consider. The links between genomic differences, gene expression and their functional and behavioural outcomes are obvious. Differences among individuals are perpetuated and amplified at all levels, from genes to functionality into behaviour, making a case for diversity.

2.4.5 Final remarks

In summary, this experimental research protocol, besides implementing a valid animal model for brain trauma, has shown that NPY is a promising therapeutic agent in TBI. Different experimental tasks demonstrate an overall beneficial effect of NPY by attenuating short-term microglial activation and afterwards shifting microglia phenotype profile, attenuating astrocyte activation and neuronal death, hampering post-traumatic BBB impairment and neuroinflammatory response.

Secondary injury and post-traumatic penumbra area can, in theory, be attenuated if early intervention is in place, namely in a pre-hospital context and upon admission, hopefully impacting mortality and long-term impairment. Despite this being a study based on acute short-term findings (48h and 7 days), TBI should not be considered an acute disease (nor a static one), neither for clinical or scientific purposes.

An initial NPY single dose was given in the present experimental protocol, but one can speculate on optimized prolonged therapeutic protocols. This NPY administration might be helpful in different types of TBI and their typical injuries at different timings. It is clear that equally or even more important than treating primary injury (a somewhat inglorious effort) is addressing the secondary damage.

Another shortcoming of this type of pre-clinical studies is the frequent focus on one agent or a single step in a complex environment. Effective translation into clinical trials should involve multi-modality therapies or, at least, multipurpose agents, following a rigorous assessment of pharmacodynamics, pharmacokinetics and possible interactions.[273, 784] Unfortunately, the necessary and desirable contribution of adjacent fields of knowledge (pharmacotherapy, bioinformatics, bioengineering) is frequently undervalued.

Despite significant concordance between our findings and our working hypothesis, in line with previous reports and pre-existing knowledge in the field, much remains to be elucidated in promising and exciting future research directions.

CHAPTER III

Clinical studies

3.1 Introduction

We hypothesized that significant TBI leads to a multistage neuropeptide response, with significant roles for SP and NPY. Following previously described findings in animal models of trauma, displaying a neuroprotective role for NPY, the next logical step would be to assess the possibility of modulating this same response in TBI human patients. In order to further explore this potential therapeutic approach, this study aimed at assessing neuropeptide response among human TBI victims, including those with and without obvious macroscopic brain injury, its temporal profile and relation to S100B and Mg^{2+} levels, knowingly affected by TBI.[12, 785]

3.2 Materials and Methods

3.2.1 Study design

A prospective, single-center analysis of patients with a clinical diagnosis of TBI and indication for head CT imaging was performed from January 2017 to July 2019 at the *Centro Hospitalar e Universitário de Coimbra* (Coimbra Hospital and University Centre).

A thorough Informed Consent Form and the protocol for selection of patients, preservation of anonymity and handling of clinical data was approved by the Ethics Committee both in the Coimbra Hospital and University Centre and Faculty of Medicine, University of Coimbra.

Diagnosis of TBI was confirmed upon anamnesis, with corroboration of significant head trauma, and clinical examination. Timing of TBI and time interval until clinical observation was confirmed by the patient (when appropriate), accompanying persons and medical teams involved in pre-hospital management. Indication for performing initial CT scan was in accordance to the Portuguese National Protocol in Traumatic Brain Injury: moderate to severe TBI, according to the GCS score; abnormal neurological examination; significant LOC; suspected fracture; known risk factors (>65 yr, alcoholism, epilepsy, coagulopathy or hypocoagulable state, previous cranial surgery) (*Protocolo Nacional para a abor-*

dagem dos Traumatismos Crânio-Encefálicos; Ministério da Saúde, Direcção Geral de Saúde; Circular Normativa, 1999).[21, 786] CT scans (Siemens SOMATOM go-All) were evaluated by an independent radiologist (from a group of 6 dedicated neuroradiologists) and classified according to the presence or not of 1 or more lesions described as cerebral haemorrhagic contusions.

Exclusion criteria included:

- pediatric patients (17 years or less);
- patients > 80 years;
- active or recent infection;
- any recent surgical procedure (neurosurgical or other) prior to TBI;
- any surgical procedure while still included in the study (minimally invasive procedures not considered for this purpose);
- acute/chronic renal, liver or gastrointestinal disease;
- alcohol dependence or chronic alcohol abuse;
- acute alcoholic intoxication at the time of assessment;
- uncontrolled or recently diagnosed diabetes;
- history of malignant tumour;
- chronic inflammatory systemic disease;
- ongoing acute inflammatory event;
- recent vomiting/diarrhea;
- previous/present cranial or intracranial pathologies;
- concomitant cranial/intracranial traumatic findings (skull fractures; obvious epidural or subdural haematomas; significant subarachnoid haemorrhage; intraparenchymal haematomas);
- concomitant and relevant cranial/intracranial non-traumatic findings;
- simultaneous significant traumatic findings in other systems or organs (thoracic, abdomen, limbs, spine, significant scalp lacerations);
- recent traumatic injuries of any kind;
- patients prescribed with diuretics, angiotensin-converting enzyme inhibitors, gentamicin, amphotericin (or other medications capable of interfering with Mg^{2+} metabolism).

Patients who, while still included in the study, developed an infectious or other type of significant medical condition were also excluded. All previously mentioned conditions or medications can potentially interfere with inflammation pathways, neuropeptides levels or ionic balance.

Patients and controls were enrolled into the study upon meeting inclusion criteria and assigned to their corresponding group following assessment of head CT scan findings and the presence or not of cerebral haemorrhagic contusion.

Participants were then divided into 5 groups, n=35 per group (as n>30 is usually considered a minimum for large samples regarding Central Limit Theorem in statistical analysis). In bold, designation used for each group as follows:

- **Control** group: healthy volunteers (same exclusion criteria);
- **TBI** group: TBI victims without traumatic lesions (as shown on CT scans and despite significant head trauma), 6h or less after trauma;
- **C-6h** group: TBI victims with visible haemorrhagic contusions (as shown on CT scans), 6h or less after TBI;
- **C-48h** group: TBI victims with visible haemorrhagic contusions, 48h after TBI;
- **C-7d** group: TBI victims with visible haemorrhagic contusions, 7 days after TBI.

Blood samples were collected via direct venipuncture and immediately processed in Hospital's laboratories.

Initially included in group C-6h, a subset of patients underwent repeated samplings in all different timings (6h, 48h and 7 days post-TBI) and is therefore included in all groups C.

3.2.2 Laboratory methods

Peripheral blood was collected to 8 mL heparin tubes (S-Monovette), homogenized and transferred within a 5 min interval to a 15 mL Falcon tube containing aprotinin, preventing protein degradation (concentration - 0.014 TIU/mL). After gently inverted, Falcon tubes were left to rest for 20 min. Falcon tubes were then centrifuged for 15 min at 1000 g and 4^0 C. Samples were stored in 200 µL aliquots at -80^0 C to prevent repetitive freeze/thaw cycles. Fifty µL of plasma were used in duplicates and absorbance was determined (BioRad model 600 plate reader). Average of duplicates readings was performed and a standard curve was generated using a four-parameter logistic curve-fit to determine plasma concentrations in pg/mL.

Determination of NPY, S100B and SP's plasma levels was performed by Enzyme-Linked Immunosorbent Assay (ELISA) [(NPY and S100B kits Merck KGaA ® (New Jersey, USA); SP kits R&D Systems ® kit (Minnesota, USA)]. Dilutions for the ELISAs took place for SP (1:2, taken into consideration for final calculations). The following detection limits were used for the different assays: 16.8-43.8 pg/mL dynamic range for SP (according to supplier); 2.7 pg/mL for S100B; 2 pg/mL for NPY.

Determination of Calcium (Ca^{2+}), Magnesium (Mg^{2+}), Sodium (Na^{+}), Potassium (K^{+}), Chloride (Cl^{-}), C-Reactive Protein (CRP) and Osmolality was undertaken. Blood samples were processed on Architect analyzers (Abbot Diagnostics®): ionogram indirect potentiomety (Na^{+}, K^{+}, Cl^{-}); enzymatic assays (Mg^{2}); immunoturbidimetry and arsenazo III Ca^{2+} complexes assays.

Normal range of values was considered as follows (according to current laboratory protocols in Coimbra Hospital and University Centre):

- Mg^{2+}: 0.66-1.07 mmol/L.
- Na^{+}: 136-146 mmol/L.
- K^{+}: 3.5-5.1 mmol/L.
- Cl^{-}: 101-109 mmol/L.
- Ca^{2+}: 8.8-10.6 mg/dL.
- CRP: 0-0.5 mg/dL.
- Osmolality: 260-302 mOsm/kg.

3.2.3 Statistical analysis

All data were analyzed using IBM SPSS Statistics version 24.0 and are presented as mean ± SEM. For comparison of parametric results between multiple groups, one-way analysis of variance (ANOVA), followed by post hoc Tukey's test (for equal sample sizes) or Tukey-Kramer test (for unequal sample sizes), were performed. When in the presence of non-parametric distribution, comparison with Kruskal-Wallis test by ranks was the chosen alternative. Concerning the sub-group of patients who underwent repeated sampling, a non-parametric Friedman Test was performed, according to specific data requirements and non-gaussian distribution. A p-value less than 0.05 ($p \leq .05$) was considered statistically significant.

3.3 Results

3.3.1 General findings

For each group, 35 patients (or controls, regarding that specific group) were enrolled as an endpoint. As some of the patients initially enrolled in group C-6h **(Figure 3.1)** were also included in subsequent groups C-48h and C-7d (undergoing repeated sampling) (23 patients), a total of 129 patients (instead of 175 patients) were included in the study, distributed in 5 distinct groups as outlined in the Methods section.

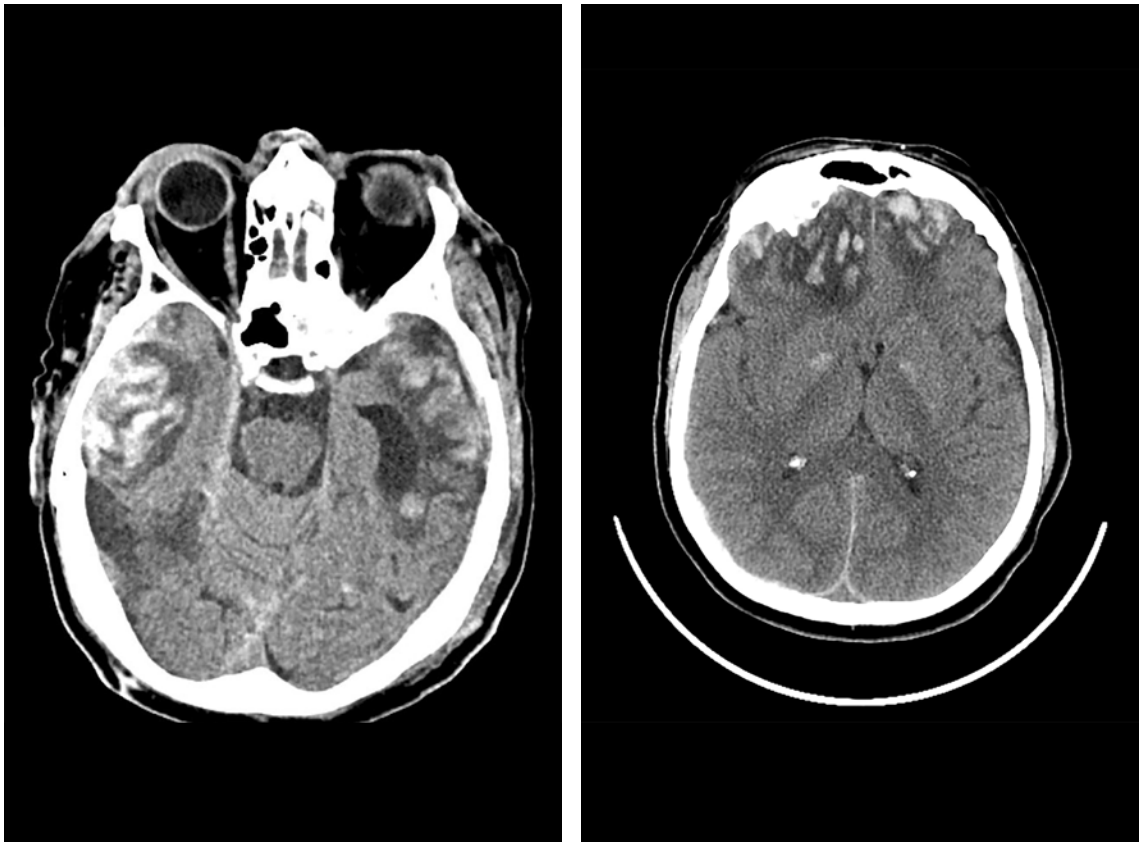


Figure 3.1 - Cerebral haemorrhagic contusions in TBI victims (CT-scans), group C-6h. Left: male patient, 62y, GCS - 8 (E1V2M5), bitemporal contusions; Right: male patient, 71y, GCS - 14 (E4V4M6), bifrontal contusions.

In total, sex distribution (male/female) was 85/44 (66%/34%), with an obvious prevalence of male patients. The average age was [years +/- standard deviation (SD)]: controls group - 48,80 yr ± 10.96; TBI group - 61,40 yr ± 15.56; C-6h group - 65.03 yr ± 12.14; C-48h group - 65.06 yr ± 13.18; C-7d group - 65.40 yr ± 13.90 (range, considering all groups, from 27 to 80 years). No other specific findings with statistical significance were obvious concerning age or gender. Two patients (in a total of 35, 5.7%) initially enrolled in group C-6h died in the first 48h following TBI.

Missed samples and exclusion of obvious outliers explain differences in group sizes concerning obtained results.

At the moment of blood sampling, the number of patients who were admitted to Neurointensive Care Unit was as follows: group C-6h - 7 patients (20%); group C-48h - 10 patients (28.6%); group C-7d - 8 patients (22.9%). As expected, a correct and realistic GCS score assessment was not possible in patients requiring sedation.

A general overview of results obtained in different groups is displayed in **Table 3.1.**

Table 3.1 - General view on results concerning different groups. Legend: CRP, C-reactive protein; C-6h, assessment at 6h post-TBI; C-48h, assessment at 48h post-TBI; C-7d, assessment at 7 days post-TBI; GCS, Glasgow Coma Scale score; n, number of patients; NPY, Neuropeptide Y; SD, standard deviation; SEM, standard error of the mean; SP, Substance P; TBI, traumatic brain injury.

	Controls	TBI group	Group C-6h	Group C-48h	Group C-7d
Age (years) (mean ± SD)	48,80 ± 10.96	61,40 ±15.56	65.03 ± 12.14	65.06 ± 13.18	65.40 ± 13.90
Male/female (%)	60/40	69/31	66/34	66/34	63/37
GCS (n) 14-15 9-13 3-8	35	25 10	16 14 5	16 9 10	19 8 8
Deaths (in 7 first days post-TBI)	-	-	2	-	-
NPY (pg/mL) (mean ± SEM) (n)	19.702 ± 1.462 (n=31)	29.567 ± 5.427 (n=29)	45.997 ± 4.968 (n=32)	32.395 ± 4.056 (n=32)	43.268 ± 6.260 (n=30)
SP (pg/mL) (mean ± SEM) (n)	441.441 ± 22.572 (n=31)	825.606 ± 23.690 (n=30)	613.463 ± 49.055 (n=26)	587.576 ± 48.363 (n=26)	620.083 ± 46.743 (n=27)
S100B (pg/mL) (mean ± SEM) (n)	30.187 ± 3.347 (n=31)	42.303 ± 6.302 (n=29)	95.668 ± 14.102 (n=22)	71.778 ± 9.556 (n=23)	58.860 ± 13.708 (n=22)
Magnesium (mmol/L) (mean ± SEM) (n)	0.897 ± 0.021 (n=35)	0.861 ± 0.039 (n=29)	0.754 ± 0.015 (n=33)	0.811 ± 0.019 (n=34)	0.925 ± 0.039 (n=34)
Calcium (mg/dL) (mean ± SEM) (n)	9.460 ± 0.063 (n=35)	9.100 ± 0.102 (n=35)	8.730 ± 0.149 (n=35)	8.630 ± 0.098 (n=35)	8.710 ± 0.135 (n=35)
CRP (mg/dL) (mean ± SEM) (n)	0.461 ± 0.244 (n=35)	1.435 ± 0.518 (n=35)	1.674 ± 0.469 (n=35)	7.706 ± 1.106 (n=35)	6.348 ± 1.244 (n=35)
Sodium (mmol/L) (mean ± SEM) (n)	140.066 ± 0.415 (n=35)	138.566 ± 0.570 (n=35)	137.766 ± 0.682 (n=35)	139.200 ± 0.718 (n=35)	137.533 ± 0.816 (n=35)
Potassium (mmol/L) (mean ± SEM) (n)	4.550 ± 0.354 (n =33)	4.080 ± 0.454 (n =34)	4.060 ± 0.364 (n =28)	3.890 ± 0.454 (n =31)	3.940 ± 0.576 (n =31)
Chloride (mmol/L) (mean ± SEM) (n)	105.193 ± 0.338 (n=35)	105.500 ± 0.630 (n=35)	102.966 ± 0.552 (n=35)	103.933 ± 0.828 (n=35)	102.933 ± 0.854 (n=35)
Osmolality (mOsm/kg) (mean ± SEM) (n)	280.677 ± 0.983 (n=35)	280.066 ± 1.168 (n=35)	281.833 ± 1.465 (n=35)	283.500 ± 2.204 (n=35)	282.000 ± 1.629 (n=34)

As mentioned, from initial group C-6h, 23 patients were carried over and included in subsequent groups C-48h and C-7d, forming a specific set of patients with consecutive sampling at 6h, 48h and 7 days following TBI **(Table 3.2)**.

Table 3.2 - Subset of patients, initially in group C-6h, undergoing repeated sampling; patients demographics and initial GCS score. Legend: 6h post-TBI, sampling at 6h post-TBI; 48h post-TBI, sampling at 48h post-TBI; 7d post-TBI, sampling at 7 days post-TBI; GCS, Glasgow Coma Scale score; n, number of patients; SEM, standard error of the mean; TBI, traumatic brain injury.

	Repeated sampling
n	23
Age (mean ± SEM)	63,80 ± 2.596
Male/female (n)	14/9
GCS (n)	**GCS 14-15 / 9-13 / 3-8**
6h post-TBI	13 / 6 / 4
48h post-TBI	13 / 5 / 5
7d post-TBI	15 / 4 / 4

3.3.2 Neuropeptide Y

Our results show a significant increase in NPY levels (pg/mL) upon TBI among different groups [$F(4, 151) = 4{,}76$, $p = .0012$], post hoc Tukey-Kramer method test **(Figure 3.2)**, namely in the presence of parenchymal lesions. At 48h, there is a significant decrease in NPY levels, no longer noticeable at 7 days post-TBI. TBI victims, with and without parenchymal lesion at 6h, display higher NPY levels than controls (TBI and C-6h vs. controls) (although with statistical significance only concerning C-6h). When comparing TBI victims with and without parenchymal lesion (at 6h post-TBI) (C-6h vs. TBI), significant differences are also present, with higher NPY levels in the former. NPY is also significantly increased when comparing TBI with a parenchymal lesion at 6h and 48h post-TBI (C-6h vs. C-48h), with a significant decrease at 48h post-TBI, followed by another evident increase, noticeable at 7 days post-TBI.

The values obtained in these experiments were the following (pg/mL, mean +/- SEM): **Controls**, n=31, 19.702 +/- 1.462; **TBI**, n=29, 29.567 +/- 5.427; **C-6h**, n=32, 45.997 +/- 4.968; **C-48h**, n=32, 32.395 +/- 4.056; **C-7d**, n=30, 43.268 +/- 6.260 **(Figure 3.2)**.

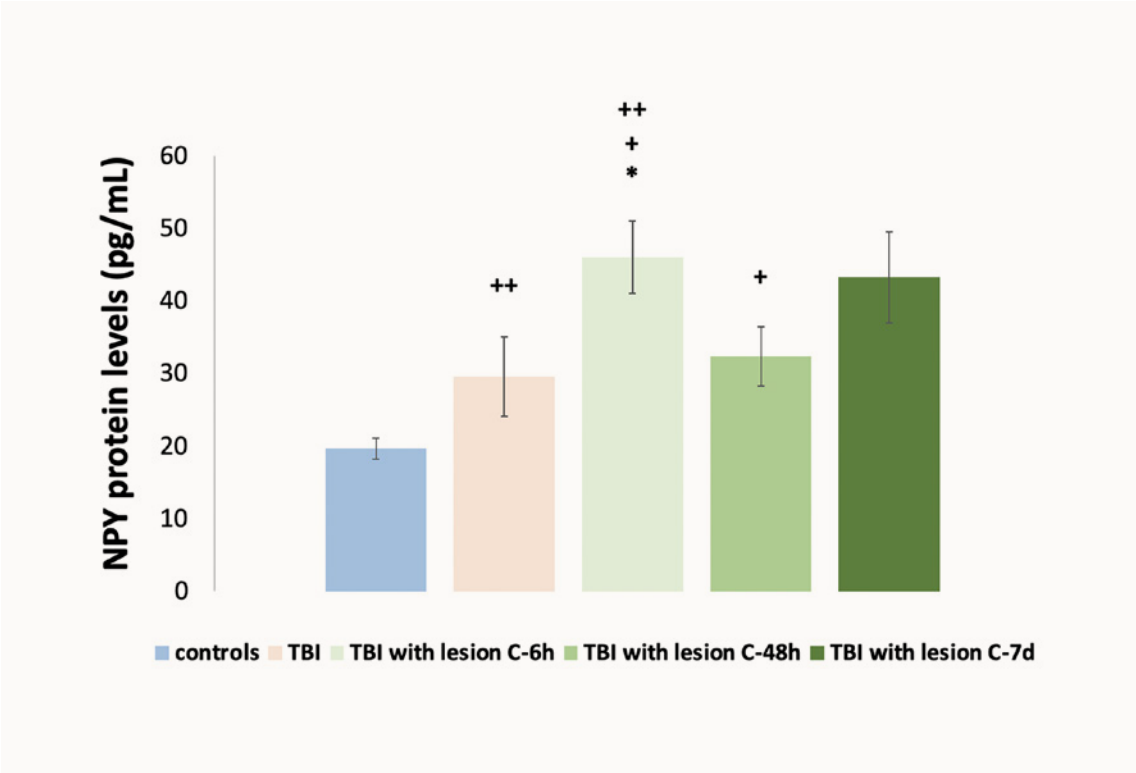


Figure 3.2 - Response to TBI concerning NPY levels (pg/mL). TBI induces an increase in NPY levels. *p<0.05 significantly different from controls. $^{+}$p<0.05; $^{++}$p<0.01 significantly different from each other. **Legend**: C-6h, assessment 6h post-TBI; C-48h, assessment at 48h post-TBI; C-7d, assessment at 7 days post-TBI; NPY, Neuropeptide Y; TBI, traumatic brain injury.

Considering the subset of patients with paired samples (repeated blood sampling in the same patient at 6h, 48h and 7 days post-TBI), a similar pattern in NPY levels is displayed **(Figure 3.3)**: significantly increased levels within the first 6h, with NPY levels declining out to 48h and rising again until 7 days following TBI. These differences in mean values did not reach statistical significance upon non-parametric Friedman Test for repeated measures: χ^2 - 5.826087 (**α** - 0.05; dF - 2; χ^2 critical value - 5.99147).

Results were as follows (n=21, pg/mL, mean ± SEM): **C-6h**, 39.924 ± 6.487; **C-48h**, 28.929 ± 4.867; **C-7d**, 43.467 ± 8.072 **(Figure 3.3)**.

In sum, TBI (with a parenchymal lesion) induced an early increase in NPY levels (at 6h post-TBI), followed by a steep decline (at 48h post-TBI) and a later resurgence in NPY plasma concentrations (as displayed at 7 days post-TBI).

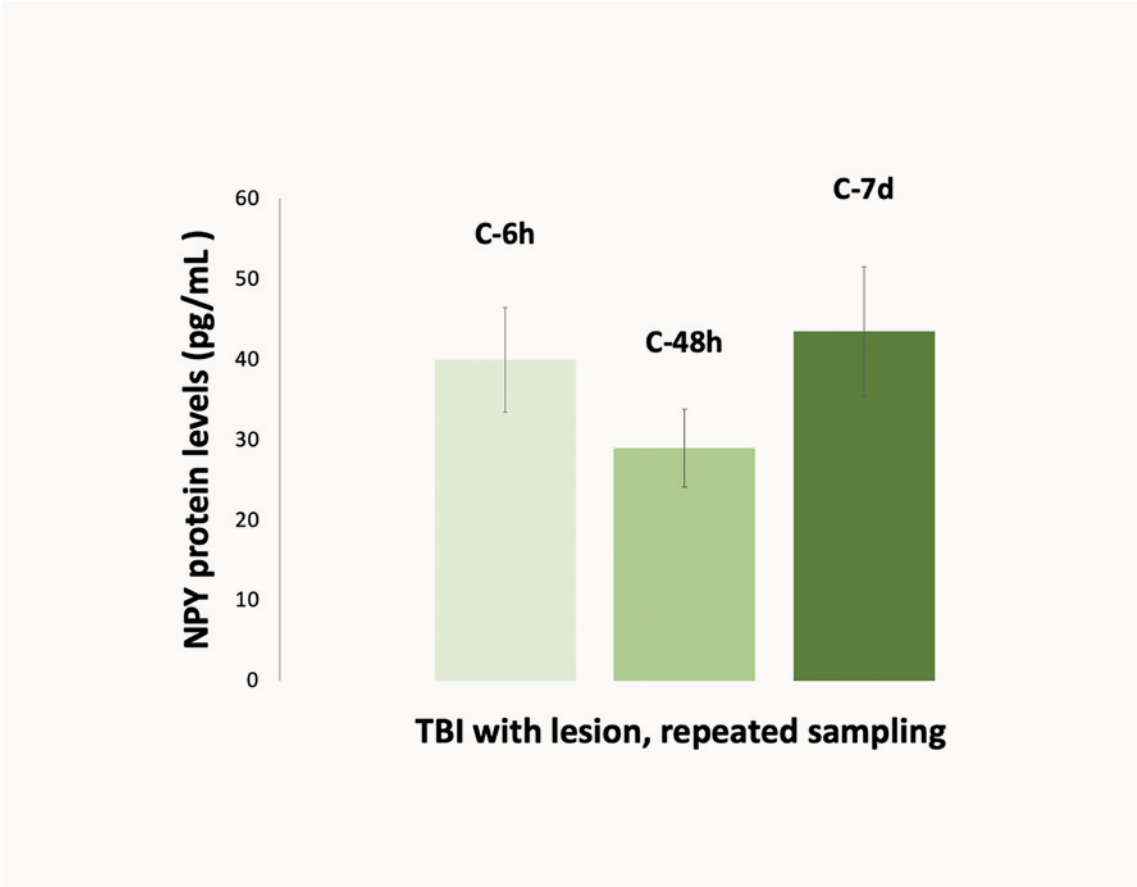


Figure 3.3 - Response to TBI concerning NPY levels (pg/mL), repeated sampling in patients with haemorrhagic contusion. NPY levels decrease at 48h (trend with no statistical significance). **Legend**: C-6h, assessment 6h post-TBI; C-48h, assessment at 48h post-TBI; C-7d, assessment at 7 days post-TBI; NPY, Neuropeptide Y; TBI, traumatic brain injury.

Concerning NPY levels and their relation to initial GCS scores in group C-6h, the group of patients classified as suffering from a severe TBI (GCS 3-8) was the least represented (n=4, 12,5%) and presented with higher NPY levels **(Figure 3.4)**. Those differences did not reach statistical significance upon non-parametric 3-groups comparison with Kruskal-Wallis test by ranks: χ^2 - 1.180461 (**α** - 0.05; dF - 2; χ^2 critical value - 5.99147).

The following results were obtained (pg/mL, mean ± SEM): **GCS 3-8**, n=4, 53.210 ± 11.910; **GCS 9-13**, n=14, 29.460 ± 3.950, **GCS 14-15**, n=14, 40.114 ± 11.435 **(Figure 3.4)**.

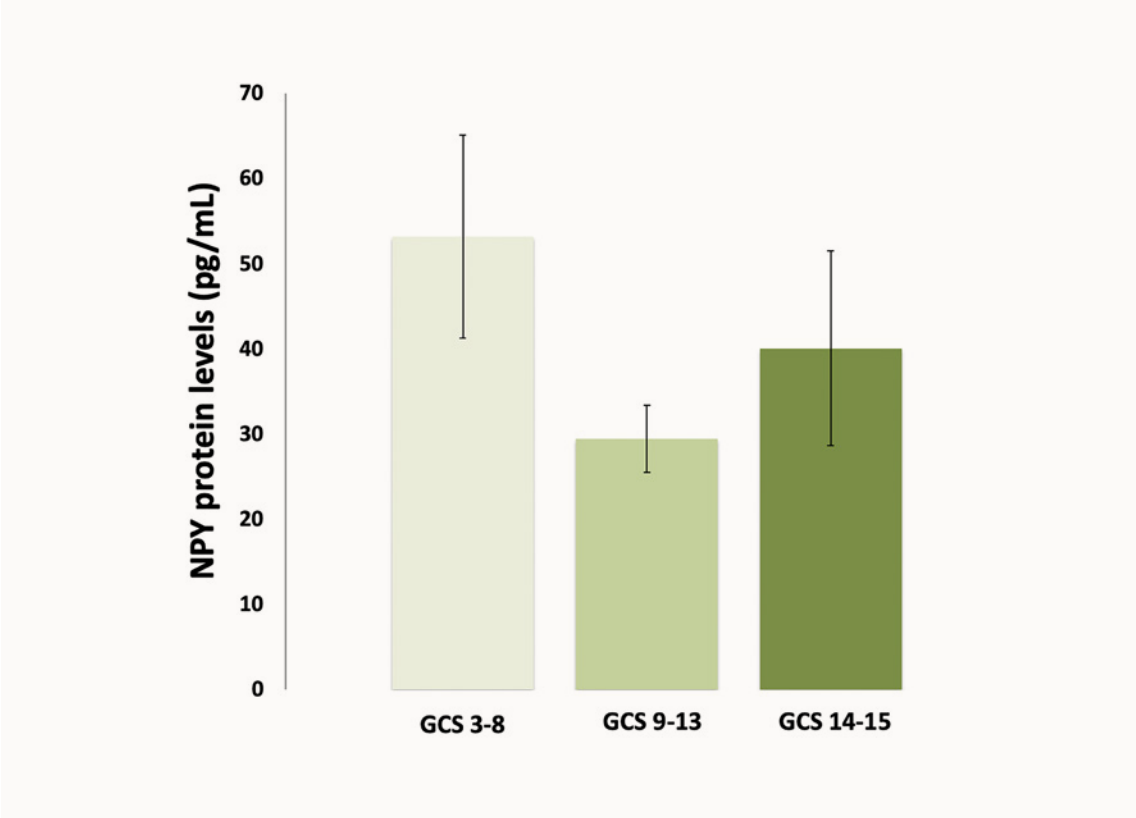


Figure 3.4 - Response to TBI concerning NPY levels (pg/mL), group C-6h, according to initial GCS scores. Legend: GCS, Glasgow Coma Scale score; NPY, Neuropeptide Y; TBI, traumatic brain injury.

3.3.3 Substance P

Herein, we also demonstrated a significant effect of TBI on SP levels (pg/mL) among different groups - [$F(4, 100) = 8.190$, $p < .001$], post hoc Tukey-Kramer method test **(Figure 3.5)**. Significant increases in SP levels are observed in the presence of TBI, with TBI victims (with and without parenchymal lesion at 6h) displaying significantly higher SP levels compared to controls (TBI and C-6h vs. controls). When comparing TBI victims with and without parenchymal lesion (at 6h post-TBI) (C-6h vs. TBI), a discrepancy in SP levels is also present, with higher levels in the latter. Concerning the other time points, SP levels remained relatively stable in all patient groups.

The values obtained were the following (pg/mL, mean ± SEM): **Controls**, n=31, 441.441 ± 22.572; **TBI**, n=30, 825.606 ± 23.690; **C-6h**, n=26, 613.463 ± 49.055; **C-48h**, n=26, 587.576 ± 48.363; **C-7d**, n=27, 620.083 ± 46.743 **(Figure 3.5)**.

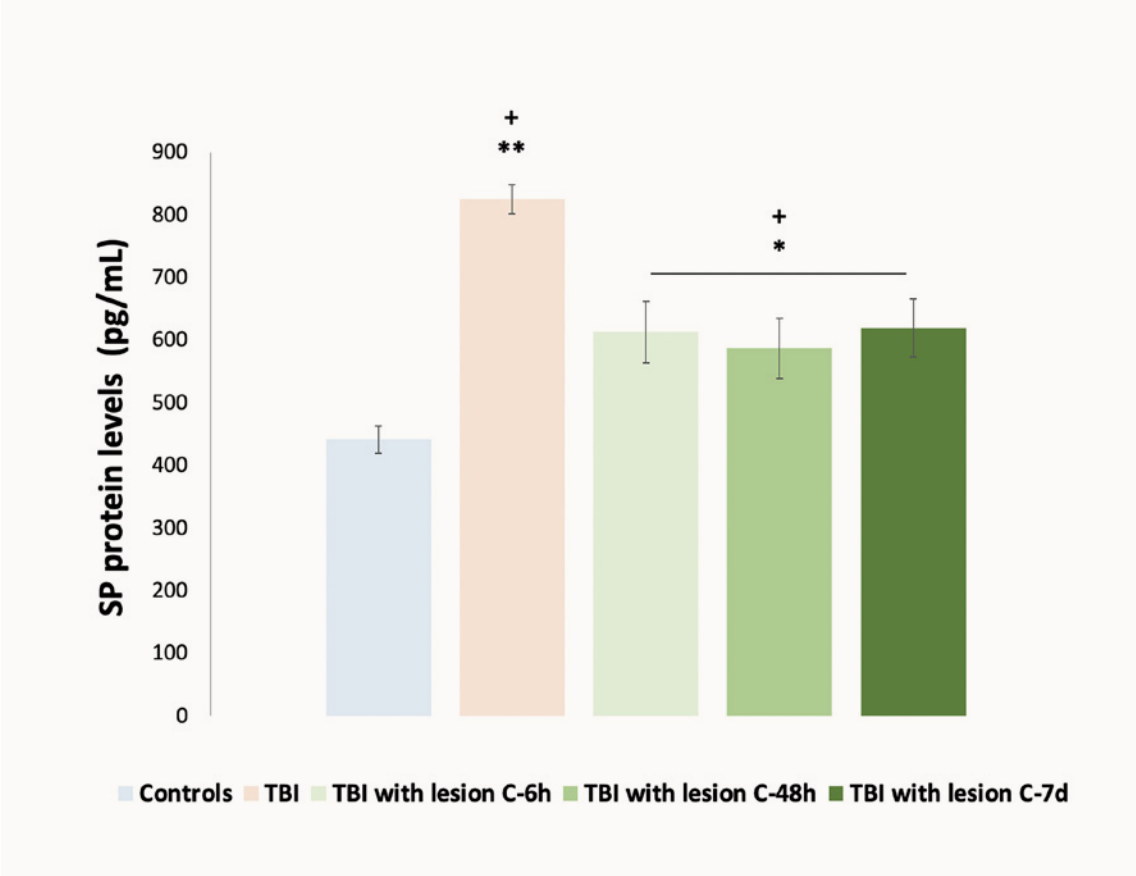


Figure 3.5 - Response to TBI concerning SP levels (pg/mL). TBI induces an increase in SP levels. *p<0.05; **p<0.01 significantly different from controls. $^{+}$p<0.05 significantly different from each other. Groups under the bar display similar statistical findings when compared to the TBI group and controls. **Legend**: C-6h, assessment at 6h post-TBI; C-48h, assessment at 48h post-TBI; C-7d, assessment at 7 days post-TBI; SP, substance P; TBI, traumatic brain injury.

Considering the subset of patients undergoing repeated sampling in group C-6h (blood sampling in the same patient at 6h, 48h and 7 days post-TBI), a similar trend in SP levels is present **(Figure 3.6)**: significantly increased levels within the first 6h, with SP levels declining in 48h and increasing again until 7 days following TBI. These differences in mean values did not reach statistical significance upon non-parametric Friedman Test for repeated measures: χ^2 - 0.5 (**α** - 0.05; dF - 2; χ^2 critical value - 5.99147).

The results obtained were as follows (n=16, pg/mL, mean ± SEM): **C-6h**, 618.548 ± 58.283; **C-48h**, 558.175 ± 59.988; **C-7d**, 616.595 ± 60.596 **(Figure 3.6)**.

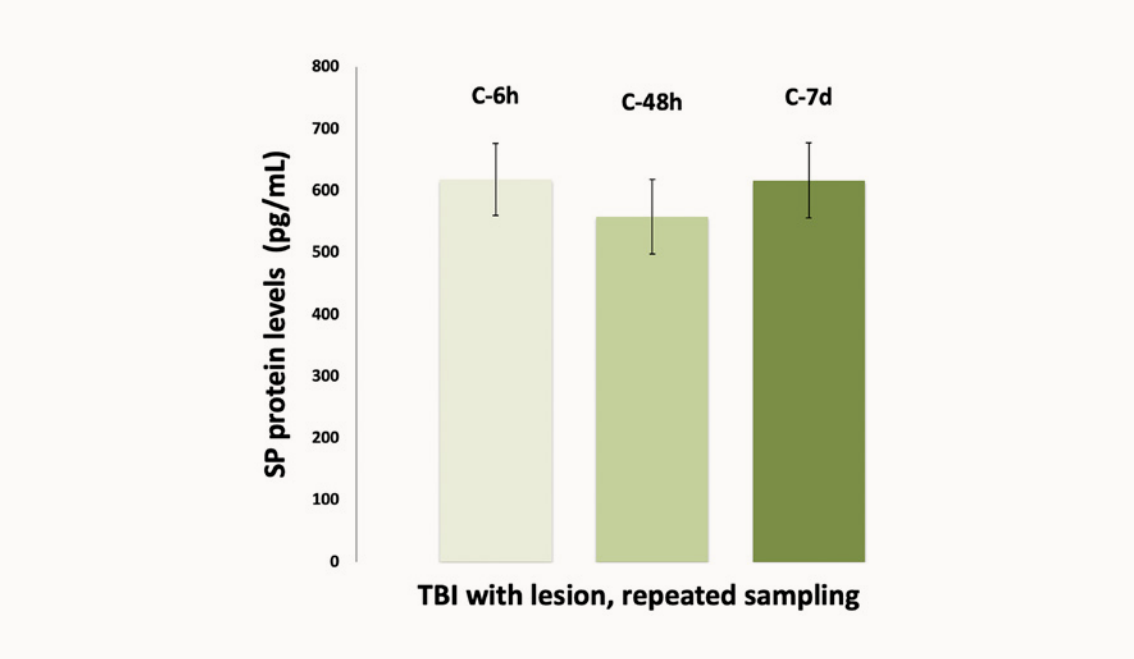


Figure 3.6 - Response to TBI concerning SP levels (pg/mL), repeated sampling in patients with haemorrhagic contusion. Legend: C-6h, assessment at 6h post-TBI; C-48h, assessment at 48h post-TBI; C-7d, assessment at 7 days post-TBI; SP, substance P; TBI, traumatic brain injury.

In summary, TBI induced an early and obvious increase in SP concentrations (even without parenchymal lesions) (at 6h post-TBI), with a decline in SP plasma levels in the following hours and an unexpected later increase (evident at 7 days).

No relevant findings were present when assessing SP levels in relation to the GCS score.

3.3.4 S100B

There was a noteworthy effect of TBI on S100B levels (pg/mL) among different groups - [$F(4, 95) = 4{,}959$, $p = .0011$], post hoc Tukey-Kramer method test **(Figure 3.7)**. In the presence of parenchymal lesion, a significant increase in S100B takes place in the first 6h post-TBI when compared to controls (C-6h vs. controls), followed by sustained progression to baseline values in the next 7 days. S100B is also significantly increased in the presence of post-traumatic brain parenchymal lesion when comparing to TBI with no visible lesion in CT scan (both at 6h) (C-6h vs. TBI).

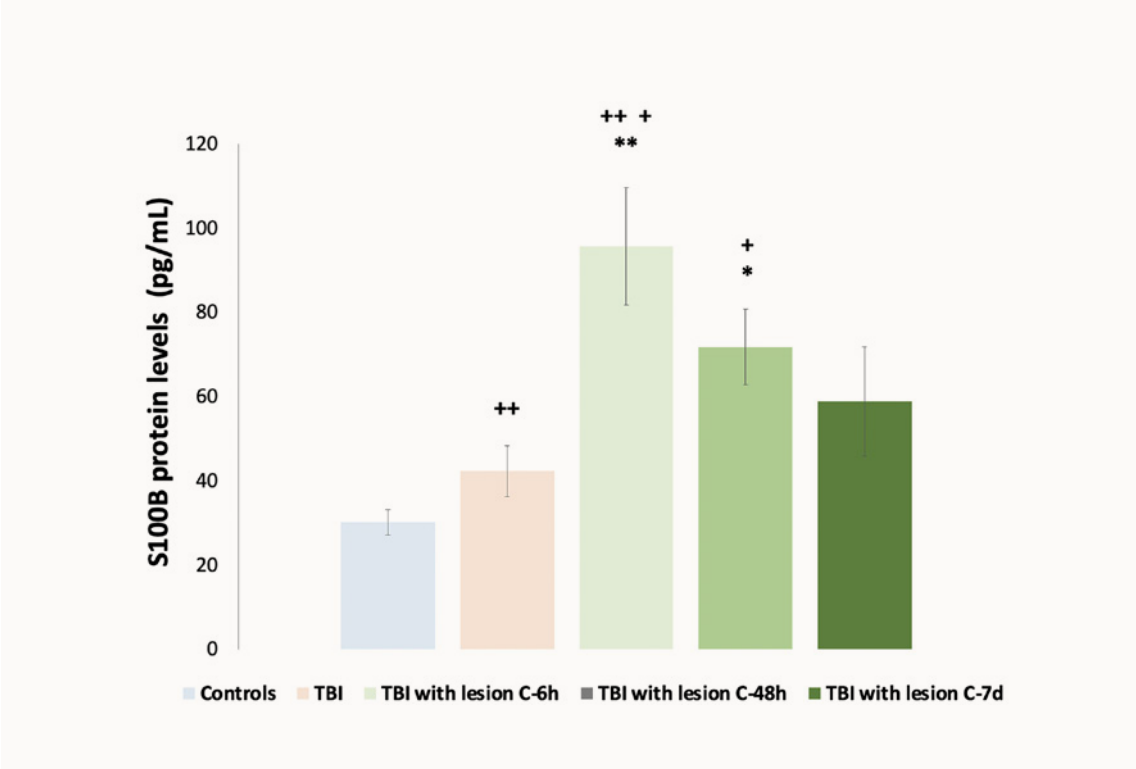


Figure 3.7 - Response to TBI concerning S100B levels (pg/mL). TBI induces an increase in S100B levels, declining with time. *p<0.05; **p<0.01 significantly different from controls. $^{+}$p<0.05; $^{++}$p<0.01 significantly different from each other. **Legend**: C-6h, assessment at 6h post-TBI; C-48h, assessment at 48h post-TBI; C-7d, assessment at 7 days post-TBI; TBI, traumatic brain injury.

Obtained results were (pg/mL, mean ± SEM): **Controls**, n=31, 30.187 ± 3.347; **TBI**, n=29, 42.303 ± 6.302; **C-6h**, n=22, 95.668 ± 14.102; **C-48h**, n=23, 71.778 ± 9.556; **C-7d**, n=22, 58.860 ± 13.708 **(Figure 3.7)**.

Considering the subset of patients undergoing repeated sampling in group C-6h (blood sampling in the same patient at 6h, 48h and 7 days post-TBI), a similar trend in S100B levels is present **(Figure 3.8)**: significantly increased levels within the first 6h, with progressive decline afterwards, as measured at 48h and 7 days post-TBI. These differences in mean values display statistical significance upon non-parametric Friedman Test for repeated measures: χ^2 - 10 (**α** - 0.05; dF - 2; χ^2 critical value - 5.99147).

The results were the following (n=15, pg/mL, mean ± SEM): **C-6h**, 155.106 ± 38.416; **C-48h**, 92.360 ± 14.864; **C-7d**, 36.961 ± 6.124 **(Figure 3.8)**.

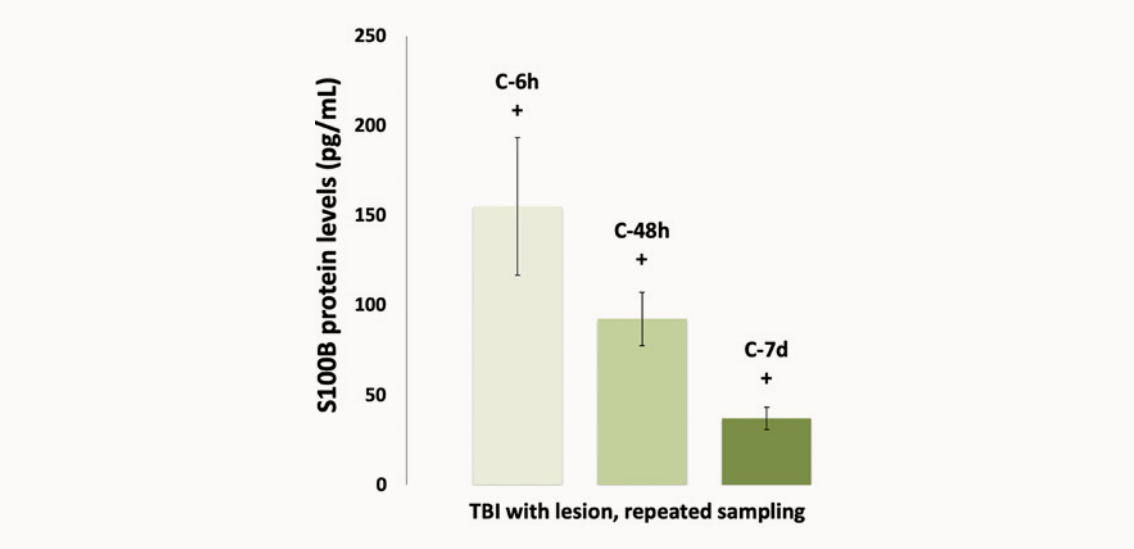


Figure 3.8 - Response to TBI concerning S100B levels (pg/mL), repeated sampling in patients with haemorrhagic contusion. Post-TBI S100B levels significantly decline with time. $^{+}p<0.05$ significantly different from each other. **Legend**: C-6h, sampling at 6h post-TBI; C-48h, sampling at 48h post-TBI; C-7d, sampling at 7 days post-TBI; TBI, traumatic brain injury.

In conclusion, TBI (with a parenchymal lesion) induced an obvious increase in S100B levels (at 6h post-TBI), followed by a progressive decline in its plasma concentrations in the following days.

No relevant findings were present when assessing S100B levels in relation to the GCS score.

3.3.5 Magnesium

There was a significant effect of TBI on circulating total Mg^{2+} levels (mmol/L) among different groups - [$F(4, 145) = 5{,}682$, $p < .001$], post hoc Tukey-Kramer method test **(Figure 3.9)**. A statistically significant decrease in Mg^{2+} levels is present when comparing TBI victims with a parenchymal lesion at 6h and controls (C-6h vs. controls) and when comparing different timings in all groups of TBI with a parenchymal lesion (C-6h vs. C-48h vs. group C-7d), with progressive recovery of Mg^{2+} levels following TBI. Average levels of Mg^{2+} are also visibly different when comparing TBI victims with and without parenchymal lesion (at 6h) (C-6h vs. TBI), with lower levels in the former. On average, all groups presented with Mg^{2+} levels were still above the clinically accepted threshold for hypomagnesemia (0.66 mmol/L).[787]

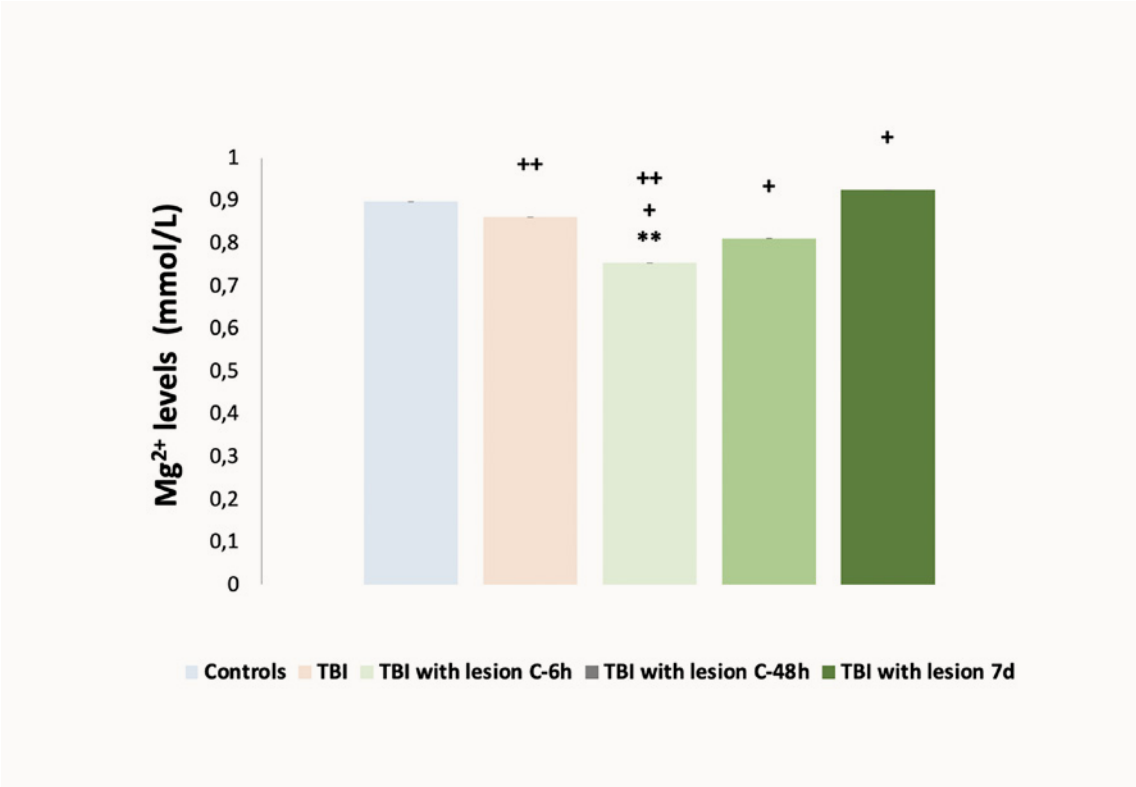


Figure 3.9 - Response to TBI concerning Mg^{2+} levels (mmol/L). TBI induces a decrease in Mg^{2+} levels, recovering with time. **p<0.01 significantly different from controls. $^{+}$p<0.05; $^{++}$p<0.01 significantly different from each other. **Legend**: C-6h, assessment at 6h post-TBI; C-48h, assessment at 48h post-TBI; C-7d, assessment at 7 days post-TBI; Mg^{2+}, magnesium ion; TBI, traumatic brain injury.

The results obtained were (mmol/L, mean ± SEM): **Controls**, n=35, 0.897 ± 0.021; **TBI**, n=29, 0.861 ± 0.039; **C-6h**, n=33, 0.754 ± 0.015; **C-48h**, n=34, 0.811 ± 0.019; **C-7d**, n=34, 0.925 ± 0.039 **(Figure 3.9).**

Considering the subset of patients undergoing repeated sampling in group C-6h (blood sampling in the same patient at 6h, 48h and 7 days post-TBI), a similar trend regarding Mg^{2+} is obvious **(Figure 3.10)**: significantly lower levels within the first 6h, with Mg^{2+} levels progressively increasing afterwards, as measured at 48h and 7 days following TBI. On one-way ANOVA test, there was a noteworthy effect of TBI on Mg^{2+} levels among different groups - [$F(2, 87) = 10.415$, $p < .001$], post hoc Tukey's method test.

Obtained results were as follows (n=22, mmol/L, mean ± SEM): **C-6h**, 0.754 ± 0.015; **C-48h**, 0.811 ± 0.019; **C-7d**, 0.924 ± 0.039 **(Figure 3.10)**.

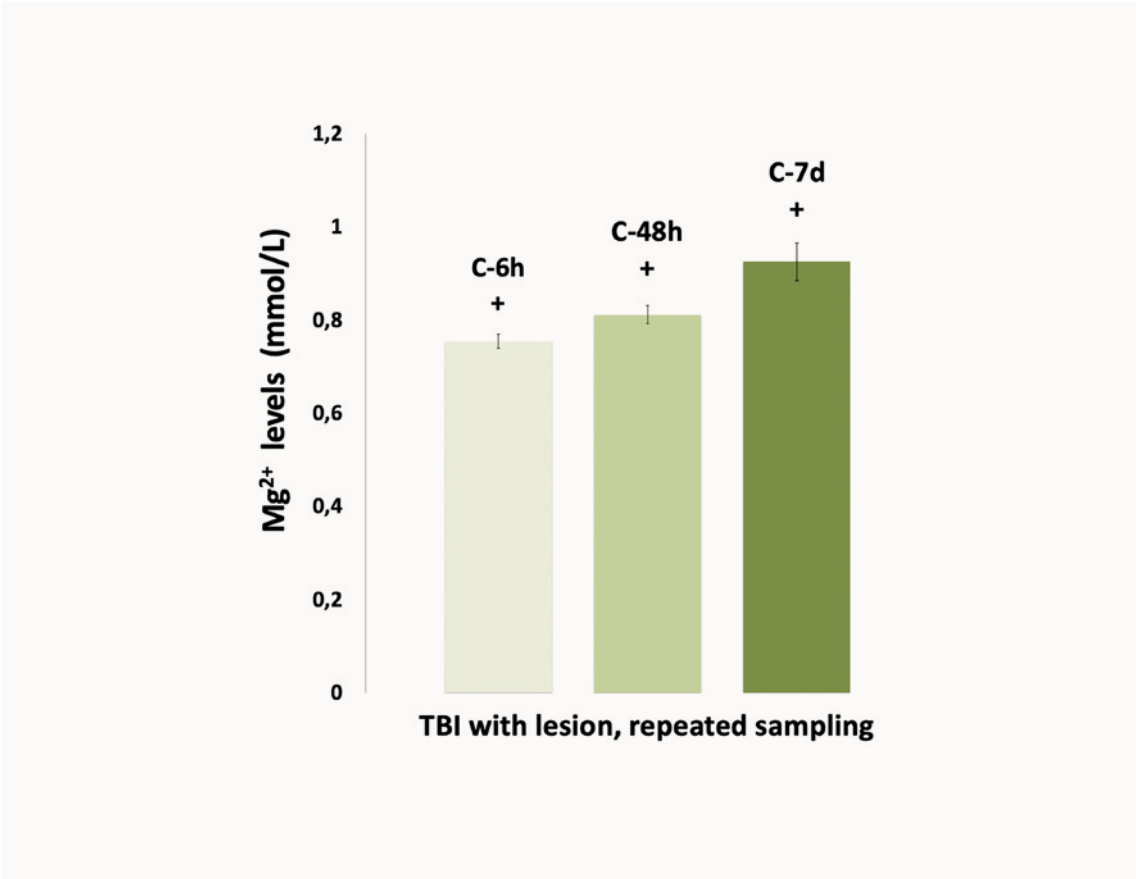


Figure 3.10 - Response to TBI concerning Mg^{2+} levels (mmol/L), repeated sampling in patients with haemorrhagic contusion. Post-TBI Mg^{2+} levels increase with time. $^{+}p<0.05$ significantly different from each other. **Legend**: C-6h, assessment at 6h post-TBI; C-48h, assessment at 48h post-TBI; C-7d, assessment at 7 days post-TBI; Mg^{2+}, magnesium ion; TBI, traumatic brain injury.

Briefly, TBI (with a parenchymal lesion) induced an early noticeable decrease in Mg^{2} plasma levels (at 6h post-TBI), followed by an increase in its concentrations in the following days.

No relevant findings were present when assessing Mg^{2} levels in relation to the GCS score.

Figure 3.11 summarizes our overall findings concerning NPY, SP, S100B and Mg^{2+}.

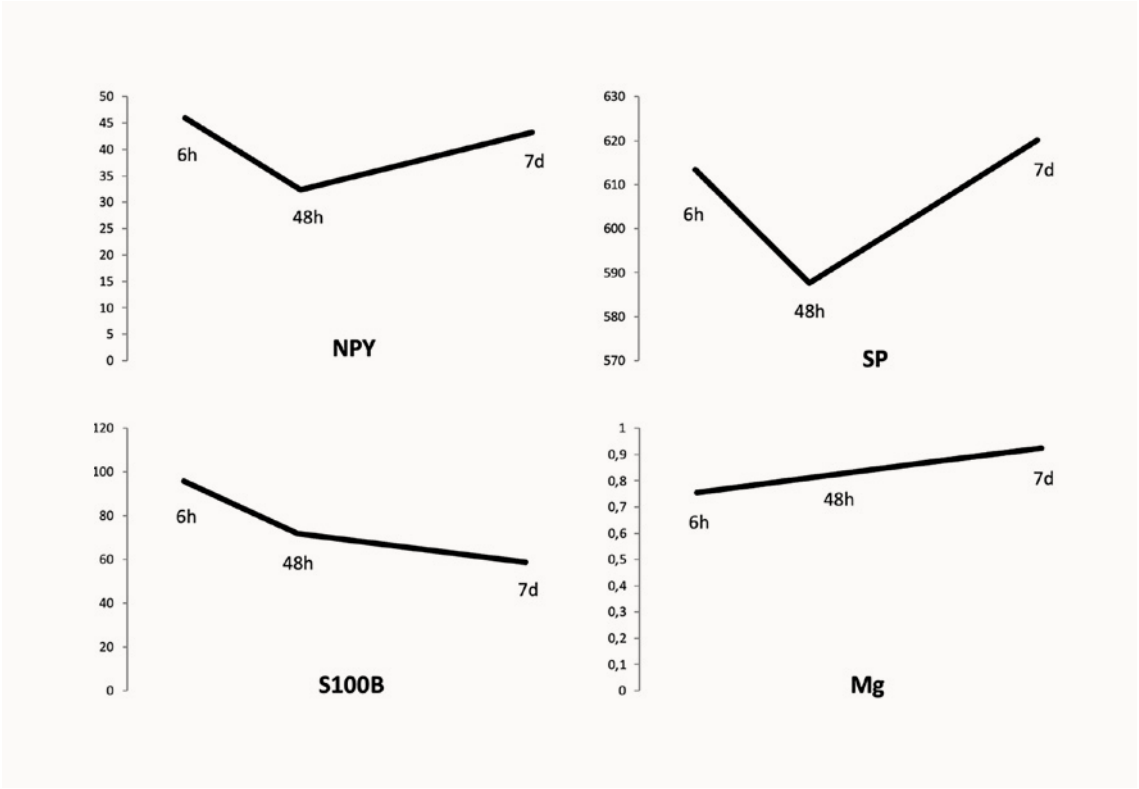


Figure 3.11 - Schematic representation of multistage response to TBI with haemorrhagic contusion, with different timings for each element involved. Legend: Mg, magnesium; NPY, Neuropeptide Y; SP, substance P; TBI, traumatic brain injury.

3.3.6 Calcium

There was an important effect of TBI on total serum Calcium ion (Ca^{2+}) levels (mg/dL) among different groups - [$F(4, 146) = 9{,}593$, $p < .001$], post hoc Tukey's method test **(Figure 3.12)**. A significant decrease in Ca^{2+} levels is obvious when comparing controls and TBI victims in all groups, with even lower Ca^{2+} levels in patients with a parenchymal lesion [hypocalcemia (Ca^{2+} < 8.8mg/dL) in all subgroups)].

The results, concerning calcium, were (n=35, mg/dL, mean ± SEM): **Controls**, 9.460 ± 0.063; **TBI**, 9.100 ± 0.102; **C-6h**, 8.730 ± 0.149; **C-48h**, 8.630 ± 0.098; **C-7d**, 8.710 ± 0.135 **(Figure 3.12)**.

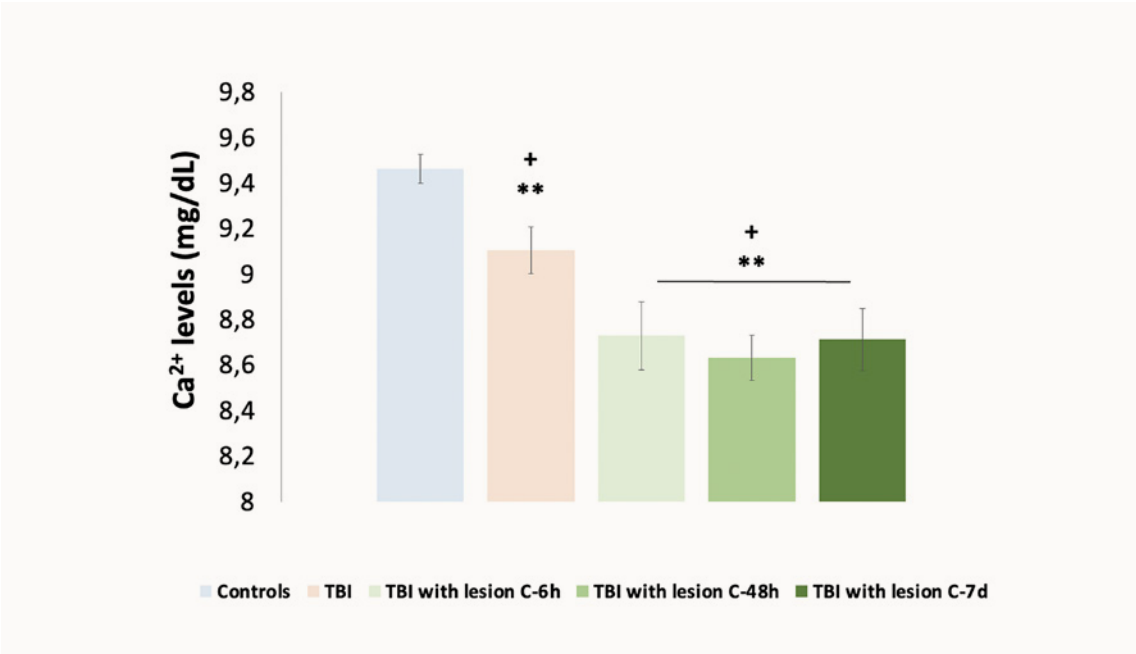


Figure 3.12 - Response to TBI concerning Ca^{2+} levels (mg/dL). Post-TBI Ca^{2+} levels are lower compared to controls. **p<0.01 significantly different from controls. $^{+}$p<0.05 significantly different from each other. Groups under the bar display similar statistical findings when compared to the TBI and controls group. **Legend**: Ca^{2+}, total serum calcium ion; C-6h, assessment at 6h post-TBI; C-48h, assessment at 48h post-TBI; C-7d, assessment at 7 days post-TBI; TBI, traumatic brain injury.

Considering the subset of patients undergoing repeated sampling in group C-6h (blood sampling in the same patient at 6h, 48h and 7 days post-TBI), no relevant findings were perceptible (n=23).

3.3.7 C-reactive protein

There was a significant effect of TBI on C-reactive protein (CRP) levels (mg/dL) - [F(4, 143) = 16,056, *p* <.001], post hoc Tukey's method test **(Figure 3.13)**. A significant increase occurs when comparing TBI victims with a parenchymal lesion at 48h post-TBI to the following groups: controls, TBI (with no parenchymal lesion), TBI with a parenchymal lesion at 6h post-TBI (C-48h vs. controls/TBI/C-6h). An overall post-traumatic increase in CRP levels was still noticeable at 7 days post-TBI.

Concerning CRP, obtained results were the following (n=35, mg/dL, mean ± SEM): **Controls**, 0.461 ± 0.244; **TBI**, 1.435 ± 0.518; **C-6h**, 1.674 ± 0.469; **C-48h**, 7.706 ± 1.106; **C-7d**, 6.348 ± 1.244 **(Figure 3.13)**.

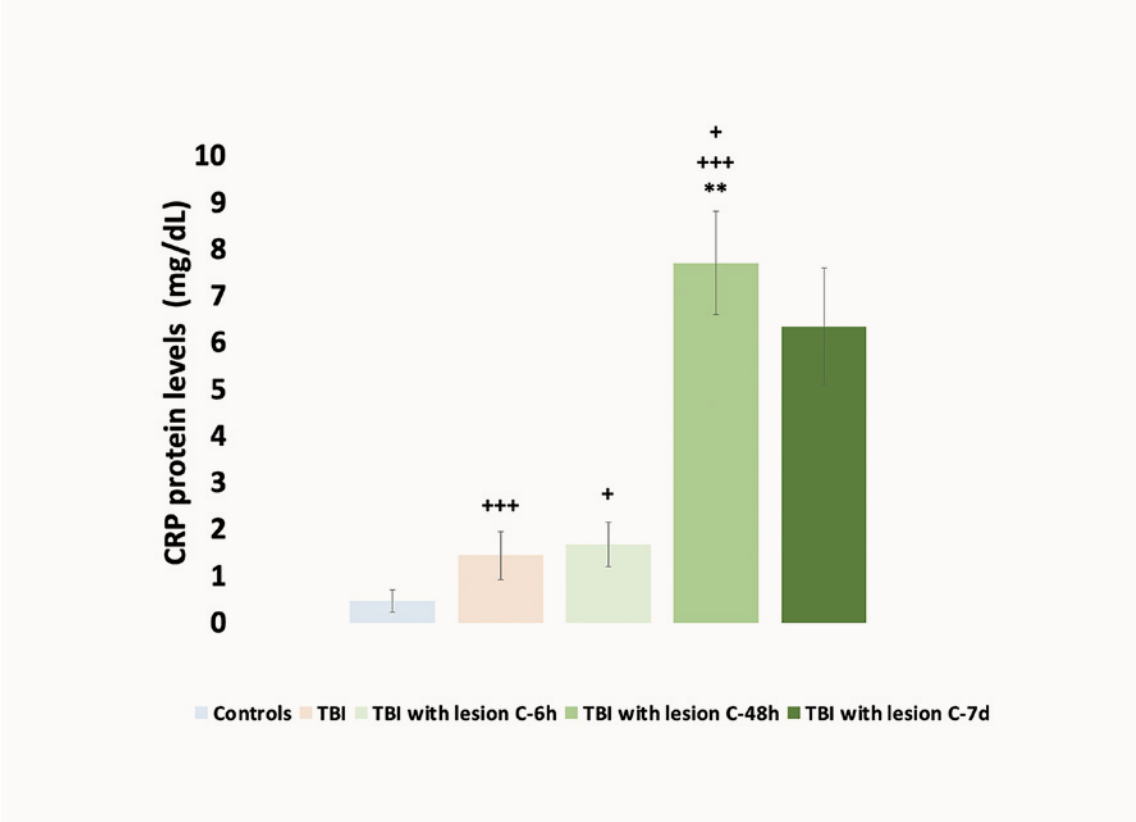


Figure 3.13 - Response to TBI concerning CRP levels (mg/dL). Post-TBI CRP levels are higher compared to controls, namely with haemorrhagic contusion at 48h post-TBI. **p<0.01 significantly different from controls. $^{+}$p<0.05, $^{+++}$p<0.001 significantly different from each other. **Legend**: CRP, C-reactive protein; C-6h, assessment at 6h post-TBI; C-48h, assessment at 48h post-TBI; C-7d, assessment at 7 days post-TBI; TBI, traumatic brain injury.

Considering the subset of patients undergoing repeated sampling in group C-6h (blood sampling in the same patient at 6h, 48h and 7 days post-TBI), a similar trend regarding CRP is present **(Figure 3.14)**, with obviously higher CRP levels at 48h post-TBI. An overall post-traumatic increase in CRP levels was still noticeable at 7 days post-TBI. These findings display statistical significance upon non-parametric Friedman Test for repeated measures: χ^2 - 10 (**α** - 0.05; dF - 2; χ^2 critical value - 5.99147).

Obtained results were as follows (n=23, mg/dL, mean ± SEM): **C-6h**, 1.628 ± 0.473; **C-48h**, 7.638 ± 1.098; **C-7d**, 5.983 ± 1.098 **(Figure 3.14)**.

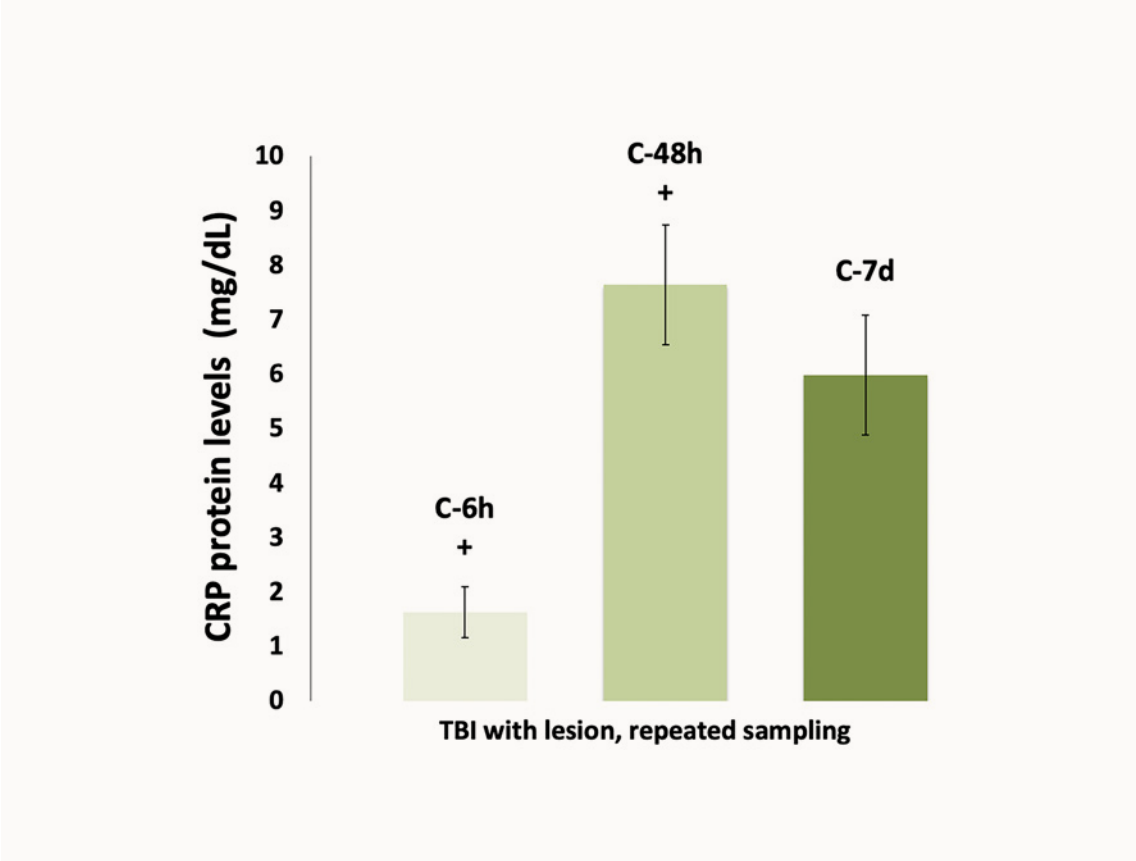


Figure 3.14 - Response to TBI concerning CRP levels (mg/dL), repeated sampling in patients with a parenchymal lesion. Post-TBI CRP levels are higher at 48h. $^{+}p<0.05$ significantly different from each other. **Legend**: CRP, C-reactive protein; C-6h, sampling at 6h post-TBI; C-48h, sampling at 48h post-TBI; C-7d, sampling at 7 days post-TBI; TBI, traumatic brain injury.

3.3.8 Clinical laboratory tests

No meaningful differences were detected among groups regarding mean values of Sodium, Potassium, Chloride and Osmolality **(Table 3.3)**.

Table 3.3 - Response to TBI regarding standard clinical laboratory tests. No relevant findings were present. **Legend**: C-6h, assessment at 6h post-TBI; C-48h, assessment at 48h post-TBI; C-7d, assessment at 7 days post-TBI; n, number of patients; SEM, standard error of the mean; TBI, traumatic brain injury.

	Sodium (mmol/L) (mean ± SEM) (n)	Potassium (mmol/L) (mean ± SEM) (n)	Chloride (mmol/L)(mean ± SEM) (n)	Osmolality (mOsm/kg) (mean ± SEM) (n)
Controls	140.066 ± 0.415 (n =35)	4.550 ± 0.354 (n =33)	105.193 ± 0.338 (n =35)	280.677 ± 0.983 (n =35)
TBI	138.566 ± 0.570 (n =35)	4.080 ± 0.454 (n =34)	105.500 ± 0.630 (n =35)	280.066 ± 1.168 (n =35)
Group C-6h	137.766 ± 0.682 (n =35)	4.060 ± 0.364 (n =28)	102.966 ± 0.552 (n =35)	281.833 ± 1.465 (n =35)
Group C-48h	139.200 ± 0.718 (n =35)	3.890 ± 0.454 (n =31)	103.933 ± 0.828 (n =35)	283.500 ± 2.204 (n =35)
Group C-7d	137.533 ± 0.816 (n =35)	3.940 ± 0.576 (n =31)	102.933 ± 0.854 (n =35)	282.000 ± 1.629 (n =34)

3.4 Discussion

3.4.1 Overview

Despite growing interest in long-term consequences of TBI,[788] several therapeutic protocols failed the test of facing modern evidence-based medicine. As discussed before, a main reason for this relative non-success is the persistent lack of knowledge regarding many aspects of TBI's complex mechanisms of response.

We hypothesized that TBI leads to a multistage neuropeptide response, with an immediate response concerning SP, followed by compensatory NPY upregulation. This response is divided, based on previous literature and according to our working model, into 3 different moments:

- An hyper acute response exacerbated by SP, as part of initial inflammatory response in the first hours following TBI, promoting cerebral vasogenic edema and inflammatory processes (as shown in the extensive literature on the subject);

- An acute response determined by excitotoxic phenomena, partially mediated by SP, and a peak in S100B levels as a sign of neuronal/glial disturbance and progressing inflammation;

- Finally, a delayed response with a predominant increase in NPY levels (and possibly others peptides) as a reinforcement to neuroprotective and regenerative pathways (as shown in our animal model of trauma), attenuating excitotoxicity and inflammatory phenomena, with ancillary progressive recovery in Mg^{2+} levels.

Therefore, it was important to confirm this supposed multistage neuropeptide response in actual human TBI victims.

As depicted in the Results section, evidence of an early and delayed neuropeptide response to TBI is relatively evident in human patients. These findings are in line with the perceived role for neuropeptides and neurogenic inflammation as key components of post-TBI inflammation, along with SP's role in many aspects of the classical inflammatory response (activation of microglia and astrocytes, leukocyte migration, degranulation of mast cells).[133] An initial increase in NPY was followed by an expected and significant decrease at 48h post-TBI (coinciding with usual timing for peak clinical deterioration and known deleterious secondary injury on a cellular level)[138, 160, 172] Unexpectedly, in contrary to what was delineated in our working model and mentioned in previous literature, SP levels at 7 days were again increased. The schematic representation displayed in **Figure 3.11** is solely based on the said 3 time points and is primarily a theoretical interpretation of our findings, considering that a straight line between two time points might not be the accurate representation of involved kinetics.

In order to better assess the relevance and adequacy of our working model, namely regarding NPY, this research protocol contemplated the possibility of comparing distinct patients among different groups (control, TBI with no parenchymal lesions, TBI with parenchymal lesions in different timings) and, on a specific sub-group of patients with post-TBI haemorrhagic contusions, compare the results obtained in the same patient upon different timings. Similar trends in NPY fluctuating levels were confirmed in both contexts, although an insufficient number of patients in the paired-samples (repeated measurements) analysis might have prevented it from reaching statistical significance. This, in our opinion, reinforces our conclusions, as the statistical relevance of comparing specific, separate groups of unrelated patients is reinforced by the notion of biological continuity in the individual, considering the similar objective response in the subset of patients assessed upon different timings.

3.4.2 Study design and timings

This staged neuropeptide response is also in agreement to well-known timings in brain injury and its biomarkers, with well-described post-traumatic hypomagnesemia and S100B levels peaking in the first 48h post-TBI and subsequently normalizing (assuming stabilization of the clinical picture and no sustained progression in traumatic lesions).[789]

The decision on different timings for blood sampling was based on clinical grounds and previous research. An initial assessment at 6h or less following TBI is realistic, concerning previously described primary injury and earlier secondary injury phenomena (see Chapter I) and usual time course in this type of patients, considering pre-hospital and hospital management. Dividing obtained data into subgroups (e.g., 30min vs. 2h vs. 6h) should provide additional useful information concerning rapid fluctuations in SP levels. Given this, it was decided to maintain the 6h threshold, again based on clinical reasons: as in most tertiary hospitals, many patients arrive at the Emergency Department several hours after initial trauma; as all doctors know, patient and family reports are frequently unreliable concerning timeline; it would be relatively impractical to repeatedly collect blood samples in a trauma patient in such a narrow time frame. Significantly, our data indeed display significant changes at 6h post-TBI, assuming an eventual underrepresentation of specific timing-dependent phenomena.

Post-traumatic edema peaks around the 3rd day post-TBI, with conflicting reports on the actual contribution of vasogenic and cytotoxic edema (see Chapter I).[304] Several pathological phenomena have been mentioned to peak at 48h, including a decline in cellular groups of up to 60% following increased cell death by apoptosis,[132, 160] and immune system activation and recruitment.[138, 201, 347] Considering all this, a 48h time point seems suitable for an adequate, comprehensive assessment of most pathological phenomena, on their peak or close to it upon this specific timing. This is in line with many research and clinical protocols and biomarkers profiling assays, using time points of 6h and 48h for an early assessment,[3, 522, 555] as it seems an appropriate threshold, inevitably arbitrary to some extent, concerning biological phenomena and real-life clinical scenarios.

A 7-days time point provides, in our opinion, a good long-term notion, when secondary injury phenomena are still present but are already receding in intensity and reach. The 7-days threshold seems to be a clinically adequate time period for assessment, considering the clinical perception that most patients, at this time, while not fully recovered, will be stable and the acute lesions should be progressing to their chronic state or resolution (stable peri-lesional edema, blood resorption).[790] In animal models of trauma, most relevant phenomena

(BBB's impairment, cerebral edema, ICP) are shown to be largely normalized or stabilized within 7 days following moderate TBI.[328]

Equally interesting would be to assess the same variables on a longer period of time following TBI (e.g., 4 weeks post-TBI). This would eventually complement our findings and shed new light on specific issues, arguably differentiating NPY and SP's dynamics in the long term, as the latter demonstrated an unexpected rise in its levels in the 7-days assessment (as previously discussed).

The decision on not taking additional blood samples in the TBI group (no haemorrhagic contusion) (namely at 48h or 7 days following TBI) was based mainly on clinical reasons, considering the nature of this study, the early discharges (as no traumatic findings were present) and the unnecessary blood sampling. Future studies might confirm and further explore our relevant findings in this specific subgroup.

Concerning the exclusion criteria, the intention was to rule out any possible source of interference with neuropeptides and biomarkers under scrutiny, their levels and mechanisms of action: medication capable of interfering with known TBI pathophysiology, past medical conditions, other potential confounding factors or sources of bias (including polytrauma patients with non-CNS trauma injuries or non-traumatic CNS conditions). Special care was taken to eliminate, as much as possible, a likely interference from alcohol dependence and acute alcoholic intoxication. These two contexts, unfortunately so common in the Portuguese society, would most likely interfere with our multimodal study in different ways: alcoholic intoxication will certainly interfere with neurological status assessment; alcohol chronic abuse interferes, in more advanced stages, with hepatic and renal metabolism (with all sort of implications concerning protein synthesis, metabolism and renal excretion); NPY and its receptors are known to play a significant role in drug and alcohol abuse disorders.[791]

3.4.3 Neuropeptide Y

Although other neurotransmitters are likely to be involved in post-traumatic neurogenic inflammation, namely CGRP (which might potentiate SP's action),[133] this research project focused on SP and NPY, both ubiquitous and potent neuropeptides.[792]

Given all evidence pointing to a neuroprotective action of NPY in different contexts, including the described findings in our animal model, it is plausible to consider a vital role for NPY in the brain's response to TBI. Not surprisingly, brain NPY levels and function are reduced in the elderly,[793] a significantly vulnerable group to TBI, both in its incidence and biological consequences. This age-related

NPY decay, although normal and expected, will eventually preclude an optimal neuroprotective response, underlining the importance and feasibility of artificially potentiating this response. Therefore, reinforcing NPY's role in TBI is a potential therapeutic strategy, possibly along with SP's modulation, considering previously mentioned NPY's pro-neurogenic, pro-migratory and neuroprotective properties.[198, 424] As previously discussed, NPY is known to act as an antagonist to SP's activity (and other neuropeptides), inhibiting SP's release with anti-hyperalgesic effect via Y1 receptor signalling in the dorsal horn.[794] NPY supplementation protocols (namely by intranasal delivery) are included in phase II and phase III clinical trials concerning other clinical contexts.[458, 795]

Significantly, our protocol clearly demonstrates an initial post-traumatic increase in NPY, followed by an expected and significant decrease at 48h post-TBI (coinciding with peak acute deleterious response). As secondary injury subsides and widespread phenomena start to settle down (as shown, for example, in declining S100B levels at 7 days), NPY levels climb into substantially higher values. According to our view and working model, this can be interpreted as part of a broader, encompassing neuroprotective response.

Even so, unexpected findings concerning early increase in NPY should be clearly assessed. Followed by an apparent decrease in the acute phase (48h), an early rise in NPY levels (in the first few hours) might represent a possible initial neuroprotective mechanism, unsuccessful in its intent and clearly overwhelmed by ensuing excitotoxic events. Later, as neurogenic inflammation and other deleterious events settle down and regenerative/recovery processes start, NPY would reassume its role as a neuroprotective and restorative agent, which would explain its increment at 7 days post-TBI. For this reason, considering all positive effects of early NPY supplementation as described in our research protocol involving animals, a clearly beneficial effect should come from artificially upregulating and prolonging NPY's action through the more acute noxious stages.

Assessment of NPY levels according to initial GCS scores did not provide any additional information, a fact most likely related to significant clinical variability upon initial presentation and small-sized groups. Future research can and should focus on clinical variability and outcomes in relation to neuropeptide response.

NPY's role in the gut-brain axis, acting both as a neural and an endocrine messenger (along with other neuropeptides), is another poorly understood context, although undoubtedly relevant in the context of known post-traumatic gastrointestinal dysfunction.[796] The gut-brain axis, a network connecting the central and enteric nervous systems, functions upon bidirectional pathways and feedback mechanisms and much remains to be elucidated.[797]

3.4.4 Substance P

SP levels in the TBI group with no intracranial lesions visible on CT scans are increased when compared to controls, an interesting finding that reinforces the relevance of TBI-related deleterious phenomena even in often overlooked CT-negative patients.[529] This fact is in line with reports of clinical symptoms and biochemical deregulation in minor TBI CT-negative patients, with all its implications if one considers the typical dismissal of these patients´ complaints, namely when assessing long-term impairment.[529, 798]

Somewhat counterintuitively, SP levels are also further increased in the TBI group compared to group C-6h, in which brain parenchymal lesions are present. This fact, never reported before, can be explained if considering a scenario in which inflammatory pathways upon TBI are surpassed by more relevant and disrupting events, including cell death and haemorrhages, in the context of direct brain injury with significant parenchymal damage. This would preclude the expected inflammatory response, somewhat dependent on more intact underlying brain structures.

Considering all groups C, an expected decrease at 48h was followed by an unexpected increase in SP levels at 7 days, unlike the typical pattern described in the literature.[362] It is important to mention that studies reporting post-traumatic SP increased levels are mostly focused on the first hours (namely 24h) following TBI,[425] despite sporadic reports mentioning increased SP mRNA levels, as determined by PCR analysis, lasting for at least 3 days.[799] In regard to this late resurgence in SP levels in our study, as verified at 7 days post-TBI sampling, this unexpected finding is not aligned with what most authors describe concerning SP's behaviour in TBI. This late but sustained increase in SP, never reported before, is difficult to frame in the expected profile of acute inflammation, with early deployment and progressive attenuation. A possible explanation for this may lie in the fact that, as mentioned when trying to explain the also unexpected higher SP levels in the TBI group (without parenchymal lesions) in relation to group C-6h, inflammatory pathways might be transiently surpassed by more relevant and disrupting events, including cell death, excitotoxicity and haemorrhages. A more chronic neuroinflammation status might eventually ensue upon attenuation of hyperacute phenomena, as mentioned in works describing chronic microglial activation in a more prolonged mild neuroinflammation status (see Discussion in Chapter II, Microglia section). A more straightforward interpretation of these findings would be to consider this late increase in SP as part of a late-onset second peak in post-traumatic inflammation and overall secondary injury - a scenario contradicted by S100B profile. This late increase in SP is of uncertain significance and, if confirmed in future studies, its nature and purpose must be better elucidated.

3.4.5 S100B

As the most common and well-studied biomarker for TBI,[3] S100B was a suitable adjunct reference element in assessing the intrinsic adequacy of our protocol and, simultaneously, the distinct dynamics and timings in neuropeptide response. S100B is a comprehensive biomarker, allowing mild TBI patients screening and the need for CT scan, detecting lesion progression and secondary injury development and evaluating treatment efficacy while being able to predict outcome in moderate and severe TBI.[3] It is sensitive enough to detect different intracranial lesions, from cerebral contusions to subdural and epidural haematomas.[800] Importantly, S100B appears to be more responsive to focal than diffuse injuries, especially in the presence of brain contusions, which present a direct correlation in their volume to S100B levels.[516, 801] Thelin and team[3] summarize all extrapolations to be made from these findings in two crucial notions concerning S100B (and biomarkers in general) and TBI: the amount of injured tissue is more important than specific spatial location when assessing brain injury; combining biomarkers assessment with imaging is mandatory to have a proper perception of inflicted damage.[3] In designing this study, these two notions were considered and deemed extremely relevant. Correlating imaging with biological markers was considered crucial and the presence of parenchymal lesions in TBI was defined as a group-defining independent variable. As the presence of parenchymal lesions was a defining variable, its specific anatomical location was considered non-relevant - as mentioned and discussed previously, TBI and secondary injury is a diffuse, whole-brain phenomenon. Measuring the volume of post-traumatic parenchymal lesions (or even extra-axial lesions) and correlating it with biological elements as NPY and others, not contemplated in this study, is arguably of interest and thus represents another line of research to further explore.

Our findings concerning S100B are well correlated with the profile usually described in the literature, with a noticeable increase in the first few hours following TBI and a sustained decrease to basal values in the following days. In our findings, and unlike SP, S100B levels are clearly related not only to TBI but specifically to the presence of parenchymal lesions (S100B levels are increased in group C-6h compared to controls and TBI patients with no parenchymal lesion). This finding is in line with the notion of S100B being a specific and reliable biomarker, derived from astrocyte injury and correlated to the amount of injured brain tissue.

As displayed in **Figure 1.15**, initial S100B release in polytrauma patients is most likely originated from extracranial tissue, with a rapid wash-out in the first few hours following injury. Known extracerebral sources of S100B include adipocytes, Langerhans cells, chondrocytes, epithelial cells, cardiac and skel-

etal muscle cells.[3, 802] Concerning polytrauma patients, with potential multiple injuries to the limbs, thorax and internal organs, elevated S100B concentrations have been shown in the absence of CNS injury,[803] displaying an apparent faster wash-out compared to TBI-derived S100B increased levels.[518, 804] Cerebral release, more prolonged in time, is usually masked in the beginning by extracerebral contributions. In controlled experimental conditions, an extracranial contribution is negligible. In carefully monitored patients with no other lesions and rigorous kinetic mapping, S100B peak was documented around 27h post-TBI.[789] Given this, our results indeed confirm a peak before 48h, but we have no means, with the available data, to determine if the actual peak was prior, at or after 6h.

3.4.6 Magnesium

Our findings concerning hypomagnesemia in TBI were in line with previous reports.[488, 805] When compared to controls, TBI victims (even without parenchymal lesions) display significantly lower levels of seric Mg^{2+}. Of interest, several reports mention the impact of hypomagnesemia in long-term outcome (up to 6-months).[617, 805]

One can mention several theories explaining post-traumatic hypomagnesemia: a possible syndrome of inappropriate secretion of antidiuretic hormone (contradicted by the absence of other ion abnormalities)[488]; enhanced renal excretion of Mg^{2+} (highly unlikely given its rapid onset); adrenergic control of plasma Mg^{2+} levels, with adrenaline-induced mediation of hypomagnesemia. [488, 806]

Immediate post-traumatic hypomagnesemia, although of interest from a therapeutic perspective,[805] will hardly contribute as a clinically valid biomarker guiding therapeutic protocols in TBI. However, when considering all described properties of Mg^{2+} concerning brain trauma and secondary injury, one can easily assess the relevance of post-traumatic ionic imbalance.[491, 805] Physiological extracellular Mg^{2+} concentrations modulate glutamate release inhibition,[807] restore BBB integrity, theoretically decrease brain edema to a certain degree[808] and non-competitively antagonize NMDA receptor activation via blockage of voltage-dependent calcium channels. In brain injury models, intracellular Mg^{2+} has been linked to changes in cerebral energy metabolism and inhibition of mitochondrial function.[809] Finally, early reports show Mg^{2+}'s competition with calcium at voltage-gated calcium channels, impeding calcium influx into ischemic neurons and preventing a recognized final common pathway for cell death.[810]

Our results display significantly lower levels of Mg^{2+} specifically in the group of patients with relevant lesions. Lower Mg^{2+} levels in the TBI group with no parenchymal lesions were also evident, although significantly lesser compared to group C-6h. As discussed concerning SP and NPY, this fact again stresses the relevance of secondary injury (although with no obvious macroscopic primary damage) in CT-negative patients. These patients are discharged home, many times with no specialist referral and presenting with a myriad of symptoms (many times underdiagnosed or underappreciated), with no specific treatment or therapeutic protocols and usually in need of returning to their professional activities. These patients, with minor or even no obvious macroscopic parenchymal lesion, develop increased urinary excretion of Mg^{2+}, possibly following increased lipolysis (in the context of stress-induced catecholamine surge) and free fatty acids binding.[811]

Mendez et al.[488] have shown a decrease in total Mg^{2+} in mild to severe TBI, irrespective of the presence of parenchymatous lesions, which is in agreement with what we describe in our data. Interestingly, the same team has described a short-lived increase (24h) in ionized Mg^{2+}, limited to severe head injury patients.[488]

Several other confounding factors can eventually explain baseline hypomagnesemia in specific TBI patients. Inadequate dietary intake is a possible and common cause for hypomagnesemia,[812] albeit almost impossible to accurately exclude in this clinical setting. But, if we consider the trend for Mg^{2+} levels recovery in TBI groups in our protocol, inadequate Mg^{2+} intake should not significantly affect our observations. Other pathological contexts for hypomagnesemia (gastrointestinal disorders, renal impairment, electrolytes abnormalities, chronic alcohol abuse, sepsis),[787, 813] would constitute an excluding criteria and, therefore, are not a confounding factor in our data.

3.4.7 Other findings

Although of undeniable clinical relevance, other findings are somewhat expected and deemed not relevant in this context. An obvious post-traumatic hypocalcemia is present, most likely in relation to well-described iatrogenic hemodilution mechanisms,[814] as expected in patients subjected to Neurointensive Care protocols, frequently associated with aggressive fluid management. Other causes for hypocalcemia in trauma patients, including severe shock or ischemia-reperfusion mechanisms,[815] are not in place in this context, considering our initial exclusion criteria.

Significantly higher CRP levels were also present in TBI patients. Despite several research teams exploring the possibility of using CRP as a biomarker for TBI,[816] this possibility, beyond the scope of this work, does not seem valid or useful, given CRP's heterogeneity, clinical ubiquity and lack of specificity in complex patients as in polytrauma.[817]

3.4.8 Limitations and future directions

Some issues can be raised concerning this research protocol. First, CT scans were initially classified by 6 experienced radiologists. Although a possible source of bias, it is unlikely that significant errors may arise from a simple assessment on having or not brain contusions, an objective and rather obvious finding in scans. The presence of intraparenchymal lesions was the only variable and the number or volume of those lesions were not considered for this study. Even if the distinction between intraparenchymal contusion and haematoma is sometimes rather unclear (depending on the amount of intervening brain tissue and necrosis),[818] when in the presence of a single obvious haematoma, the patient was excluded from the study, for diagnostic clarity and data coherence. Variations in size and severity of those lesions might influence neuropeptide response. Still, we intended to demonstrate an encompassing phenomenon, regardless of severity and distribution, considering the diffuse nature of post-TBI changes. If deemed useful, future studies can easily correlate the degree of post-traumatic neuropeptide response to findings in imaging studies, as the latter can be objectively described using standardized scales.[819, 820]

Likewise, it was not our intention to assess a possible relation between neuropeptide response, clinical status and outcome. Assessment of NPY levels according to initial GCS scores did not provide any additional information, a fact most likely related to significant clinical variability upon initial presentation and small-sized groups. This research project was not designed for pure clinical evaluation of neurological status (as measured by GCS score, for example), clinical outcome (e.g., assessed by GOS score) or even qualification/quantification of a new disease biomarker. Instead, it focused on documenting a so-far not described specific time-dependent phenomenon involving different neuropeptide elements in a broader response. As such, specific data that could be relevant for more clinically-oriented reasoning is missing or non-existent. It is undeniable that, when considering our overall results, a more patient-oriented clinical study or trial is not only reasonable but also potentially enriching, as long as a more broad clinical research protocol is developed to cope with intrinsic variability and uncertainty in patients' profile and response. Possibly, the contribution of imaging studies and their different modalities might also be reinforced.

Many studies actually do not report GCS as a parameter upon TBI clinical evolvement. Although of irrefutable clinical value, it is flawed, prone to errors, underestimates several phenomena and it does not correlate well with intrinsic neurological damage pathophysiology.[821] An explicit limitation is that those in the severe TBI group will require, as defined by current clinical protocols, intubation and sedation, preventing any direct rigorous assessment of neurological status. Unknown or underappreciated alcohol intoxication might as well be a confounding factor. For all these reasons, the GCS score is a helpful but not unquestionable tool in brain trauma research.[822] Perhaps more suitable than using similar alternatives to the GCS score, namely when assessing therapeutic efficacy, would be to shift our focus to more objectively employ GOS score or other long-term assessment tools on performance.[823]

As previously mentioned, none of the measured proteins is brain-specific. Given this, as the traumatic event is well identified and time-specific, any significant changes in a specific protein should be a direct consequence of TBI. Possible confounding factors and events have been thoroughly assessed and we believe that previously mentioned exclusion criteria have prevented the most relevant ones from interfering with our findings. Older and pediatric patients were also excluded from this study, halting extreme responses from specific biological contexts, from a diminished response in elderly patients to extreme variability in biomarkers' normal range and pathological response in pediatric populations (e.g., extreme variation of S100B reference levels in children and teenagers).[519, 824]

Another undeniable source of interference with the validity of these results would be non-related concomitant traumatic lesions, not only those affecting distant organs and systems but also facial trauma, cranial fractures or significant scalp lacerations. All these lesions have been considered objective exclusion criteria and were ruled out in primary and secondary trauma surveys.

Reported variability of SP and NPY serum and plasma levels is another possible bias.[825] Distinct sample preparation, qualitative differences in reagents, diverse analytical methods and SP's plasma/serum free and bound states could lead to wrong estimates.[825] Besides intrinsic difficulties in dealing with complex patients and environments (e.g., lost or damaged samples), there was considerable difficulty obtaining valid results concerning NPY, SP and S100B, with missed samples and outliers explaining most discrepancies in groups size. As some issues with outliers were present, a tendency to spurious results should also be kept in mind.

Another significant issue is, without any doubt, the degree of parallelism between the levels of a particular biomarker in traumatized brain tissue and its levels in peripheral blood. If that correspondence does exist, how do cytosolic pro-

teins released from injured brain tissue, such as the well-studied S100B, reach peripheral blood? First of all and most significantly, studies show that CSF: serum ratio concerning S100B is a coherent reflection of post-traumatic changes, even in early stages of TBI.[3, 826] Previous studies have also confirmed that, for example, serum levels of glutamate are positively correlated with CSF's glutamate levels.[249] This notion, reinforcing the intrinsic validity of measuring biomarkers for neurotrauma in peripheral blood, raises again the possibility of S100B being directly released from CSF to serum (eventually through arachnoid villi).[3] As post-traumatic BBB disruption ensues, easily quantified by albumin CSF:serum ratio,[827] some authors speculate on S100B release into the serum being another consequence of BBB impairment.[828] However, besides intrinsic flaws in these research protocols, all attempts have failed in showing a significant and reliable correlation between disrupted BBB and peak serum levels of S100B.[829, 830]

Interesting studies with murine TBI models mention a possible role for glymphatic pathways, transporting molecules into the bloodstream, namely via the cervical lymphatic system, and driving the removal of different elements (albumin, amyloid, paramagnetic contrast agents) from the brain's interstitial space.[148, 242] The glymphatic system, involving para-arterial influx, interstitial fluid, venous outflow and CSF, is reliant on the connection between glial cells and AQP4.[3, 685] The proposed mechanism is based on CSF's access to brain parenchyma via peri-arterial space, partially driven by arterial pulsatility and interacting with interstitial fluid, with the latter then recirculating into CSF or draining either into arachnoid granulations or along myelin sheaths of cranial nerves into perineural lymphatic structures.[685, 831] Glymphatic system has been suggested to play a significant role in biomarkers extravasation, possibly explaining the previously mentioned mismatch between BBB integrity/disruption and biomarkers levels.[3, 242] Specifically in TBI, several factors might affect glymphatic system clearance rates, from post-traumatic AQP4-containing podocytes depolarization in perivascular astrocytes[685, 832] to therapeutic sedation and CSF drainage.[242, 833] Glymphatic system's role in TBI is still to be elucidated and much research is still needed, considering the difficulty of developing reliable *in vivo* monitoring tools. One problem with this model is that, while seric S100B is increased within minutes of the initial trauma,[834] glymphatic system's activity appears to be of much later onset.[242, 831]

All these considerations concerning biomarkers and their transport across CNS structures, despite being more focused on classical biomarkers such as S100B, are also applicable to other molecules. Considering all evidence, classical biomarkers, such as S100B, and other elements, namely NPY, most likely reach the serum via more than one mechanism, with different rates and speeds (slower glymphatic system vs. faster trans-BBB route) and depending on many other factors.[834]

Although more specific in its purpose and possible gains, an arguably interesting line of research would be to assess the impact of surgery (craniotomy, decompressive craniectomy, haemorrhage drainage) in serum and CSF levels of these elements (namely SP, NPY and S100B). Possible findings from this hypothetical study would undoubtedly be valuable and contribute to the body of knowledge in the field. Even so, our intention was to demonstrate this phenomenon in its "pure state", with the least confounding factors possible and despite an unavoidable interindividual variability. Careful selection of sample preparation and choice of analytical methods may attenuate variability reported in the literature, namely in what concerns SP quantification.[825]

Another significant issue of the present study is the option of designating, *ab initio*, brain haemorrhagic contusions as the only finding defining "CT-positive" patients to be enrolled in the study. Other relevant findings, namely frequent subdural and epidural haematomas, were not included in the study and were considered criteria for exclusion. These lesions, although frequently synchronous with brain contusions and haematomas, are very different in their nature: extra-axial lesions causing sudden rises in ICP, pressure gradients and brain damage due to extrinsic compression (although causing significant primary brain damage).[5, 120] These lesions are indeed related to changes in known biomarkers, namely subdural haematomas and S100B.[835] Even so, their mechanisms of action and intrinsic nature would make it a mistake, in our opinion, to group different pathologies in this kind of study. This reasoning is even more evident concerning skull fractures, quite different (in every aspect) from intrinsic brain injury, although capable of interfering with brain biomarkers expression, namely S100B (see Biomarkers section). Again, although not the intention of the present study, future research might as well focus on this specific topic.

A frequently raised question in clinical studies, of them taking place in only one clinical center and whether this is beneficial or not, does not strictly apply in this case, as the study is merely descriptive and not an interventional one. Importantly, being a prospective study is undoubtedly advantageous, allowing better control of all variables and exclusion criteria.

As mentioned in Chapter II (General Discussion section), the human brain shares many similarities with rodents, bearing significant structural and functional parallelism. Studying these systems in mice and rats, under controlled conditions, will unquestionably provide insights into the nature of cellular and physiopathologic processes, delineating admissible representations of similar phenomena in humans.[836] However, as discussed before, one should be parsimonious when making direct extrapolations.

Regarding the statistical analysis, as the sizes of different groups are well specified and quite similar, it is acceptable to use SE, as an inferential statistic and instead of SD, in order to allow an intuitive comparison between estimated populations.[837] Being a theoretical estimate of variability of samples´ means, SE is a better measure of the precision with which the sample mean reflects the true mean in the population.[838]

In this experimental protocol, as depicted in the Results section, evidence of an acute and ensuing delayed neuropeptide response to TBI is therefore shown. These findings are in line with what is believed to be the role for neuropeptides and neurogenic inflammation as crucial components of initial post-TBI inflammation.[133, 353]

CHAPTER IV

Global Perspective on Neurotrauma research

4.1 Introduction

Acknowledging the indisputable progress in medical science and cumulative knowledge on brain trauma,[39, 839] one should not hesitate in classifying as a relative failure all conjoined efforts in translating this knowledge into successful clinical protocols, improving TBI patients care and prognosis.[839] Frequent appeals for broader and standardized research and treatment protocols and multicentric studies are simultaneously indisputable and inconsequential.[39, 839] The fact that only in recent years have long-term effects of otherwise seemingly minor traumas increasingly become the subject of relevant studies should in part explain the relatively late-onset of significant Neurotrauma research funding.[840] The specific nature of traumatic lesions, with a sudden and unexpected impact and no primary risk factors, makes it rather difficult to invest resources in biological prevention of this disease (public health prevention policies are fundamental but beyond the scope of this work), limiting the comprehensiveness and impact of focused mechanistic research.

Traumatic brain injuries pose a challenge as they are unannounced and sudden, deranging the most specialized tissue/organ in the human body, with a still debated but undoubtedly limited regeneration capability (both functionally and structurally).[841] Following initial damage and secondary injury cascades, traumatic lesions will undergo a predictable and inexorable progression (contusion, blood resorption, encephalomalacia) with few exceptions. Single components of much larger and complex response mechanisms to an external aggression should not be addressed separately and will hardly impact the overall prognosis by themselves. Mechanistic studies, while extremely valuable for a better understanding of TBI, will primarily focus on isolated pathways or factors on a cellular/molecular scale, necessarily missing the big picture. Attempting to bind basic research to such a complex clinical picture, efforts have been put in *in vitro* models of trauma.[842] However, one should be careful in interpreting possible findings in this type of research.

Several aspects must be considered when addressing the current status of brain trauma research:

- Relatively unknown biological mechanisms of disease;
- Significant knowledge gaps regarding post-traumatic neurogenesis and whether this represents a viable therapeutic target for functional recovery of the brain;

- Related to the previous point and unlike other organs, the fact that, although functional recovery might occur, significant structural recovery of the brain (a highly specialized and topographically organized organ) will hardly happen;
- A rather diverse type of injury in location, extent and nature, with heterogeneous behaviour and consequences. Brain trauma is not a unidimensional injury;
- Significant knowledge gaps regarding the true spatial and temporal extent of secondary damage;
- Unrealistic and insufficient animal models of TBI;
- Highly variable outcomes, ranging from death or profound disability to full recovery[843];
- Unsuccessful monotherapies targeting one single step or element in a much more complex process, opposed to combination therapies focusing on multiple targets[844];
- Over-extended time lapse between initial injury and neuroprotective drugs administration (with most clinical trials assuming a window of opportunity of 8h)[590];
- Role of BBB as a deterrent for therapeutic agents (at least partially, as its function is impaired in TBI).

4.2 Clinical research

Clinical research focuses mostly on developing therapeutic agents and demonstrating the validity of biomarkers, along with innovative neuromonitoring techniques and modalities.

Given the specific nature of brain trauma, a word of caution is advisable concerning an evergrowing research trend on disease biomarkers. One should be careful when assessing the real impact of biomarkers in brain trauma, as they represent, in our opinion, a significant misconception in research and a source of misguided investment (in logistics, financial and human resources). First of all, unlike any other pathological event, the initial insult to the brain is purely extrinsic to the individual (despite inter-individual variations in response to it), there is no prodrome as well as no direct biological risk factors involved and, by definition, initial diagnosis (head/brain trauma) is rather indisputable (with basic imaging tools displaying easily recognizable traumatic lesions, with few exceptions). Its timeline is more or less well defined and predictable, along with its invariable progression: initial lesion, initial damage, secondary injury, peak disturbance, progressive and long functional recovery (total, partial or even absent) along with

histological reorganization (on a macroscopic and microscopic level). Therefore, the specific context of neurotrauma empties a significant part of biomarkers´ classical role as a diagnostic and disease-progression assessment tool. The possibility of eliminating the need for diagnostic Computed Tomographies scans by quantifying specific biomarkers is also, in our opinion and according to several scientific committees, highly disputable.[501, 845]

Biomarkers in general are helpful in monitoring the progression of disease, namely for prognosis assessment (partially futile in brain trauma, as addressed before, as its time progression is relatively predictable). But given the fundamental fact that there are no proper therapeutic protocols to be initiated or adjusted following a hypothetical biomarker fluctuation, the latter intrinsic relevance should be questioned. One can always argue that, despite the absence of effective therapeutic agents, adjuvant medical support and Intensive Care protocols could benefit from biomarkers guidance.[789, 846]

In respect to clinical trials, ever-present difficulties in this type of research protocols (namely gold-standard randomized controlled clinical trials) are invariably an issue in Neurotrauma research.[587, 847] When promising therapeutic protocols are put to the test in Phase III trials, they consistently fail to make a significant impact in day-to-day clinical practice.[603, 848] Discernible reasons for this include, among others, troublesome selection of patients, ethical issues concerning randomization and a non-uniform population of patients. The urgency in treating these patients will, in some cases, make it even harder to delineate an adequate randomized prospective study properly.[60] Even when suitably structured, Phase III clinical trials may suffer (and usually do) from a typical combination of overenthusiastic estimation and insufficient peer-review of positive findings in pre-clinical and laboratory studies data.[849]

4.2.1 Designing better clinical trials

Regarding clinical trials, their intrinsic structure and endpoints are part of the solution and also part of the problem. The absence of mensurable disease progression indicators implies that objective therapeutic efficacy can only be asserted via clinical appreciation of functional outcome, depending on standardized scores and other tools, always subjective and sometimes non-reliable. First, currently used classifications (namely the GCS score and related categories of mild, moderate and severe TBI), although practical and valuable as a uniformizing tool, are too broad and unspecific and obviously fail to take into account too many details in patients condition and its clinical and radiological findings.[850]

Outcome measurements, as quality-of-life assessment tools, are described by Stein and colleagues as useful but blunt instruments.[26] Endpoints for treatment efficacy estimation, besides overall survival, are necessarily subjective (as in GOS) or useless (e.g., one should not determine contusion resorption as a goal, as it is expected nevertheless).[851] Cognitive performance, memory and return-to-work metrics are unquestionably non-objective variables and prone to under/over appreciation (namely by the patients themselves) and personal/populational/cultural variability.[852] More rigorous quantitative outcome assessment tools are in development or already being implemented in specialized centres.[853] Other factors, including genetic variance and pre-event nutritional status, should also be considered. Heterogeneity in patients´ response to injury and treatment interventions is a significant issue to take into consideration.[854]

4.2.2 Connecting basic research to day-to-day clinical issues

Basic research is, by definition, a field in which the objectives and procedures are well defined and standardized, performed by highly specialized researchers (frequently with no clinical background). Its standard mechanistic approach tipically focuses on one element or event of a much larger chain of events.[855] Its contribution is undeniable and its potential is nearly unlimited.[257] Even so, close cooperation between basic researchers and clinicians, who can bring extremely valuable feedback from their clinical practice, should help set more realistic goals and, at the same time, provide a more objective purpose, guiding research protocols in fruitful directions.[587, 847]

4.2.3 Reaching for translational research's potential

Nowadays, the potential for translational research, namely upon animal models of disease, is broadly recognized.[719, 856] The purpose of "from bench to bedside" research projects, providing a fruitful interaction between basic researchers and clinical context, is only achievable if the inevitable artificiality of all experimental models is addressed and attenuated.[363, 857] This will only be possible with the invaluable contribution of academic clinicians (who must overcome their own suspicions on this type of research), adjusting experimental models to the closest resemblance possible to reality and providing a solid framework for diverse results interpretation. Even in the presence of a single straightforward event as a TBI, different animal models provide distinct types of lesions and consequences.[843]
Clinicians have the responsibility to integrate this seemingly disadvantageous variability and realistically extrapolate it into useful clinical knowledge.

4.2.4 Implementing new therapeutic protocols

When testing for new drugs, it is mandatory to refine patient's selection and better stratify them concerning age and type of injury, in order to quantify recovery based on reliable quantitative outcome measures.[858] Implementing innovative research protocols, as in adaptive trial designs (using Bayesian computer modelling), with useful multi-criteria prediction models and proportional odds/sliding dichotomy models, is another growing trend in larger studies.[859] Applying Comparative Effectiveness Research protocols, able to compare treatments and Clinical Centres without strict inclusion/exclusion criteria, is another valid option, taking advantage of "big data" assessment tools, capable of assuring continuity in research between acute and post-acute studies.[860] Clinical trials should focus on perfecting enrollment criteria for clinical trials, selecting fewer patients with homogeneous injuries and clinical pictures. This approach will require more time and money but could prove more effective. Concerning therapeutic protocols, it should be helpful to consider post-treatment concentration levels intervals as primary endpoints instead of a single and constant therapeutic administration.

New routes for CNS drug administration are being developed and might prove more effective, of which the transnasal route is a good example.[861] Single drugs targeting multiple pathways or combination therapies might be the path to success. Another possible approach would be to combine two or more therapeutic protocols, distinct in their nature but complementary in their actions and targets (e.g., associating specific drugs with hypothermia protocols). New monitoring and clinical assessment techniques in the pre-hospital context should provide teams with significant amounts of data from crucial, earlier stages of brain trauma. In pre-hospital environment, earlier administration of neuroprotective drugs (ideally within minutes, not hours from initial trauma) should help prevent type II errors when evaluating drug efficacy. Considering the long-term effects of TBI, recent approaches in the field of endogenous and exogenous stem cell therapies seem promising as a stand-alone or adjunctive therapy, aiming at enhanced brain plasticity and repair of the injured brain.[13]

4.2.5 Imaging

Imaging is experiencing tremendous technological advances, focusing not only on primary diagnosis as it did before but also shedding light on structural and metabolic changes and functional impairments.[862] Its contribution towards a better understanding of pathophysiological intricacies of brain trauma and improving patient's management is undeniable. One can speculate on a possible future role

for more complex neuroimaging techniques, including DTI and resting-state functional MRI, able to assess functional impairments and network-level damage.[863]

4.3 Civil society

Concerning civil society, a greater awareness for TBI and its long-term cognitive sequelae, is becoming evident and still evolving, as demonstrated in all the controversies and discussions concerning sports activities.[104, 864, 865] Systematic and standardized prevention of TBI and its long-term consequences is mandatory. One shouldn´t forget that 10% of all TBI's and spinal injuries take place in the context of sports activity.[866]

Several state and private-funded programs, influencing and promoting public policies, are increasingly prominent in today's society, namely by focusing on specific niches and epidemiological contexts, ranging from the elderly population to road traffic accident prevention.

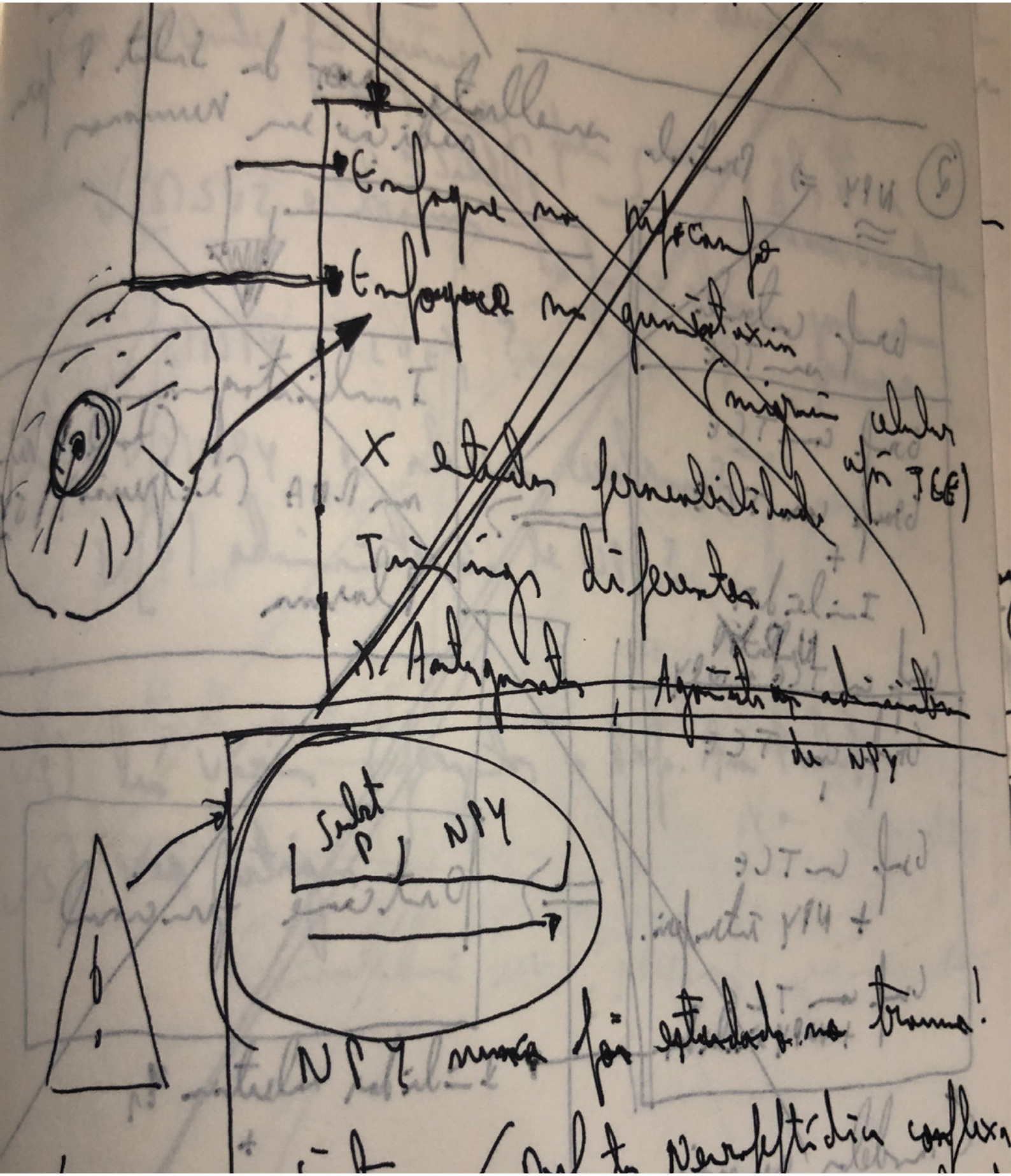
CHAPTER V

Conclusion

A multistage neuropeptide response to TBI is shown in human patients and its implications are discussed in the present work. Administration of exogenous NPY in an animal model of TBI has shown an apparently beneficial response at many levels, with diverse synergistic effects (involving distinct structural and functional elements) and attenuation of deleterious post-traumatic secondary injury.

Future research projects involving animal models of trauma (including ongoing projects in our Research group) should confirm the possibility of potentiating this neuropeptide response involving NPY and NPY agonists. Ensuing experimental studies will identify new therapeutic targets and agents and their ideal timing of action, aiming at maximum functional recovery. Promising scenarios for future research should include making a distinction between different neuroinflammatory processes in the context of pathologically diverse types of TBI, allowing tailored research protocols, pre-clinical and clinical trials, with specific timings and selected targets.

Brain trauma research should focus on solid and purposeful multicentric translational research, with major contributions from basic scientists and clinicians, in order to provide substantial evidence on the benefits of new therapies and diagnostic tools. A more precise definition of TBI and its sub-types is paramount, along with refined quantitative outcomes measures and effective clinical trials design. Innovative therapeutic protocols, using multi-targeted combination therapies upon new routes of drug administration, are needed. On another level, prevention of brain trauma and its sequelae, including large population-based awareness programs combined with targeted programs for specific vulnerable groups, is undoubtedly part of the answer.


Substance P
NPY

REFERENCES

1. Gustavsson A, Svensson M, Jacobi F, Allgulander C, Alonso J, Beghi E, et al. Cost of disorders of the brain in Europe 2010. Eur Neuropsychopharmacol. 2011;21(10):718-79.

2. Roozenbeek B, Maas AI, Menon DK. Changing patterns in the epidemiology of traumatic brain injury. Nat Rev Neurol. 2013;9(4):231-6.

3. Thelin EP, Nelson DW, Bellander BM. A review of the clinical utility of serum S100B protein levels in the assessment of traumatic brain injury. Acta Neurochir (Wien). 2017;159(2):209-25.

4. Alluri H, Shaji CA, Davis ML, Tharakan B. A Mouse Controlled Cortical Impact Model of Traumatic Brain Injury for Studying Blood-Brain Barrier Dysfunctions. Methods Mol Biol. 2018;1717:37-52.

5. Jang SH. Diagnostic Problems in Diffuse Axonal Injury. Diagnostics (Basel). 2020;10(2).

6. Alluri H, Wiggins-Dohlvik K, Davis ML, Huang JH, Tharakan B. Blood-brain barrier dysfunction following traumatic brain injury. Metab Brain Dis. 2015;30(5):1093-104.

7. de Freitas Cardoso MG, Faleiro RM, de Paula JJ, Kummer A, Caramelli P, Teixeira AL, et al. Cognitive Impairment Following Acute Mild Traumatic Brain Injury. Front Neurol. 2019;10:198.

8. Algethamy H. Baseline Predictors of Survival, Neurological Recovery, Cognitive Function, Neuropsychiatric Outcomes, and Return to Work in Patients after a Severe Traumatic Brain Injury: an Updated Review. Mater Sociomed. 2020;32(2):148-57.

9. Brooks JC, Strauss DJ, Shavelle RM, Paculdo DR, Hammond FM, Harrison-Felix CL. Long-term disability and survival in traumatic brain injury: results from the National Institute on Disability and Rehabilitation Research Model Systems. Arch Phys Med Rehabil. 2013;94(11):2203-9.

10. Fu TS, Jing R, McFaull SR, Cusimano MD. Health & Economic Burden of Traumatic Brain Injury in the Emergency Department. Can J Neurol Sci. 2016;43(2):238-47.

11. Berkner J, Mannix R, Qiu J. Clinical Traumatic Brain Injury in the Preclinical Setting. Methods Mol Biol. 2016;1462:11-28.

12. Sorby-Adams AJ, Marcoionni AM, Dempsey ER, Woenig JA, Turner RJ. The Role of Neurogenic Inflammation in Blood-Brain Barrier Disruption and Development of Cerebral Oedema Following Acute Central Nervous System (CNS) Injury. Int J Mol Sci. 2017;18(8).

13. Dewan MC, Rattani A, Gupta S, Baticulon RE, Hung YC, Punchak M, et al. Estimating the global incidence of traumatic brain injury. J Neurosurg. 2018:1-18.

14. Wilson MZ, P.; Griffin, C.; Lockey, D.; Tolias, C.; Verma, V. . The future of traumatic brain injury research. Scand J Trauma Resus Emerg. 2014;22:A7 62.

15. Majdan M, Plancikova D, Brazinova A, Rusnak M, Nieboer D, Feigin V, et al. Epidemiology of traumatic brain injuries in Europe: a cross-sectional analysis. Lancet Public Health. 2016;1(2):e76-e83.

16. Peeters W, van den Brande R, Polinder S, Brazinova A, Steyerberg EW, Lingsma HF, et al. Epidemiology of traumatic brain injury in Europe. Acta Neurochir (Wien). 2015;157(10):1683-96.

17. Faul MC, V. . Epidemiology of traumatic brain injury. In: Grafman JS, A., editor. Handbook of Clinical Neurology. Netherlands: Elsevier Publishers; 2015. p. 3-13.

18. Horvat CM, Bell MJ. Bringing attention into higher focus within the traumatic brain injury research agenda. Transl Pediatr. 2015;4(4):320-2.

19. Maas AI, Stocchetti N, Bullock R. Moderate and severe traumatic brain injury in adults. Lancet Neurol. 2008;7(8):728-41.

20. Santos ME, Agrela N. Traumatic brain injury in Portugal: progress in incidence and mortality. Brain Inj. 2019;33(12):1552-5.

21. Oliveira E, Lavrador JP, Santos MM, Lobo Antunes J. [Traumatic brain injury: integrated approach]. Acta Med Port. 2012;25(3):179-92.

22. Dias C, Rocha J, Pereira E, Cerejo A. Traumatic brain injury in Portugal: trends in hospital admissions from 2000 to 2010. Acta Med Port. 2014;27(3):349-56.

23. Coronado VG, Xu L, Basavaraju SV, McGuire LC, Wald MM, Faul MD, et al. Surveillance for traumatic brain injury-related deaths--United States, 1997-2007. MMWR Surveill Summ. 2011;60(5):1-32.

24. Coronado VT, D.; Greenspan, A.; Weissman, B. . Epidemiology. In: Jallo JL, C., editor. Neurotrauma and critical care of the brain. New York: Thieme Medical Publishers; 2009. p. 23-41.

25. Skandsen T, Einarsen CE, Normann I, Bjoralt S, Karlsen RH, McDonagh D, et al. The epidemiology of mild traumatic brain injury: the Trondheim MTBI follow-up study. Scand J Trauma Resusc Emerg Med. 2018;26(1):34.

26. Stein SC, Georgoff P, Meghan S, Mizra K, Sonnad SS. 150 years of treating severe traumatic brain injury: a systematic review of progress in mortality. J Neurotrauma. 2010;27(7):1343-53.

27. Rosenfeld JV, Maas AI, Bragge P, Morganti-Kossmann MC, Manley GT, Gruen RL. Early management of severe traumatic brain injury. Lancet. 2012;380(9847):1088-98.

28. Logsdon AF, Lucke-Wold BP, Turner RC, Huber JD, Rosen CL, Simpkins JW. Role of Microvascular Disruption in Brain Damage from Traumatic Brain Injury. Compr Physiol. 2015;5(3):1147-60.

29. Dash HH, Chavali S. Management of traumatic brain injury patients. Korean J Anesthesiol. 2018;71(1):12-21.

30. Picetti E, Rossi S, Abu-Zidan FM, Ansaloni L, Armonda R, Baiocchi GL, et al. WSES consensus conference guidelines: monitoring and management of severe adult traumatic brain injury patients with polytrauma in the first 24 hours. World J Emerg Surg. 2019;14:53.

31. Carney N, Totten AM, O'Reilly C, Ullman JS, Hawryluk GW, Bell MJ, et al. Guidelines for the Management of Severe Traumatic Brain Injury, Fourth Edition. Neurosurgery. 2017;80(1):6-15.

32. Silverberg ND, Duhaime AC, Iaccarino MA. Mild Traumatic Brain Injury in 2019-2020. JAMA. 2020;323(2):177-8.

33. Algamal M, Saltiel N, Pearson AJ, Ager B, Burca I, Mouzon B, et al. Impact of Repetitive Mild Traumatic Brain Injury on Behavioral and Hippocampal Deficits in a Mouse Model of Chronic Stress. J Neurotrauma. 2019;36(17):2590-607.

34. Teasdale G, Jennett B. Assessment of coma and impaired consciousness. A practical scale. Lancet. 1974;2(7872):81-4.

35. Shukla D, Devi BI. Mild traumatic brain injuries in adults. J Neurosci Rural Pract. 2010; 1(2):82-8.

36. Ganti L, Stead T, Daneshvar Y, Bodhit AN, Pulvino C, Ayala SW, et al. GCS 15: when mild TBI isn't so mild. Neurol Res Pract. 2019;1:6.

37. Joseph B, Pandit V, Aziz H, Kulvatunyou N, Zangbar B, Green DJ, et al. Mild traumatic brain injury defined by Glasgow Coma Scale: Is it really mild? Brain Inj. 2015; 29(1):11-6.

38. Gravesteijn BY, Sewalt CA, Ercole A, Akerlund C, Nelson D, Maas AIR, et al. Toward a New Multi-Dimensional Classification of Traumatic Brain Injury: A Collaborative European NeuroTrauma Effectiveness Research for Traumatic Brain Injury Study. J Neurotrauma. 2020;37(7):1002-10.

39. Kaur P, Sharma S. Recent Advances in Pathophysiology of Traumatic Brain Injury. Curr Neuropharmacol. 2018;16(8):1224-38.

40. Stevens RD, Shoykhet M, Cadena R. Emergency Neurological Life Support: Intracranial Hypertension and Herniation. Neurocrit Care. 2015;23 Suppl 2:S76-82.

41. Ng SY, Lee AYW. Traumatic Brain Injuries: Pathophysiology and Potential Therapeutic Targets. Front Cell Neurosci. 2019;13:528.

42. Jang SH, Kim OL, Kim SH, Kim JB. The Relation Between Loss of Consciousness, Severity of Traumatic Brain Injury, and Injury of Ascending Reticular Activating System in Patients With Traumatic Brain Injury. Am J Phys Med Rehabil. 2019;98(12):1067-71.

43. Waseem M, Iyahen P, Jr., Anderson HB, Kapoor K, Kapoor R, Leber M. Isolated LOC in head trauma associated with significant injury on brain CT scan. Int J Emerg Med. 2017;10(1):30.

44. Roy D, Peters ME, Everett A, Leoutsakos JM, Yan H, Rao V, et al. Loss of consciousness and altered mental state predicting depressive and post-concussive symptoms after mild traumatic brain injury. Brain Inj. 2019;33(8):1064-9.

45. Aggarwal SS, Ott SD, Padhye NS, Schulz PE. Sex, race, ADHD, and prior concussions as predictors of concussion recovery in adolescents. Brain Inj. 2020;34(6):809-17.

46. Mullally WJ. Concussion. Am J Med. 2017;130(8):885-92.

47. Scorza KA, Cole W. Current Concepts in Concussion: Initial Evaluation and Management. Am Fam Physician. 2019;99(7):426-34.

48. Sharp DJ, Jenkins PO. Concussion is confusing us all. Pract Neurol. 2015;15(3):172-86.

49. Bouley J, Chung DY, Ayata C, Brown RH, Jr., Henninger N. Cortical Spreading Depression Denotes Concussion Injury. J Neurotrauma. 2019;36(7):1008-17.

50. Kundu S, Ghodadra A, Fakhran S, Alhilali LM, Rohde GK. Assessing Postconcussive Reaction Time Using Transport-Based Morphometry of Diffusion Tensor Images. AJNR Am J Neuroradiol. 2019;40(7):1117-23.

51. Capi M, Pomes LM, Andolina G, Curto M, Martelletti P, Lionetto L. Persistent Post-Traumatic Headache and Migraine: Pre-Clinical Comparisons. Int J Environ Res Public Health. 2020;17(7).

52. Chandran A, Kerr ZY, Roby PR, Nedimyer AK, Arakkal A, Pierpoint LA, et al. Concussion Symptom Characteristics and Resolution in 20 United States High School Sports, 2013/14-2017/18 Academic Years. Neurosurgery. 2020;87(3):573-83.

53. Maruta J, Lee SW, Jacobs EF, Ghajar J. A unified science of concussion. Ann N Y Acad Sci. 2010;1208:58-66.

54. Tator CH. Concussions and their consequences: current diagnosis, management and prevention. CMAJ. 2013;185(11):975-9.

55. McCrory PM, WH.; Aubry, M.; Cantu, RC.; Dvorak, J.; Echemendia, RJ., et al. . Consensus statement on concussion in sport. J Athletic Training. 2012;32:780-90.

56. Torrente D, Cabezas R, Avila MF, Garcia-Segura LM, Barreto GE, Guedes RC. Cortical spreading depression in traumatic brain injuries: is there a role for astrocytes? Neurosci Lett. 2014;565:2-6.

57. Mouzon BC, Bachmeier C, Ferro A, Ojo JO, Crynen G, Acker CM, et al. Chronic neuropathological and neurobehavioral changes in a repetitive mild traumatic brain injury model. Ann Neurol. 2014;75(2):241-54.

58. Chung S, Wang X, Fieremans E, Rath JF, Amorapanth P, Foo FA, et al. Altered Relationship between Working Memory and Brain Microstructure after Mild Traumatic Brain Injury. AJNR Am J Neuroradiol. 2019;40(9):1438-44.

59. Cassidy JD, Cancelliere C, Carroll LJ, Cote P, Hincapie CA, Holm LW, et al. Systematic review of self-reported prognosis in adults after mild traumatic brain injury: results of the International Collaboration on Mild Traumatic Brain Injury Prognosis. Arch Phys Med Rehabil. 2014;95(3 Suppl):S132-51.

60. Losoi H, Silverberg ND, Waljas M, Turunen S, Rosti-Otajarvi E, Helminen M, et al. Recovery from Mild Traumatic Brain Injury in Previously Healthy Adults. J Neurotrauma. 2016;33(8):766-76.

61. Cassidy JD, Boyle E, Carroll LJ. Population-based, inception cohort study of the incidence, course, and prognosis of mild traumatic brain injury after motor vehicle collisions. Arch Phys Med Rehabil. 2014;95(3 Suppl):S278-85.

62. Szaflarski JP, Nazzal Y, Dreer LE. Post-traumatic epilepsy: current and emerging treatment options. Neuropsychiatr Dis Treat. 2014;10:1469-77.

63. Lucke-Wold BP, Nguyen L, Turner RC, Logsdon AF, Chen YW, Smith KE, et al. Traumatic brain injury and epilepsy: Underlying mechanisms leading to seizure. Seizure. 2015;33:13-23.

64. Abou-Abbass H, Bahmad H, Ghandour H, Fares J, Wazzi-Mkahal R, Yacoub B, et al. Epidemiology and clinical characteristics of traumatic brain injury in Lebanon: A systematic review. Medicine (Baltimore). 2016;95(47):e5342.

65. Christensen J, Pedersen MG, Pedersen CB, Sidenius P, Olsen J, Vestergaard M. Long-term risk of epilepsy after traumatic brain injury in children and young adults: a population-based cohort study. Lancet. 2009;373(9669):1105-10.

66. Glushakov AV, Glushakova OY, Dore S, Carney PR, Hayes RL. Animal Models of Posttraumatic Seizures and Epilepsy. Methods Mol Biol. 2016;1462:481-519.

67. Shandra O, Winemiller AR, Heithoff BP, Munoz-Ballester C, George KK, Benko MJ, et al. Repetitive Diffuse Mild Traumatic Brain Injury Causes an Atypical Astrocyte Response and Spontaneous Recurrent Seizures. J Neurosci. 2019;39(10):1944-63.

68. Ferguson PL, Smith GM, Wannamaker BB, Thurman DJ, Pickelsimer EE, Selassie AW. A population-based study of risk of epilepsy after hospitalization for traumatic brain injury. Epilepsia. 2010;51(5):891-8.

69. Christensen J. Traumatic brain injury: risks of epilepsy and implications for medicolegal assessment. Epilepsia. 2012;53 Suppl 4:43-7.

70. Robert SM, Buckingham SC, Campbell SL, Robel S, Holt KT, Ogunrinu-Babarinde T, et al. SLC7A11 expression is associated with seizures and predicts poor survival in patients with malignant glioma. Sci Transl Med. 2015;7(289):289ra86.

71. Shandra O, Moshe SL, Galanopoulou AS. Inflammation in Epileptic Encephalopathies. Adv Protein Chem Struct Biol. 2017;108:59-84.

72. Ritter AC, Wagner AK, Fabio A, Pugh MJ, Walker WC, Szaflarski JP, et al. Incidence and risk factors of posttraumatic seizures following traumatic brain injury: A Traumatic Brain Injury Model Systems Study. Epilepsia. 2016;57(12):1968-77.

73. Mendonca GS, Sander JW. Post traumatic epilepsy: there is still much to learn. Arq Neuropsiquiatr. 2019;77(6):373-4.

74. Christensen J. The Epidemiology of Posttraumatic Epilepsy. Semin Neurol. 2015;35(3):218-22.

75. Lingsma HF, Yue JK, Maas AI, Steyerberg EW, Manley GT, Investigators T-T. Outcome prediction after mild and complicated mild traumatic brain injury: external validation of existing models and identification of new predictors using the TRACK-TBI pilot study. J Neurotrauma. 2015;32(2):83-94.

76. Frankowski JC, Kim YJ, Hunt RF. Selective vulnerability of hippocampal interneurons to graded traumatic brain injury. Neurobiol Dis. 2019;129:208-16.

77. McAllister TW, Flashman LA, Maerlender A, Greenwald RM, Beckwith JG, Tosteson TD, et al. Cognitive effects of one season of head impacts in a cohort of collegiate contact sport athletes. Neurology. 2012;78(22):1777-84.

78. Anderson KM, K.; Fugaccia, K.; Scheff, S. Differential sensitivity of Hippocampal neurons to traumatic injury as determined by fluro-jade B staining. Exp Neurol. 2005;193:125-30.

79. Spain A, Daumas S, Lifshitz J, Rhodes J, Andrews PJ, Horsburgh K, et al. Mild fluid percussion injury in mice produces evolving selective axonal pathology and cognitive deficits relevant to human brain injury. J Neurotrauma. 2010;27(8):1429-38.

80. Reeves TM, Lyeth BG, Povlishock JT. Long-term potentiation deficits and excitability changes following traumatic brain injury. Exp Brain Res. 1995;106(2):248-56.

81. Dikmen S, Machamer J, Temkin N. Mild Traumatic Brain Injury: Longitudinal Study of Cognition, Functional Status, and Post-Traumatic Symptoms. J Neurotrauma. 2017;34(8):1524-30.

82. Moore DR, Pindus DM, Raine LB, Drollette ES, Scudder MR, Ellemberg D, et al. The persistent influence of concussion on attention, executive control and neuroelectric function in preadolescent children. Int J Psychophysiol. 2016;99:85-95.

83. Mannix R, Levy R, Zemek R, Yeates KO, Arbogast K, Meehan WP, et al. Fluid Biomarkers of Pediatric Mild Traumatic Brain Injury: A Systematic Review. J Neurotrauma. 2020;37(19):2029-44.

84. Neumane S, Camara-Costa H, Francillette L, Araujo M, Toure H, Brugel D, et al. Functional outcome after severe childhood traumatic brain injury: Results of the TGE prospective longitudinal study. Ann Phys Rehabil Med. 2020:101375.

85. Broussard JI, Acion L, De Jesus-Cortes H, Yin T, Britt JK, Salas R, et al. Repeated mild traumatic brain injury produces neuroinflammation, anxiety-like behaviour and impaired spatial memory in mice. Brain Inj. 2018;32(1):113-22.

86. Guilhaume-Correa F, Cansler SM, Shalosky EM, Goodman MD, Evanson NK. Greater neurodegeneration and behavioral deficits after single closed head traumatic brain injury in adolescent versus adult male mice. J Neurosci Res. 2020;98(3):557-70.

87. Girgis F, Pace J, Sweet J, Miller JP. Hippocampal Neurophysiologic Changes after Mild Traumatic Brain Injury and Potential Neuromodulation Treatment Approaches. Front Syst Neurosci. 2016;10:8.

88. Jenkins PO, De Simoni S, Bourke NJ, Fleminger J, Scott G, Towey DJ, et al. Stratifying drug treatment of cognitive impairments after traumatic brain injury using neuroimaging. Brain. 2019;142(8):2367-79.

89. Calvillo M, Irimia A. Neuroimaging and Psychometric Assessment of Mild Cognitive Impairment After Traumatic Brain Injury. Front Psychol. 2020;11:1423.

90. Qin XY, Fang H, Shan QH, Qi CC, Zhou JN. All-trans Retinoic Acid-induced Abnormal Hippocampal Expression of Synaptic Genes SynDIG1 and DLG2 is Correlated with Anxiety or Depression-Like Behavior in Mice. Int J Mol Sci. 2020;21(8).

91. Bigler ED, Tsao JW. Mild traumatic brain injury in soldiers returning from combat. Neurology. 2017;88(16):1490-2.

92. Lindquist LK, Love HC, Elbogen EB. Traumatic Brain Injury in Iraq and Afghanistan Veterans: New Results From a National Random Sample Study. J Neuropsychiatry Clin Neurosci. 2017;29(3):254-9.

93. Bryant RA, O'Donnell ML, Creamer M, McFarlane AC, Clark CR, Silove D. The psychiatric sequelae of traumatic injury. Am J Psychiatry. 2010;167(3):312-20.

94. Pavlovic D, Pekic S, Stojanovic M, Popovic V. Traumatic brain injury: neuropathological, neurocognitive and neurobehavioral sequelae. Pituitary. 2019;22(3):270-82.

95. Silverberg ND, Panenka WJ. Antidepressants for depression after concussion and traumatic brain injury are still best practice. BMC Psychiatry. 2019;19(1):100.

96. Bahraini NH, Breshears RE, Hernandez TD, Schneider AL, Forster JE, Brenner LA. Traumatic brain injury and posttraumatic stress disorder. Psychiatr Clin North Am. 2014;37(1):55-75.

97. McMahon P, Hricik A, Yue JK, Puccio AM, Inoue T, Lingsma HF, et al. Symptomatology and functional outcome in mild traumatic brain injury: results from the prospective TRACK-TBI study. J Neurotrauma. 2014;31(1):26-33.

98. Hou R, Moss-Morris R, Peveler R, Mogg K, Bradley BP, Belli A. When a minor head injury results in enduring symptoms: a prospective investigation of risk factors for postconcussional syndrome after mild traumatic brain injury. J Neurol Neurosurg Psychiatry. 2012;83(2):217-23.

99. Corman SR, Adame BJ, Tsai JY, Ruston SW, Beaumont JS, Kamrath JK, et al. Socioecological influences on concussion reporting by NCAA Division 1 athletes in high-risk sports. PLoS One. 2019;14(5):e0215424.

100. Iverson GS, N.; Lange, RT.; Zasler, ND. . Conceptualizing Outcome from Mild Traumatic Brain Injury. In: Zasler N.; Katz D.; Zafonte R, editor. Brain Injury Medicine: Principles and Practice, 2nd Edition. New York: Demos Medical Publishing; 2012. p. 470-97.

101. Mendez MF. The neuropsychiatric aspects of boxing. Int J Psychiatry Med. 1995;25(3):249-62.

102. Alosco ML, Mez J, Tripodis Y, Kiernan PT, Abdolmohammadi B, Murphy L, et al. Age of first exposure to tackle football and chronic traumatic encephalopathy. Ann Neurol. 2018;83(5):886-901.

103. Fesharaki-Zadeh A. Chronic Traumatic Encephalopathy: A Brief Overview. Front Neurol. 2019;10:713.

104. Ling H, Morris HR, Neal JW, Lees AJ, Hardy J, Holton JL, et al. Mixed pathologies including chronic traumatic encephalopathy account for dementia in retired association football (soccer) players. Acta Neuropathol. 2017;133(3):337-52.

105. Edwards G, 3rd, Zhao J, Dash PK, Soto C, Moreno-Gonzalez I. Traumatic Brain Injury Induces Tau Aggregation and Spreading. J Neurotrauma. 2020;37(1):80-92.

106. McKee AC, Alosco ML, Huber BR. Repetitive Head Impacts and Chronic Traumatic Encephalopathy. Neurosurg Clin N Am. 2016;27(4):529-35.

107. Zhang Y, Wu F, Iqbal K, Gong CX, Hu W, Liu F. Subacute to chronic Alzheimer-like alterations after controlled cortical impact in human tau transgenic mice. Sci Rep. 2019;9(1):3789.

108. Tucker B, Aston J, Dines M, Caraman E, Yacyshyn M, McCarthy M, et al. Early Brain Edema is a Predictor of In-Hospital Mortality in Traumatic Brain Injury. J Emerg Med. 2017;53(1):18-29.

109. Wilson MH. Monro-Kellie 2.0: The dynamic vascular and venous pathophysiological components of intracranial pressure. J Cereb Blood Flow Metab. 2016;36(8):1338-50.

110. Hawthorne C, Piper I. Monitoring of intracranial pressure in patients with traumatic brain injury. Front Neurol. 2014;5:121.

111. Canac N, Jalaleddini K, Thorpe SG, Thibeault CM, Hamilton RB. Review: pathophysiology of intracranial hypertension and noninvasive intracranial pressure monitoring. Fluids Barriers CNS. 2020;17(1):40.

112. Ropper AH. Management of raised intracranial pressure and hyperosmolar therapy. Pract Neurol. 2014;14(3):152-8.

113. Freeman WD. Management of Intracranial Pressure. Continuum (Minneap Minn). 2015;21(5 Neurocritical Care):1299-323.

114. Winkler EA, Minter D, Yue JK, Manley GT. Cerebral Edema in Traumatic Brain Injury: Pathophysiology and Prospective Therapeutic Targets. Neurosurg Clin N Am. 2016;27(4):473-88.

115. Gottlieb M, Bailitz J. Does Mannitol Reduce Mortality From Traumatic Brain Injury? Ann Emerg Med. 2016;67(1):83-5.

116. Hutchinson PJ, Kolias AG, Timofeev IS, Corteen EA, Czosnyka M, Timothy J, et al. Trial of Decompressive Craniectomy for Traumatic Intracranial Hypertension. N Engl J Med. 2016;375(12):1119-30.

117. Jha RM, Elmer J, Zusman BE, Desai S, Puccio AM, Okonkwo DO, et al. Intracranial Pressure Trajectories: A Novel Approach to Informing Severe Traumatic Brain Injury Phenotypes. Crit Care Med. 2018;46(11):1792-802.

118. Smith DH, Johnson VE, Stewart W. Chronic neuropathologies of single and repetitive TBI: substrates of dementia? Nat Rev Neurol. 2013;9(4):211-21.

119. Omalu BI, Fitzsimmons RP, Hammers J, Bailes J. Chronic traumatic encephalopathy in a professional American wrestler. J Forensic Nurs. 2010;6(3):130-6.

120. McKee AC, Daneshvar DH. The neuropathology of traumatic brain injury. Handb Clin Neurol. 2015;127:45-66.

121. Djordjevic J, Sabbir MG, Albensi BC. Traumatic Brain Injury as a Risk Factor for Alzheimer's Disease: Is Inflammatory Signaling a Key Player? Curr Alzheimer Res. 2016;13(7):730-8.

122. Hosomi S, Ohnishi M, Ogura H, Shimazu T. Traumatic brain injury-related inflammatory projection: beyond local inflammatory responses. Acute Med Surg. 2020;7(1):e520.

123. Yoon JE, Lee CY, Sin EG, Song J, Kim HW. Clinical Feature and Outcomes of Secondary Hydrocephalus Caused by Head Trauma. Korean J Neurotrauma. 2018;14(2):86-92.

124. Chen KH, Lee CP, Yang YH, Yang YH, Chen CM, Lu ML, et al. Incidence of hydrocephalus in traumatic brain injury: A nationwide population-based cohort study. Medicine (Baltimore). 2019;98(42):e17568.

125. Wheble JL, Menon DK. TBI-the most complex disease in the most complex organ: the CENTER-TBI trial-a commentary. J R Army Med Corps. 2016;162(2):87-9.

126. DeKosky ST, Asken BM. Injury cascades in TBI-related neurodegeneration. Brain Inj. 2017;31(9):1177-82.

127. Hoffman JR, Zuckerman A, Ram O, Sadot O, Stout JR, Ostfeld I, et al. Behavioral and inflammatory response in animals exposed to a low-pressure blast wave and supplemented with beta-alanine. Amino Acids. 2017;49(5):871-86.

128. Hinzman JM, Thomas TC, Burmeister JJ, Quintero JE, Huettl P, Pomerleau F, et al. Diffuse brain injury elevates tonic glutamate levels and potassium-evoked glutamate release in discrete brain regions at two days post-injury: an enzyme-based microelectrode array study. J Neurotrauma. 2010;27(5):889-99.

129. Dobrachinski F, da Rosa Gerbatin R, Sartori G, Ferreira Marques N, Zemolin AP, Almeida Silva LF, et al. Regulation of Mitochondrial Function and Glutamatergic System Are the Target of Guanosine Effect in Traumatic Brain Injury. J Neurotrauma. 2017;34(7):1318-28.

130. Donkin JJ, Vink R. Mechanisms of cerebral edema in traumatic brain injury: therapeutic developments. Curr Opin Neurol. 2010;23(3):293-9.

131. Prakash R, Carmichael ST. Blood-brain barrier breakdown and neovascularization processes after stroke and traumatic brain injury. Curr Opin Neurol. 2015;28(6):556-64.

132. Akamatsu Y, Hanafy KA. Cell Death and Recovery in Traumatic Brain Injury. Neurotherapeutics. 2020;17(2):446-56.

133. Corrigan F, Mander KA, Leonard AV, Vink R. Neurogenic inflammation after traumatic brain injury and its potentiation of classical inflammation. J Neuroinflammation. 2016;13(1):264.

134. Isokuortti H, Iverson GL, Silverberg ND, Kataja A, Brander A, Ohman J, et al. Characterizing the type and location of intracranial abnormalities in mild traumatic brain injury. J Neurosurg. 2018;129(6):1588-97.

135. Saatman KE, Duhaime AC, Bullock R, Maas AI, Valadka A, Manley GT, et al. Classification of traumatic brain injury for targeted therapies. J Neurotrauma. 2008;25(7):719-38.

136. Marshall LF, Marshall SB, Klauber MR, Van Berkum Clark M, Eisenberg H, Jane JA, et al. The diagnosis of head injury requires a classification based on computed axial tomography. J Neurotrauma. 1992;9 Suppl 1:S287-92.

137. Maas AI, Hukkelhoven CW, Marshall LF, Steyerberg EW. Prediction of outcome in traumatic brain injury with computed tomographic characteristics: a comparison between the computed tomographic classification and combinations of computed tomographic predictors. Neurosurgery. 2005;57(6):1173-82; discussion -82.

138. Hellewell S, Semple BD, Morganti-Kossmann MC. Therapies negating neuroinflammation after brain trauma. Brain Res. 2016;1640(Pt A):36-56.

139. Harish G, Mahadevan A, Pruthi N, Sreenivasamurthy SK, Puttamallesh VN, Keshava Prasad TS, et al. Characterization of traumatic brain injury in human brains reveals distinct cellular and molecular changes in contusion and pericontusion. J Neurochem. 2015;134(1):156-72.

140. Klima D. Traumatic brain injury. In: Umphred DC, C. , editor. Neurorehabilitation for the Physical Therapist Assistant. New Jersey: Slack Publishers; 2006. p. 155-72.

141. Sweeney MD, Zhao Z, Montagne A, Nelson AR, Zlokovic BV. Blood-Brain Barrier: From Physiology to Disease and Back. Physiol Rev. 2019;99(1):21-78.

142. Arango-Lievano M, Dromard Y, Fontanaud P, Lafont C, Mollard P, Jeanneteau F. Regeneration of the neurogliovascular unit visualized in vivo by transcranial live-cell imaging. J Neurosci Methods. 2020;343:108808.

143. Loane DJ, Stoica BA, Faden AI. Neuroprotection for traumatic brain injury. Handb Clin Neurol. 2015;127:343-66.

144. Cheng G, Kong RH, Zhang LM, Zhang JN. Mitochondria in traumatic brain injury and mitochondrial-targeted multipotential therapeutic strategies. Br J Pharmacol. 2012;167(4):699-719.

145. Sajja VS, Hlavac N, VandeVord PJ. Role of Glia in Memory Deficits Following Traumatic Brain Injury: Biomarkers of Glia Dysfunction. Front Integr Neurosci. 2016;10:7.

146. Tan YL, Yuan Y, Tian L. Microglial regional heterogeneity and its role in the brain. Mol Psychiatry. 2020;25(2):351-67.

147. Russo MV, Latour LL, McGavern DB. Distinct myeloid cell subsets promote meningeal remodeling and vascular repair after mild traumatic brain injury. Nat Immunol. 2018;19(5):442-52.

148. Burda JE, Bernstein AM, Sofroniew MV. Astrocyte roles in traumatic brain injury. Exp Neurol. 2016;275 Pt 3:305-15.

149. Javid S, Rezaei A, Karami G. A micromechanical procedure for viscoelastic characterization of the axons and ECM of the brainstem. J Mech Behav Biomed Mater. 2014;30:290-9.

150. Zhou Y, Shao A, Yao Y, Tu S, Deng Y, Zhang J. Dual roles of astrocytes in plasticity and reconstruction after traumatic brain injury. Cell Commun Signal. 2020;18(1):62.

151. Pan H, Wang H, Wang X, Zhu L, Mao L. The absence of Nrf2 enhances NF-kappaB-dependent inflammation following scratch injury in mouse primary cultured astrocytes. Mediators Inflamm. 2012;2012:217580.

152. Wu MY, Gao F, Yang XM, Qin X, Chen GZ, Li D, et al. Matrix metalloproteinase-9 regulates the blood brain barrier via the hedgehog pathway in a rat model of traumatic brain injury. Brain Res. 2020;1727:146553.

153. Giaume C, Koulakoff A, Roux L, Holcman D, Rouach N. Astroglial networks: a step further in neuroglial and gliovascular interactions. Nat Rev Neurosci. 2010;11(2):87-99.

154. Sun LQ, Gao JL, Cui CM, Cui Y, Jing XB, Zhao MM, et al. Astrocytic p-connexin 43 regulates neuronal autophagy in the hippocampus following traumatic brain injury in rats. Mol Med Rep. 2014;9(1):77-82.

155. Rovegno M, Soto PA, Saez PJ, Naus CC, Saez JC, von Bernhardi R. Connexin43 hemichannels mediate secondary cellular damage spread from the trauma zone to distal zones in astrocyte monolayers. Glia. 2015;63(7):1185-99.

156. Huang C, Han X, Li X, Lam E, Peng W, Lou N, et al. Critical role of connexin 43 in secondary expansion of traumatic spinal cord injury. J Neurosci. 2012;32(10):3333-8.

157. Sun L, Gao J, Zhao M, Cui J, Li Y, Yang X, et al. A novel cognitive impairment mechanism that astrocytic p-connexin 43 promotes neuronic autophagy via activation of P2X7R and down-regulation of GLT-1 expression in the hippocampus following traumatic brain injury in rats. Behav Brain Res. 2015;291:315-24.

158. De Bock M, Decrock E, Wang N, Bol M, Vinken M, Bultynck G, et al. The dual face of connexin-based astroglial Ca(2+) communication: a key player in brain physiology and a prime target in pathology. Biochim Biophys Acta. 2014;1843(10):2211-32.

159. Graham NS, Sharp DJ. Understanding neurodegeneration after traumatic brain injury: from mechanisms to clinical trials in dementia. J Neurol Neurosurg Psychiatry. 2019;90(11):1221-33.

160. Park MS, Oh HA, Ko IG, Kim SE, Kim SH, Kim CJ, et al. Influence of mild traumatic brain injury during pediatric stage on short-term memory and hippocampal apoptosis in adult rats. J Exerc Rehabil. 2014;10(3):148-54.

161. Johnson VE, Stewart W, Arena JD, Smith DH. Traumatic Brain Injury as a Trigger of Neurodegeneration. Adv Neurobiol. 2017;15:383-400.

162. Lifshitz J, Lisembee AM. Neurodegeneration in the somatosensory cortex after experimental diffuse brain injury. Brain Struct Funct. 2012;217(1):49-61.

163. Carron SF, Yan EB, Alwis DS, Rajan R. Differential susceptibility of cortical and subcortical inhibitory neurons and astrocytes in the long term following diffuse traumatic brain injury. J Comp Neurol. 2016;524(17):3530-60.

164. Blennow K, Hardy J, Zetterberg H. The neuropathology and neurobiology of traumatic brain injury. Neuron. 2012;76(5):886-99.

165. Greer JE, Hanell A, McGinn MJ, Povlishock JT. Mild traumatic brain injury in the mouse induces axotomy primarily within the axon initial segment. Acta Neuropathol. 2013;126(1):59-74.

166. Vieira RC, Paiva WS, de Oliveira DV, Teixeira MJ, de Andrade AF, de Sousa RM. Diffuse Axonal Injury: Epidemiology, Outcome and Associated Risk Factors. Front Neurol. 2016;7:178.

167. Mu J, Li M, Wang T, Li X, Bai M, Zhang G, et al. Myelin Damage in Diffuse Axonal Injury. Front Neurosci. 2019;13:217.

168. Povlishock JT, Katz DI. Update of neuropathology and neurological recovery after traumatic brain injury. J Head Trauma Rehabil. 2005;20(1):76-94.

169. Conforti L, Gilley J, Coleman MP. Wallerian degeneration: an emerging axon death pathway linking injury and disease. Nat Rev Neurosci. 2014;15(6):394-409.

170. Maxwell WB, E.; Morgan, H. . Wallerian degeneration in the optic nerve stretch-injury model of TBI: a stereological analysis. J Neurotrauma. 2014;32:780-90.

171. Armstrong RC, Mierzwa AJ, Marion CM, Sullivan GM. White matter involvement after TBI: Clues to axon and myelin repair capacity. Exp Neurol. 2016;275 Pt 3:328-33.

172. Dikranian K, Cohen R, Mac Donald C, Pan Y, Brakefield D, Bayly P, et al. Mild traumatic brain injury to the infant mouse causes robust white matter axonal degeneration which precedes apoptotic death of cortical and thalamic neurons. Exp Neurol. 2008;211(2):551-60.

173. Carron SF, Yan EB, Allitt BJ, Rajan R. Immediate and Medium-term Changes in Cortical and Hippocampal Inhibitory Neuronal Populations after Diffuse TBI. Neuroscience. 2018;388:152-70.

174. Ding MC, Wang Q, Lo EH, Stanley GB. Cortical excitation and inhibition following focal traumatic brain injury. J Neurosci. 2011;31(40):14085-94.

175. Cantu D, Walker K, Andresen L, Taylor-Weiner A, Hampton D, Tesco G, et al. Traumatic Brain Injury Increases Cortical Glutamate Network Activity by Compromising GABAergic Control. Cereb Cortex. 2015;25(8):2306-20.

176. Huusko N, Romer C, Ndode-Ekane XE, Lukasiuk K, Pitkanen A. Loss of hippocampal interneurons and epileptogenesis: a comparison of two animal models of acquired epilepsy. Brain Struct Funct. 2015;220(1):153-91.

177. Blaiss CA, Yu TS, Zhang G, Chen J, Dimchev G, Parada LF, et al. Temporally specified genetic ablation of neurogenesis impairs cognitive recovery after traumatic brain injury. J Neurosci. 2011;31(13):4906-16.

178. Hong S, Washington PM, Kim A, Yang CP, Yu TS, Kernie SG. Apolipoprotein E Regulates Injury-Induced Activation of Hippocampal Neural Stem and Progenitor Cells. J Neurotrauma. 2016;33(4):362-74.

179. Maldonado PD, Chanez-Cardenas ME, Fernandez-Lopez A. Mechanisms of Cell Damage in Neurological Diseases and Putative Neuroprotective Strategies. Oxid Med Cell Longev. 2018;2018:9784319.

180. Flygt J, Ruscher K, Norberg A, Mir A, Gram H, Clausen F, et al. Neutralization of Interleukin-1beta following Diffuse Traumatic Brain Injury in the Mouse Attenuates the Loss of Mature Oligodendrocytes. J Neurotrauma. 2018;35(23):2837-49.

181. Au NPB, Ma CHE. Recent Advances in the Study of Bipolar/Rod-Shaped Microglia and their Roles in Neurodegeneration. Front Aging Neurosci. 2017;9:128.

182. Fernandez-Arjona MDM, Grondona JM, Granados-Duran P, Fernandez-Llebrez P, Lopez-Avalos MD. Microglia Morphological Categorization in a Rat Model of Neuroinflammation by Hierarchical Cluster and Principal Components Analysis. Front Cell Neurosci. 2017;11:235.

183. Cserep C, Posfai B, Lenart N, Fekete R, Laszlo ZI, Lele Z, et al. Microglia monitor and protect neuronal function through specialized somatic purinergic junctions. Science. 2020;367(6477):528-37.

184. Ransohoff RM. A polarizing question: do M1 and M2 microglia exist? Nat Neurosci. 2016;19(8):987-91.

185. Izzy S, Liu Q, Fang Z, Lule S, Wu L, Chung JY, et al. Time-Dependent Changes in Microglia Transcriptional Networks Following Traumatic Brain Injury. Front Cell Neurosci. 2019;13:307.

186. Anderson WD, Greenhalgh AD, Takwale A, David S, Vadigepalli R. Novel Influences of IL-10 on CNS Inflammation Revealed by Integrated Analyses of Cytokine Networks and Microglial Morphology. Front Cell Neurosci. 2017;11:233.

187. Greenhalgh AD, David S, Bennett FC. Immune cell regulation of glia during CNS injury and disease. Nat Rev Neurosci. 2020;21(3):139-52.

188. Glass CK, Saijo K, Winner B, Marchetto MC, Gage FH. Mechanisms underlying inflammation in neurodegeneration. Cell. 2010;140(6):918-34.

189. Hernandez-Ontiveros DG, Tajiri N, Acosta S, Giunta B, Tan J, Borlongan CV. Microglia activation as a biomarker for traumatic brain injury. Front Neurol. 2013;4:30.

190. Kumar A, Alvarez-Croda DM, Stoica BA, Faden AI, Loane DJ. Microglial/Macrophage Polarization Dynamics following Traumatic Brain Injury. J Neurotrauma. 2016;33(19):1732-50.

191. Kettenmann H, Kirchhoff F, Verkhratsky A. Microglia: new roles for the synaptic stripper. Neuron. 2013;77(1):10-8.

192. Scheff SW, Price DA, Schmitt FA, Roberts KN, Ikonomovic MD, Mufson EJ. Synapse stability in the precuneus early in the progression of Alzheimer's disease. J Alzheimers Dis. 2013;35(3):599-609.

193. Orihuela R, McPherson CA, Harry GJ. Microglial M1/M2 polarization and metabolic states. Br J Pharmacol. 2016;173(4):649-65.

194. Frugier T, Morganti-Kossmann MC, O'Reilly D, McLean CA. In situ detection of inflammatory mediators in post mortem human brain tissue after traumatic injury. J Neurotrauma. 2010;27(3):497-507.

195. Cherry JD, Olschowka JA, O'Banion MK. Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. J Neuroinflammation. 2014;11:98.

196. Pal G, Vincze C, Renner E, Wappler EA, Nagy Z, Lovas G, et al. Time course, distribution and cell types of induction of transforming growth factor betas following middle cerebral artery occlusion in the rat brain. PLoS One. 2012;7(10):e46731.

197. Ramlackhansingh AF, Brooks DJ, Greenwood RJ, Bose SK, Turkheimer FE, Kinnunen KM, et al. Inflammation after trauma: microglial activation and traumatic brain injury. Ann Neurol. 2011;70(3):374-83.

198. Johnson VE, Stewart JE, Begbie FD, Trojanowski JQ, Smith DH, Stewart W. Inflammation and white matter degeneration persist for years after a single traumatic brain injury. Brain. 2013;136(Pt 1):28-42.

199. Kumar A, Henry RJ, Stoica BA, Loane DJ, Abulwerdi G, Bhat SA, et al. Neutral Sphingomyelinase Inhibition Alleviates LPS-Induced Microglia Activation and Neuroinflammation after Experimental Traumatic Brain Injury. J Pharmacol Exp Ther. 2019;368(3):338-52.

200. Cacci E, Ajmone-Cat MA, Anelli T, Biagioni S, Minghetti L. In vitro neuronal and glial differentiation from embryonic or adult neural precursor cells are differently affected by chronic or acute activation of microglia. Glia. 2008;56(4):412-25.

201. Loane DJ, Kumar A. Microglia in the TBI brain: The good, the bad, and the dysregulated. Exp Neurol. 2016;275 Pt 3:316-27.

202. Fan LW, Pang Y. Dysregulation of neurogenesis by neuroinflammation: key differences in neurodevelopmental and neurological disorders. Neural Regen Res. 2017;12(3):366-71.

203. Zhang QG, Laird MD, Han D, Nguyen K, Scott E, Dong Y, et al. Critical role of NADPH oxidase in neuronal oxidative damage and microglia activation following traumatic brain injury. PLoS One. 2012;7(4):e34504.

204. Wu HM, Huang SC, Vespa P, Hovda DA, Bergsneider M. Redefining the pericontusional penumbra following traumatic brain injury: evidence of deteriorating metabolic derangements based on positron emission tomography. J Neurotrauma. 2013;30(5):352-60.

205. Sun GZ, Gao FF, Zhao ZM, Sun H, Xu W, Wu LW, et al. Endoplasmic reticulum stress-induced apoptosis in the penumbra aggravates secondary damage in rats with traumatic brain injury. Neural Regen Res. 2016;11(8):1260-6.

206. Shi W, Nie D, Jin G, Chen W, Xia L, Wu X, et al. BDNF blended chitosan scaffolds for human umbilical cord MSC transplants in traumatic brain injury therapy. Biomaterials. 2012;33(11):3119-26.

207. Sofroniew MV. Astrocyte barriers to neurotoxic inflammation. Nat Rev Neurosci. 2015;16(5):249-63.

208. Simon DW, McGeachy MJ, Bayir H, Clark RS, Loane DJ, Kochanek PM. The far-reaching scope of neuroinflammation after traumatic brain injury. Nat Rev Neurol. 2017;13(3):171-91.

209. Jassam YN, Izzy S, Whalen M, McGavern DB, El Khoury J. Neuroimmunology of Traumatic Brain Injury: Time for a Paradigm Shift. Neuron. 2017;95(6):1246-65.

210. Hickman S, Izzy S, Sen P, Morsett L, El Khoury J. Microglia in neurodegeneration. Nat Neurosci. 2018;21(10):1359-69.

211. Al Nimer F, Lindblom R, Strom M, Guerreiro-Cacais AO, Parsa R, Aeinehband S, et al. Strain influences on inflammatory pathway activation, cell infiltration and complement cascade after traumatic brain injury in the rat. Brain Behav Immun. 2013;27(1):109-22.

212. Rostami E, Krueger F, Zoubak S, Dal Monte O, Raymont V, Pardini M, et al. BDNF polymorphism predicts general intelligence after penetrating traumatic brain injury. PLoS One. 2011;6(11):e27389.

213. Franco-Bocanegra DK, McAuley C, Nicoll JAR, Boche D. Molecular Mechanisms of Microglial Motility: Changes in Ageing and Alzheimer's Disease. Cells. 2019;8(6).

214. Gerhard A, Schwarz J, Myers R, Wise R, Banati RB. Evolution of microglial activation in patients after ischemic stroke: a [11C](R)-PK11195 PET study. Neuroimage. 2005;24(2):591-5.

215. Jacobowitz DM, Cole JT, McDaniel DP, Pollard HB, Watson WD. Microglia activation along the corticospinal tract following traumatic brain injury in the rat: a neuroanatomical study. Brain Res. 2012;1465:80-9.

216. Karve IP, Taylor JM, Crack PJ. The contribution of astrocytes and microglia to traumatic brain injury. Br J Pharmacol. 2016;173(4):692-702.

217. Tam WM, C. . Bipolar/rod-shaped microglia are proliferating microglia with distinct M1/M2 phenotypes. Sci Rep. 2015;4.

218. Davis BM, Salinas-Navarro M, Cordeiro MF, Moons L, De Groef L. Characterizing microglia activation: a spatial statistics approach to maximize information extraction. Sci Rep. 2017;7(1):1576.

219. Matias I, Morgado J, Gomes FCA. Astrocyte Heterogeneity: Impact to Brain Aging and Disease. Front Aging Neurosci. 2019;11:59.

220. Kim Y, Park J, Choi YK. The Role of Astrocytes in the Central Nervous System Focused on BK Channel and Heme Oxygenase Metabolites: A Review. Antioxidants (Basel). 2019;8(5).

221. Hu X, Yuan Y, Wang D, Su Z. Heterogeneous astrocytes: Active players in CNS. Brain Res Bull. 2016;125:1-18.

222. Cheng X, Wang J, Sun X, Shao L, Guo Z, Li Y. Morphological and functional alterations of astrocytes responding to traumatic brain injury. J Integr Neurosci. 2019;18(2):203-15.

223. Thau-Zuchman O, Gomes RN, Dyall SC, Davies M, Priestley JV, Groenendijk M, et al. Brain Phospholipid Precursors Administered Post-Injury Reduce Tissue Damage and Improve Neurological Outcome in Experimental Traumatic Brain Injury. J Neurotrauma. 2019;36(1):25-42.

224. Walker CD, Risher WC, Risher ML. Regulation of Synaptic Development by Astrocyte Signaling Factors and Their Emerging Roles in Substance Abuse. Cells. 2020;9(2).

225. Tress O, Maglione M, May D, Pivneva T, Richter N, Seyfarth J, et al. Panglial gap junctional communication is essential for maintenance of myelin in the CNS. J Neurosci. 2012;32(22):7499-518.

226. Clarke LE, Barres BA. Emerging roles of astrocytes in neural circuit development. Nat Rev Neurosci. 2013;14(5):311-21.

227. Kucukdereli H, Allen NJ, Lee AT, Feng A, Ozlu MI, Conatser LM, et al. Control of excitatory CNS synaptogenesis by astrocyte-secreted proteins Hevin and SPARC. Proc Natl Acad Sci U S A. 2011;108(32):E440-9.

228. Liddelow SA, Barres BA. Reactive Astrocytes: Production, Function, and Therapeutic Potential. Immunity. 2017;46(6):957-67.

229. Li T, Chen X, Zhang C, Zhang Y, Yao W. An update on reactive astrocytes in chronic pain. J Neuroinflammation. 2019;16(1):140.

230. Wang D, Fawcett J. The perineuronal net and the control of CNS plasticity. Cell Tissue Res. 2012;349(1):147-60.

231. Liu Z, Li Y, Cui Y, Roberts C, Lu M, Wilhelmsson U, et al. Beneficial effects of gfap/vimentin reactive astrocytes for axonal remodeling and motor behavioral recovery in mice after stroke. Glia. 2014;62(12):2022-33.

232. Thelin EP, Hall CE, Tyzack GE, Frostell A, Giorgi-Coll S, Alam A, et al. Delineating Astrocytic Cytokine Responses in a Human Stem Cell Model of Neural Trauma. J Neurotrauma. 2020;37(1):93-105.

233. Barres BA. The mystery and magic of glia: a perspective on their roles in health and disease. Neuron. 2008;60(3):430-40.

234. Liddelow SA, Guttenplan KA, Clarke LE, Bennett FC, Bohlen CJ, Schirmer L, et al. Neurotoxic reactive astrocytes are induced by activated microglia. Nature. 2017;541(7638):481-7.

235. Thelin E, Al Nimer F, Frostell A, Zetterberg H, Blennow K, Nystrom H, et al. A Serum Protein Biomarker Panel Improves Outcome Prediction in Human Traumatic Brain Injury. J Neurotrauma. 2019;36(20):2850-62.

236. Anderson MA, Burda JE, Ren Y, Ao Y, O'Shea TM, Kawaguchi R, et al. Astrocyte scar formation aids central nervous system axon regeneration. Nature. 2016;532(7598):195-200.

237. Sofroniew MV. Astrocyte Reactivity: Subtypes, States, and Functions in CNS Innate Immunity. Trends Immunol. 2020;41(9):758-70.

238. Early AN, Gorman AA, Van Eldik LJ, Bachstetter AD, Morganti JM. Effects of advanced age upon astrocyte-specific responses to acute traumatic brain injury in mice. J Neuroinflammation. 2020;17(1):115.

239. Wu H, Mahmood A, Lu D, Jiang H, Xiong Y, Zhou D, et al. Attenuation of astrogliosis and modulation of endothelial growth factor receptor in lipid rafts by simvastatin after traumatic brain injury. J Neurosurg. 2010;113(3):591-7.

240. Madathil SK, Carlson SW, Brelsfoard JM, Ye P, D'Ercole AJ, Saatman KE. Astrocyte-Specific Overexpression of Insulin-Like Growth Factor-1 Protects Hippocampal Neurons and Reduces Behavioral Deficits following Traumatic Brain Injury in Mice. PLoS One. 2013;8(6):e67204.

241. Toivola DM, Strnad P, Habtezion A, Omary MB. Intermediate filaments take the heat as stress proteins. Trends Cell Biol. 2010;20(2):79-91.

242. Plog BA, Dashnaw ML, Hitomi E, Peng W, Liao Y, Lou N, et al. Biomarkers of traumatic injury are transported from brain to blood via the glymphatic system. J Neurosci. 2015;35(2):518-26.

243. Ren Z, Iliff JJ, Yang L, Yang J, Chen X, Chen MJ, et al. 'Hit & Run' model of closed-skull traumatic brain injury (TBI) reveals complex patterns of post-traumatic AQP4 dysregulation. J Cereb Blood Flow Metab. 2013;33(6):834-45.

244. Liang F, Luo C, Xu G, Su F, He X, Long S, et al. Deletion of aquaporin-4 is neuroprotective during the acute stage of micro traumatic brain injury in mice. Neurosci Lett. 2015;598:29-35.

245. Roth TL, Nayak D, Atanasijevic T, Koretsky AP, Latour LL, McGavern DB. Transcranial amelioration of inflammation and cell death after brain injury. Nature. 2014;505(7482):223-8.

246. Robel S. Astroglial Scarring and Seizures: A Cell Biological Perspective on Epilepsy. Neuroscientist. 2017;23(2):152-68.

247. Chitturi J, Li Y, Santhakumar V, Kannurpatti SS. Consolidated Biochemical Profile of Subacute Stage Traumatic Brain Injury in Early Development. Front Neurosci. 2019;13:431.

248. Killen MJ, Giorgi-Coll S, Helmy A, Hutchinson PJ, Carpenter KL. Metabolism and inflammation: implications for traumatic brain injury therapeutics. Expert Rev Neurother. 2019;19(3):227-42.

249. Shimmura C, Suda S, Tsuchiya KJ, Hashimoto K, Ohno K, Matsuzaki H, et al. Alteration of plasma glutamate and glutamine levels in children with high-functioning autism. PLoS One. 2011;6(10):e25340.

250. Shannon RJ, van der Heide S, Carter EL, Jalloh I, Menon DK, Hutchinson PJ, et al. Extracellular N-Acetylaspartate in Human Traumatic Brain Injury. J Neurotrauma. 2016;33(4):319-29.

251. Molla G, Chaves-Sanjuan A, Savinelli A, Nardini M, Pollegioni L. Structure and kinetic properties of human d-aspartate oxidase, the enzyme-controlling d-aspartate levels in brain. FASEB J. 2020;34(1):1182-97.

252. Dorsett CR, McGuire JL, DePasquale EA, Gardner AE, Floyd CL, McCullumsmith RE. Glutamate Neurotransmission in Rodent Models of Traumatic Brain Injury. J Neurotrauma. 2017;34(2):263-72.

253. Masse IO, Moquin L, Provost C, Guay S, Gratton A, De Beaumont L. A Novel and Translational Rat Model of Concussion Combining Force and Rotation with In Vivo Cerebral Microdialysis. J Vis Exp. 2019(149).

254. Croall I, Smith FE, Blamire AM. Magnetic Resonance Spectroscopy for Traumatic Brain Injury. Top Magn Reson Imaging. 2015;24(5):267-74.

255. Parizel PPCD. Traumatic Neuroemergency: Imaging Patients with Traumatic Brain Injury—An Introduction. In: Hodler JK-H, R.; Von Schulthness, G.K. , editor. Diseases of the Brain, Head and Neck, Spine 2020–2023: Diagnostic Imaging. New York: Springer Publishers; 2020. p. 77-92.

256. Kierans AS, Kirov, II, Gonen O, Haemer G, Nisenbaum E, Babb JS, et al. Myoinositol and glutamate complex neurometabolite abnormality after mild traumatic brain injury. Neurology. 2014;82(6):521-8.

257. Jalloh I, Helmy A, Howe DJ, Shannon RJ, Grice P, Mason A, et al. A Comparison of Oxidative Lactate Metabolism in Traumatically Injured Brain and Control Brain. J Neurotrauma. 2018;35(17):2025-35.

258. Zweckberger K, Hackenberg K, Jung CS, Hertle DN, Kiening KL, Unterberg AW, et al. Cerebral metabolism after early decompression craniotomy following controlled cortical impact injury in rats. Neurol Res. 2011;33(8):875-80.

259. Qian Z, Lin Y, Xing J, Qiu Y, Ren L. Expression and functions of glutamate and gamma-aminobutyric acid transporters in ischemic models. Mol Med Rep. 2018;17(6):8196-202.

260. Belov Kirdajova D, Kriska J, Tureckova J, Anderova M. Ischemia-Triggered Glutamate Excitotoxicity From the Perspective of Glial Cells. Front Cell Neurosci. 2020;14:51.

261. Briones TL, Rogozinska M, Woods J. Modulation of ischemia-induced NMDAR1 activation by environmental enrichment decreases oxidative damage. J Neurotrauma. 2011;28(12):2485-92.

262. Xhima K, Weber-Adrian D, Silburt J. Glutamate Induces Blood-Brain Barrier Permeability through Activation of N-Methyl-D-Aspartate Receptors. J Neurosci. 2016;36(49):12296-8.

263. Yi JH, Hazell AS. Excitotoxic mechanisms and the role of astrocytic glutamate transporters in traumatic brain injury. Neurochem Int. 2006;48(5):394-403.

264. Chamard E, Lassonde M, Henry L, Tremblay J, Boulanger Y, De Beaumont L, et al. Neurometabolic and microstructural alterations following a sports-related concussion in female athletes. Brain Inj. 2013;27(9):1038-46.

265. Jalloh I, Carpenter KL, Grice P, Howe DJ, Mason A, Gallagher CN, et al. Glycolysis and the pentose phosphate pathway after human traumatic brain injury: microdialysis studies using 1,2-(13)C2 glucose. J Cereb Blood Flow Metab. 2015;35(1):111-20.

266. Kurtz P, Rocha EEM. Nutrition Therapy, Glucose Control, and Brain Metabolism in Traumatic Brain Injury: A Multimodal Monitoring Approach. Front Neurosci. 2020;14:190.

267. Fischer TD, Hylin MJ, Zhao J, Moore AN, Waxham MN, Dash PK. Altered Mitochondrial Dynamics and TBI Pathophysiology. Front Syst Neurosci. 2016;10:29.

268. Kumar Sahel DK, M.; Raj, K.; Sharma, S.;, Singh, S. Mitochondrial dysfunctioning and neuroinflammation: Recent highlights on the possible mechanisms involved in Traumatic Brain Injury. Neurosci Lett. 2019;25(134347).

269. Venkat PC, M.; Chen, J. . New insights into coupling and uncoupling of cerebral blood flow and metabolism in the brain. Croat Med J. 2016;57:223-8.

270. Hajiaghamemar M, Kilbaugh T, Arbogast KB, Master CL, Margulies SS. Using Serum Amino Acids to Predict Traumatic Brain Injury: A Systematic Approach to Utilize Multiple Biomarkers. Int J Mol Sci. 2020;21(5).

271. Algattas H, Huang JH. Traumatic Brain Injury pathophysiology and treatments: early, intermediate, and late phases post-injury. Int J Mol Sci. 2013;15(1):309-41.

272. Koenig JB, Dulla CG. Dysregulated Glucose Metabolism as a Therapeutic Target to Reduce Post-traumatic Epilepsy. Front Cell Neurosci. 2018;12:350.

273. Xiong Y, Mahmood A, Chopp M. Animal models of traumatic brain injury. Nat Rev Neurosci. 2013;14(2):128-42.

274. Carpenter KL, Young AM, Hutchinson PJ. Advanced monitoring in traumatic brain injury: microdialysis. Curr Opin Crit Care. 2017;23(2):103-9.

275. Patet C, Suys T, Carteron L, Oddo M. Cerebral Lactate Metabolism After Traumatic Brain Injury. Curr Neurol Neurosci Rep. 2016;16(4):31.

276. Barros LF, Weber B. CrossTalk proposal: an important astrocyte-to-neuron lactate shuttle couples neuronal activity to glucose utilisation in the brain. J Physiol. 2018;596(3):347-50.

277. Lozano A, Franchi F, Seastres RJ, Oddo M, Lheureux O, Badenes R, et al. Glucose and Lactate Concentrations in Cerebrospinal Fluid After Traumatic Brain Injury. J Neurosurg Anesthesiol. 2020;32(2):162-9.

278. Lutton EM, Farney SK, Andrews AM, Shuvaev VV, Chuang GY, Muzykantov VR, et al. Endothelial Targeted Strategies to Combat Oxidative Stress: Improving Outcomes in Traumatic Brain Injury. Front Neurol. 2019;10:582.

279. Xu ZM, Yuan F, Liu YL, Ding J, Tian HL. Glibenclamide Attenuates Blood-Brain Barrier Disruption in Adult Mice after Traumatic Brain Injury. J Neurotrauma. 2017;34(4):925-33.

280. Xu L, Nirwane A, Yao Y. Basement membrane and blood-brain barrier. Stroke Vasc Neurol. 2019;4(2):78-82.

281. Abbott NJ, Patabendige AA, Dolman DE, Yusof SR, Begley DJ. Structure and function of the blood-brain barrier. Neurobiol Dis. 2010;37(1):13-25.

282. Armulik A, Genove G, Betsholtz C. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. Dev Cell. 2011;21(2):193-215.

283. Geranmayeh MH, Rahbarghazi R, Farhoudi M. Targeting pericytes for neurovascular regeneration. Cell Commun Signal. 2019;17(1):26.

284. Wong AD, Ye M, Levy AF, Rothstein JD, Bergles DE, Searson PC. The blood-brain barrier: an engineering perspective. Front Neuroeng. 2013;6:7.

285. Bhat AA, Uppada S, Achkar IW, Hashem S, Yadav SK, Shanmugakonar M, et al. Tight Junction Proteins and Signaling Pathways in Cancer and Inflammation: A Functional Crosstalk. Front Physiol. 2018;9:1942.

286. Mora P, Hollier PL, Guimbal S, Abelanet A, Diop A, Cornuault L, et al. Blood-brain barrier genetic disruption leads to protective barrier formation at the Glia Limitans. PLoS Biol. 2020;18(11):e3000946.

287. Boulay AC, Cisternino S, Cohen-Salmon M. Immunoregulation at the gliovascular unit in the healthy brain: A focus on Connexin 43. Brain Behav Immun. 2016;56:1-9.

288. Nakamura S, Irie K, Tanaka H, Nishikawa K, Suzuki H, Saitoh Y, et al. Morphologic determinant of tight junctions revealed by claudin-3 structures. Nat Commun. 2019;10(1):816.

289. Yang S, Jin H, Zhu Y, Wan Y, Opoku EN, Zhu L, et al. Diverse Functions and Mechanisms of Pericytes in Ischemic Stroke. Curr Neuropharmacol. 2017;15(6):892-905.

290. Yamazaki T, Mukouyama YS. Tissue Specific Origin, Development, and Pathological Perspectives of Pericytes. Front Cardiovasc Med. 2018;5:78.

291. Kim SH, Turnbull J, Guimond S. Extracellular matrix and cell signalling: the dynamic cooperation of integrin, proteoglycan and growth factor receptor. J Endocrinol. 2011;209(2):139-51.

292. Yurchenco PD. Basement membranes: cell scaffoldings and signaling platforms. Cold Spring Harb Perspect Biol. 2011;3(2).

293. Lepelletier FX, Mann DM, Robinson AC, Pinteaux E, Boutin H. Early changes in extracellular matrix in Alzheimer's disease. Neuropathol Appl Neurobiol. 2017;43(2):167-82.

294. Thomsen MS, Routhe LJ, Moos T. The vascular basement membrane in the healthy and pathological brain. J Cereb Blood Flow Metab. 2017;37(10):3300-17.

295. Raven EP, Lu PH, Tishler TA, Heydari P, Bartzokis G. Increased iron levels and decreased tissue integrity in hippocampus of Alzheimer's disease detected in vivo with magnetic resonance imaging. J Alzheimers Dis. 2013;37(1):127-36.

296. Montagne A, Barnes SR, Sweeney MD, Halliday MR, Sagare AP, Zhao Z, et al. Blood-brain barrier breakdown in the aging human hippocampus. Neuron. 2015;85(2):296-302.

297. Donkin JJ, Nimmo AJ, Cernak I, Blumbergs PC, Vink R. Substance P is associated with the development of brain edema and functional deficits after traumatic brain injury. J Cereb Blood Flow Metab. 2009;29(8):1388-98.

298. Shlosberg D, Benifla M, Kaufer D, Friedman A. Blood-brain barrier breakdown as a therapeutic target in traumatic brain injury. Nat Rev Neurol. 2010;6(7):393-403.

299. Turner RJ, Sharp FR. Implications of MMP9 for Blood Brain Barrier Disruption and Hemorrhagic Transformation Following Ischemic Stroke. Front Cell Neurosci. 2016;10:56.

300. Nag S, Manias JL, Stewart DJ. Expression of endothelial phosphorylated caveolin-1 is increased in brain injury. Neuropathol Appl Neurobiol. 2009;35(4):417-26.

301. Sowa G. Caveolae, caveolins, cavins, and endothelial cell function: new insights. Front Physiol. 2012;2:120.

302. Lu J, Goh SJ, Tng PY, Deng YY, Ling EA, Moochhala S. Systemic inflammatory response following acute traumatic brain injury. Front Biosci (Landmark Ed). 2009;14:3795-813.

303. Sorby-Adams AJ, Leonard AV, Hoving JW, Yassi N, Vink R, Wells AJ, et al. NK1-r Antagonist Treatment Comparable to Decompressive Craniectomy in Reducing Intracranial Pressure Following Stroke. Front Neurosci. 2019;13:681.

304. Szczygielski J, Glameanu C, Muller A, Klotz M, Sippl C, Hubertus V, et al. Changes in Posttraumatic Brain Edema in Craniectomy-Selective Brain Hypothermia Model Are Associated With Modulation of Aquaporin-4 Level. Front Neurol. 2018;9:799.

305. Chodobski A, Zink BJ, Szmydynger-Chodobska J. Blood-brain barrier pathophysiology in traumatic brain injury. Transl Stroke Res. 2011;2(4):492-516.

306. Begley CG, Ellis LM. Drug development: Raise standards for preclinical cancer research. Nature. 2012;483(7391):531-3.

307. Thal SC, Luh C, Schaible EV, Timaru-Kast R, Hedrich J, Luhmann HJ, et al. Volatile anesthetics influence blood-brain barrier integrity by modulation of tight junction protein expression in traumatic brain injury. PLoS One. 2012;7(12):e50752.

308. Szarka N, Toth L, Czigler A, Kellermayer Z, Ungvari Z, Amrein K, et al. Single Mild Traumatic Brain Injury Induces Persistent Disruption of the Blood-Brain Barrier, Neuroinflammation and Cognitive Decline in Hypertensive Rats. Int J Mol Sci. 2019;20(13).

309. Montagne A, Nikolakopoulou AM, Zhao Z, Sagare AP, Si G, Lazic D, et al. Pericyte degeneration causes white matter dysfunction in the mouse central nervous system. Nat Med. 2018;24(3):326-37.

310. Xiong Y, Zhang Y, Mahmood A, Meng Y, Zhang ZG, Morris DC, et al. Neuroprotective and neurorestorative effects of thymosin beta4 treatment initiated 6 hours after traumatic brain injury in rats. J Neurosurg. 2012;116(5):1081-92.

311. Hooper C, Pinteaux-Jones F, Fry VA, Sevastou IG, Baker D, Heales SJ, et al. Differential effects of albumin on microglia and macrophages; implications for neurodegeneration following blood-brain barrier damage. J Neurochem. 2009;109(3):694-705.

312. Ralay Ranaivo H, Wainwright MS. Albumin activates astrocytes and microglia through mitogen-activated protein kinase pathways. Brain Res. 2010;1313:222-31.

313. Kuriakose M, Younger D, Ravula AR, Alay E, Rama Rao KV, Chandra N. Synergistic Role of Oxidative Stress and Blood-Brain Barrier Permeability as Injury Mechanisms in the Acute Pathophysiology of Blast-induced Neurotrauma. Sci Rep. 2019;9(1):7717.

314. Abrahamson EE, Ikonomovic MD. Brain injury-induced dysfunction of the blood brain barrier as a risk for dementia. Exp Neurol. 2020;328:113257.

315. Jha RM, Kochanek PM, Simard JM. Pathophysiology and treatment of cerebral edema in traumatic brain injury. Neuropharmacology. 2019;145(Pt B):230-46.

316. Kochanek PM, Bramlett HM, Dixon CE, Shear DA, Dietrich WD, Schmid KE, et al. Approach to Modeling, Therapy Evaluation, Drug Selection, and Biomarker Assessments for a Multicenter Pre-Clinical Drug Screening Consortium for Acute Therapies in Severe Traumatic Brain Injury: Operation Brain Trauma Therapy. J Neurotrauma. 2016;33(6):513-22.

317. Battey TW, Karki M, Singhal AB, Wu O, Sadaghiani S, Campbell BC, et al. Brain edema predicts outcome after nonlacunar ischemic stroke. Stroke. 2014;45(12):3643-8.

318. Zhang ZY, Jiang M, Fang J, Yang MF, Zhang S, Yin YX, et al. Enhanced Therapeutic Potential of Nano-Curcumin Against Subarachnoid Hemorrhage-Induced Blood-Brain Barrier Disruption Through Inhibition of Inflammatory Response and Oxidative Stress. Mol Neurobiol. 2017;54(1):1-14.

319. Hudak AM, Peng L, Marquez de la Plata C, Thottakara J, Moore C, Harper C, et al. Cytotoxic and vasogenic cerebral oedema in traumatic brain injury: assessment with FLAIR and DWI imaging. Brain Inj. 2014;28(12):1602-9.

320. Brogan ME, Manno EM. Treatment of malignant brain edema and increased intracranial pressure after stroke. Curr Treat Options Neurol. 2015;17(1):327.

321. Stokum JA, Gerzanich V, Simard JM. Molecular pathophysiology of cerebral edema. J Cereb Blood Flow Metab. 2016;36(3):513-38.

322. Michinaga S, Koyama Y. Pathogenesis of brain edema and investigation into anti-edema drugs. Int J Mol Sci. 2015;16(5):9949-75.

323. Zhang S, He WB, Chen NH. Causes of death among persons who survive an acute ischemic stroke. Curr Neurol Neurosci Rep. 2014;14(8):467.

324. Yeoh S, Bell ED, Monson KL. Distribution of blood-brain barrier disruption in primary blast injury. Ann Biomed Eng. 2013;41(10):2206-14.

325. Readnower RD, Chavko M, Adeeb S, Conroy MD, Pauly JR, McCarron RM, et al. Increase in blood-brain barrier permeability, oxidative stress, and activated microglia in a rat model of blast-induced traumatic brain injury. J Neurosci Res. 2010;88(16):3530-9.

326. Habgood MD, Bye N, Dziegielewska KM, Ek CJ, Lane MA, Potter A, et al. Changes in blood-brain barrier permeability to large and small molecules following traumatic brain injury in mice. Eur J Neurosci. 2007;25(1):231-8.

327. Guan Y, Li L, Chen J, Lu H. Effect of AQP4-RNAi in treating traumatic brain edema: Multi-modal MRI and histopathological changes of early stage edema in a rat model. Exp Ther Med. 2020;19(3):2029-36.

328. Ren W, Jing G, Shen Q, Yao X, Jing Y, Lin F, et al. Occludin and connexin 43 expression contribute to the pathogenesis of traumatic brain edema. Neural Regen Res. 2013;8(29):2703-12.

329. Guo Q, Sayeed I, Baronne LM, Hoffman SW, Guennoun R, Stein DG. Progesterone administration modulates AQP4 expression and edema after traumatic brain injury in male rats. Exp Neurol. 2006;198(2):469-78.

330. Albertini R, Bianchi R. Aquaporins and glia. Curr Neuropharmacol. 2010;8(2):84-91.

331. Szu JI, Binder DK. The Role of Astrocytic Aquaporin-4 in Synaptic Plasticity and Learning and Memory. Front Integr Neurosci. 2016;10:8.

332. Papadopoulos MC, Verkman AS. Aquaporin-4 gene disruption in mice reduces brain swelling and mortality in pneumococcal meningitis. J Biol Chem. 2005;280(14):13906-12.

333. Huang Y, Li SN, Zhou XY, Zhang LX, Chen GX, Wang TH, et al. The Dual Role of AQP4 in Cytotoxic and Vasogenic Edema Following Spinal Cord Contusion and Its Possible Association With Energy Metabolism via COX5A. Front Neurosci. 2019;13:584.

334. Fukuda AM, Badaut J. Aquaporin 4: a player in cerebral edema and neuroinflammation. J Neuroinflammation. 2012;9:279.

335. Bergold PJ. Treatment of traumatic brain injury with anti-inflammatory drugs. Exp Neurol. 2016;275 Pt 3:367-80.

336. Parker TM, Nguyen AH, Rabang JR, Patil AA, Agrawal DK. The danger zone: Systematic review of the role of HMGB1 danger signalling in traumatic brain injury. Brain Inj. 2017;31(1):2-8.

337. Nasr IW, Chun Y, Kannan S. Neuroimmune responses in the developing brain following traumatic brain injury. Exp Neurol. 2019;320:112957.

338. Needham EJ, Helmy A, Zanier ER, Jones JL, Coles AJ, Menon DK. The immunological response to traumatic brain injury. J Neuroimmunol. 2019;332:112-25.

339. Csuka E, Morganti-Kossmann MC, Lenzlinger PM, Joller H, Trentz O, Kossmann T. IL-10 levels in cerebrospinal fluid and serum of patients with severe traumatic brain injury: relationship to IL-6, TNF-alpha, TGF-beta1 and blood-brain barrier function. J Neuroimmunol. 1999;101(2):211-21.

340. Edwards KA, Pattinson CL, Guedes VA, Peyer J, Moore C, Davis T, et al. Inflammatory Cytokines Associate With Neuroimaging After Acute Mild Traumatic Brain Injury. Front Neurol. 2020;11:348.

341. Kempuraj D, Ahmed ME, Selvakumar GP, Thangavel R, Raikwar SP, Zaheer SA, et al. Mast Cell Activation, Neuroinflammation, and Tight Junction Protein Derangement in Acute Traumatic Brain Injury. Mediators Inflamm. 2020;2020:4243953.

342. Sandhir R, Onyszchuk G, Berman NE. Exacerbated glial response in the aged mouse hippocampus following controlled cortical impact injury. Exp Neurol. 2008;213(2):372-80.

343. Pertusa M, Garcia-Matas S, Rodriguez-Farre E, Sanfeliu C, Cristofol R. Astrocytes aged in vitro show a decreased neuroprotective capacity. J Neurochem. 2007;101(3):794-805.

344. Mejias NH, Martinez CC, Stephens ME, de Rivero Vaccari JP. Contribution of the inflammasome to inflammaging. J Inflamm (Lond). 2018;15:23.

345. Rebelo-Marques A, De Sousa Lages A, Andrade R, Ribeiro CF, Mota-Pinto A, Carrilho F, et al. Aging Hallmarks: The Benefits of Physical Exercise. Front Endocrinol (Lausanne). 2018;9:258.

346. Haruwaka K, Ikegami A, Tachibana Y, Ohno N, Konishi H, Hashimoto A, et al. Dual microglia effects on blood brain barrier permeability induced by systemic inflammation. Nat Commun. 2019;10(1):5816.

347. Hsieh CL, Kim CC, Ryba BE, Niemi EC, Bando JK, Locksley RM, et al. Traumatic brain injury induces macrophage subsets in the brain. Eur J Immunol. 2013;43(8):2010-22.

348. Rhodes J. Peripheral immune cells in the pathology of traumatic brain injury? Curr Opin Crit Care. 2011;17(2):122-30.

349. Jin X, Ishii H, Bai Z, Itokazu T, Yamashita T. Temporal changes in cell marker expression and cellular infiltration in a controlled cortical impact model in adult male C57BL/6 mice. PLoS One. 2012;7(7):e41892.

350. Gadani SP, Cronk JC, Norris GT, Kipnis J. IL-4 in the brain: a cytokine to remember. J Immunol. 2012;189(9):4213-9.

351. Walsh JT, Hendrix S, Boato F, Smirnov I, Zheng J, Lukens JR, et al. MHCII-independent CD4+ T cells protect injured CNS neurons via IL-4. J Clin Invest. 2015;125(2):699-714.

352. PrabhuDas MR, Baldwin CL, Bollyky PL, Bowdish DME, Drickamer K, Febbraio M, et al. A Consensus Definitive Classification of Scavenger Receptors and Their Roles in Health and Disease. J Immunol. 2017;198(10):3775-89.

353. Corrigan F, Vink R, Turner RJ. Inflammation in acute CNS injury: a focus on the role of substance P. Br J Pharmacol. 2016;173(4):703-15.

354. Peng L, Agogo GO, Guo J, Yan M. Substance P and fibrotic diseases. Neuropeptides. 2019;76:101941.

355. Tsunoda K, Hashimoto S, Kuroda H, Ishii T, Takazawa M. Exploring the Relation between Glottal Closure and Plasma Substance P: A Study Protocol. Tohoku J Exp Med. 2019;249(4):237-40.

356. Gilhus NE, Deuschl G. Neuroinflammation - a common thread in neurological disorders. Nat Rev Neurol. 2019;15(8):429-30.

357. Wofford KL, Loane DJ, Cullen DK. Acute drivers of neuroinflammation in traumatic brain injury. Neural Regen Res. 2019;14(9):1481-9.

358. Turner RJ, Vink R. The role of substance p in ischaemic brain injury. Brain Sci. 2013;3(1):123-42.

359. Marrone MC, Morabito A, Giustizieri M, Chiurchiu V, Leuti A, Mattioli M, et al. TRPV1 channels are critical brain inflammation detectors and neuropathic pain biomarkers in mice. Nat Commun. 2017;8:15292.

360. Schlereth T, Schukraft J, Kramer-Best HH, Geber C, Ackermann T, Birklein F. Interaction of calcitonin gene related peptide (CGRP) and substance P (SP) in human skin. Neuropeptides. 2016;59:57-62.

361. Psen AG, A. . Use of magnesium in traumatic brain injury. Neurotherapeutics. 2010;7:91-9.

362. Vink R, van den Heuvel C. Substance P antagonists as a therapeutic approach to improving outcome following traumatic brain injury. Neurotherapeutics. 2010;7(1):74-80.

363. Genet GF, Bentzer P, Ostrowski SR, Johansson PI. Resuscitation with Pooled and Pathogen-Reduced Plasma Attenuates the Increase in Brain Water Content following Traumatic Brain Injury and Hemorrhagic Shock in Rats. J Neurotrauma. 2017;34(5):1054-62.

364. Genet GF, Bentzer P, Hansen MB, Ostrowski SR, Johansson PI. Effects of propranolol and clonidine on brain edema, blood-brain barrier permeability, and endothelial glycocalyx disruption after fluid percussion brain injury in the rat. J Trauma Acute Care Surg. 2018;84(1):89-96.

365. Rodriguez PL, Jiang S, Fu Y, Avraham S, Avraham HK. The proinflammatory peptide substance P promotes blood-brain barrier breaching by breast cancer cells through changes in microvascular endothelial cell tight junctions. Int J Cancer. 2014;134(5):1034-44.

366. Kubale V, Abramovic Z, Pogacnik A, Heding A, Sentjurc M, Vrecl M. Evidence for a role of caveolin-1 in neurokinin-1 receptor plasma-membrane localization, efficient signaling, and interaction with beta-arrestin 2. Cell Tissue Res. 2007;330(2):231-45.

367. Li PC, Chen WC, Chang LC, Lin SC. Substance P acts via the neurokinin receptor 1 to elicit bronchoconstriction, oxidative stress, and upregulated ICAM-1 expression after oil smoke exposure. Am J Physiol Lung Cell Mol Physiol. 2008;294(5):L912-20.

368. Cash A, Theus MH. Mechanisms of Blood-Brain Barrier Dysfunction in Traumatic Brain Injury. Int J Mol Sci. 2020;21(9).

369. Harris TC, de Rooij R, Kuhl E. The Shrinking Brain: Cerebral Atrophy Following Traumatic Brain Injury. Ann Biomed Eng. 2019;47(9):1941-59.

370. Farbota KD, Sodhi A, Bendlin BB, McLaren DG, Xu G, Rowley HA, et al. Longitudinal volumetric changes following traumatic brain injury: a tensor-based morphometry study. J Int Neuropsychol Soc. 2012;18(6):1006-18.

371. Johnstone VP, Wright DK, Wong K, O'Brien TJ, Rajan R, Shultz SR. Experimental Traumatic Brain Injury Results in Long-Term Recovery of Functional Responsiveness in Sensory Cortex but Persisting Structural Changes and Sensorimotor, Cognitive, and Emotional Deficits. J Neurotrauma. 2015;32(17):1333-46.

372. Nudo RJ. Recovery after brain injury: mechanisms and principles. Front Hum Neurosci. 2013;7:887.

373. Wolf JA, Koch PF. Disruption of Network Synchrony and Cognitive Dysfunction After Traumatic Brain Injury. Front Syst Neurosci. 2016;10:43.

374. Oehr L, Anderson J. Diffusion-Tensor Imaging Findings and Cognitive Function Following Hospitalized Mixed-Mechanism Mild Traumatic Brain Injury: A Systematic Review and Meta-Analysis. Arch Phys Med Rehabil. 2017;98(11):2308-19.

375. Chung S, Fieremans E, Wang X, Kucukboyaci NE, Morton CJ, Babb J, et al. White Matter Tract Integrity: An Indicator of Axonal Pathology after Mild Traumatic Brain Injury. J Neurotrauma. 2018;35(8):1015-20.

376. Marion CM, Radomski KL, Cramer NP, Galdzicki Z, Armstrong RC. Experimental Traumatic Brain Injury Identifies Distinct Early and Late Phase Axonal Conduction Deficits of White Matter Pathophysiology, and Reveals Intervening Recovery. J Neurosci. 2018;38(41):8723-36.

377. Filley CM, Fields RD. White matter and cognition: making the connection. J Neurophysiol. 2016;116(5):2093-104.

378. Shapiro JS, Silk T, Takagi M, Anderson N, Clarke C, Davis GA, et al. Examining Microstructural White Matter Differences between Children with Typical and Those with Delayed Recovery Two Weeks Post-Concussion. J Neurotrauma. 2020;37(11):1300-5.

379. Hill CS, Coleman MP, Menon DK. Traumatic Axonal Injury: Mechanisms and Translational Opportunities. Trends Neurosci. 2016;39(5):311-24.

380. Johnson VE, Stewart W, Smith DH. Axonal pathology in traumatic brain injury. Exp Neurol. 2013;246:35-43.

381. Tang-Schomer MD, Patel AR, Baas PW, Smith DH. Mechanical breaking of microtubules in axons during dynamic stretch injury underlies delayed elasticity, microtubule disassembly, and axon degeneration. FASEB J. 2010;24(5):1401-10.

382. Wang J, Fox MA, Povlishock JT. Diffuse traumatic axonal injury in the optic nerve does not elicit retinal ganglion cell loss. J Neuropathol Exp Neurol. 2013;72(8):768-81.

383. Reeves TM, Smith TL, Williamson JC, Phillips LL. Unmyelinated axons show selective rostrocaudal pathology in the corpus callosum after traumatic brain injury. J Neuropathol Exp Neurol. 2012;71(3):198-210.

384. McKerracher L, Rosen KM. MAG, myelin and overcoming growth inhibition in the CNS. Front Mol Neurosci. 2015;8:51.

385. Clarner T, Diederichs F, Berger K, Denecke B, Gan L, van der Valk P, et al. Myelin debris regulates inflammatory responses in an experimental demyelination animal model and multiple sclerosis lesions. Glia. 2012;60(10):1468-80.

386. Duff MC, Covington NV, Hilverman C, Cohen NJ. Semantic Memory and the Hippocampus: Revisiting, Reaffirming, and Extending the Reach of Their Critical Relationship. Front Hum Neurosci. 2019;13:471.

387. Tzakis N, Holahan MR. Social Memory and the Role of the Hippocampal CA2 Region. Front Behav Neurosci. 2019;13:233.

388. Anand KS, Dhikav V. Hippocampus in health and disease: An overview. Ann Indian Acad Neurol. 2012;15(4):239-46.

389. Wible CG. Hippocampal physiology, structure and function and the neuroscience of schizophrenia: a unified account of declarative memory deficits, working memory deficits and schizophrenic symptoms. Behav Sci (Basel). 2013;3(2):298-315.

390. Tatu L, Vuillier F. Structure and vascularization of the human hippocampus. Front Neurol Neurosci. 2014;34:18-25.

391. Takano HC, D. Imaging of hippocampal circuits in epilepsy. In: Noebels JLA, M.; Rogawski, M.A., editor. Jasper's Basic Mechanisms of the Epilepsies (4th edition). Bethesda: National Center for Biotechnology Information (US). 2012.

392. Mouzon B, Chaytow H, Crynen G, Bachmeier C, Stewart J, Mullan M, et al. Repetitive mild traumatic brain injury in a mouse model produces learning and memory deficits accompanied by histological changes. J Neurotrauma. 2012;29(18):2761-73.

393. Mao H, Elkin BS, Genthikatti VV, Morrison B, 3rd, Yang KH. Why is CA3 more vulnerable than CA1 in experimental models of controlled cortical impact-induced brain injury? J Neurotrauma. 2013;30(17):1521-30.

394. Broussard JI, Redell JB, Zhao J, Maynard ME, Kobori N, Perez A, et al. Mild Traumatic Brain Injury Decreases Spatial Information Content and Reduces Place Field Stability of Hippocampal CA1 Neurons. J Neurotrauma. 2020;37(2):227-35.

395. Tran LD, Lifshitz J, Witgen BM, Schwarzbach E, Cohen AS, Grady MS. Response of the contralateral hippocampus to lateral fluid percussion brain injury. J Neurotrauma. 2006;23(9):1330-42.

396. Rink A, Fung KM, Trojanowski JQ, Lee VM, Neugebauer E, McIntosh TK. Evidence of apoptotic cell death after experimental traumatic brain injury in the rat. Am J Pathol. 1995;147(6):1575-83.

397. Tensaouti Y, Yu TS, Kernie SG. Apolipoprotein E regulates the maturation of injury-induced adult-born hippocampal neurons following traumatic brain injury. PLoS One. 2020;15(3):e0229240.

398. Atkins CM. Decoding hippocampal signaling deficits after traumatic brain injury. Transl Stroke Res. 2011;2(4):546-55.

399. Ngwenya LB, Danzer SC. Impact of Traumatic Brain Injury on Neurogenesis. Front Neurosci. 2018;12:1014.

400. Gao X, Deng P, Xu ZC, Chen J. Moderate traumatic brain injury causes acute dendritic and synaptic degeneration in the hippocampal dentate gyrus. PLoS One. 2011;6(9):e24566.

401. Zhou H, Chen L, Gao X, Luo B, Chen J. Moderate traumatic brain injury triggers rapid necrotic death of immature neurons in the hippocampus. J Neuropathol Exp Neurol. 2012;71(4):348-59.

402. Rola R, Mizumatsu S, Otsuka S, Morhardt DR, Noble-Haeusslein LJ, Fishman K, et al. Alterations in hippocampal neurogenesis following traumatic brain injury in mice. Exp Neurol. 2006;202(1):189-99.

403. Gao H, Zhang M. Asymmetry in the brain influenced the neurological deficits and infarction volume following the middle cerebral artery occlusion in rats. Behav Brain Funct. 2008;4:57.

404. Dinocourt C, Aungst S, Yang K, Thompson SM. Homeostatic increase in excitability in area CA1 after Schaffer collateral transection in vivo. Epilepsia. 2011;52(9):1656-65.

405. Winston CN, Chellappa D, Wilkins T, Barton DJ, Washington PM, Loane DJ, et al. Controlled cortical impact results in an extensive loss of dendritic spines that is not mediated by injury-induced amyloid-beta accumulation. J Neurotrauma. 2013;30(23):1966-72.

406. Leh SE, Schroeder C, Chen JK, Mallar Chakravarty M, Park MT, Cheung B, et al. Microstructural Integrity of Hippocampal Subregions Is Impaired after Mild Traumatic Brain Injury. J Neurotrauma. 2017;34(7):1402-11.

407. Meier TB, Savitz J, Singh R, Teague TK, Bellgowan PS. Smaller Dentate Gyrus and CA2 and CA3 Volumes Are Associated with Kynurenine Metabolites in Collegiate Football Athletes. J Neurotrauma. 2016;33(14):1349-57.

408. Suvas S. Role of Substance P Neuropeptide in Inflammation, Wound Healing, and Tissue Homeostasis. J Immunol. 2017;199(5):1543-52.

409. Jeong YM, Cheng XW, Lee KH, Lee S, Cho H, Kim W. Substance P enhances the local activation of NK1R-expressing c-kit(+) cardiac progenitor cells in right atrium of ischemia/reperfusion-injured heart. BMC Mol Cell Biol. 2020;21(1):41.

410. Ebner K, Singewald N. The role of substance P in stress and anxiety responses. Amino Acids. 2006;31(3):251-72.

411. Mihaly A. The Reactive Plasticity of Hippocampal Ionotropic Glutamate Receptors in Animal Epilepsies. Int J Mol Sci. 2019;20(5).

412. Lai JP, Lai S, Tuluc F, Tansky MF, Kilpatrick LE, Leeman SE, et al. Differences in the length of the carboxyl terminus mediate functional properties of neurokinin-1 receptor. Proc Natl Acad Sci U S A. 2008;105(34):12605-10.

413. Douglas SD, Leeman SE. Neurokinin-1 receptor: functional significance in the immune system in reference to selected infections and inflammation. Ann N Y Acad Sci. 2011;1217:83-95.

414. Lundy FT, Linden GJ. Neuropeptides and Neurogenic Mechanisms in Oral and Periodontal Inflammation. Crit Rev Oral Biol Med. 2004;15(2):82-98.

415. Wang XF, Ge TT, Fan J, Yang W, Cui RJ. The role of substance P in epilepsy and seizure disorders. Oncotarget. 2017;8(44):78225-33.

416. Zieglgansberger W. Substance P and pain chronicity. Cell Tissue Res. 2019;375(1):227-41.

417. Amaral DG, Scharfman HE, Lavenex P. The dentate gyrus: fundamental neuroanatomical organization (dentate gyrus for dummies). Prog Brain Res. 2007;163:3-22.

418. Acsady L, Katona I, Gulyas AI, Shigemoto R, Freund TF. Immunostaining for substance P receptor labels GABAergic cells with distinct termination patterns in the hippocampus. J Comp Neurol. 1997;378(3):320-36.

419. Mashaghi A, Marmalidou A, Tehrani M, Grace PM, Pothoulakis C, Dana R. Neuropeptide substance P and the immune response. Cell Mol Life Sci. 2016;73(22):4249-64.

420. Wease KN, Davies SN. Substance P selectively decreases paired pulse depression in the rat hippocampal slice. BMC Neurosci. 2005;6:66.

421. Wasterlain CG, Liu H, Mazarati AM, Baldwin RA, Shirasaka Y, Katsumori H, et al. Self-sustaining status epilepticus: a condition maintained by potentiation of glutamate receptors and by plastic changes in substance P and other peptide neuromodulators. Epilepsia. 2000;41 Suppl 6:S134-43.

422. Iftikhar K, Siddiq A, Baig SG, Zehra S. Substance P: A neuropeptide involved in the psychopathology of anxiety disorders. Neuropeptides. 2020;79:101993.

423. Gabrielian L, Helps SC, Thornton E, Turner RJ, Leonard AV, Vink R. Substance P antagonists as a novel intervention for brain edema and raised intracranial pressure. Acta Neurochir Suppl. 2013;118:201-4.

424. Lorente L, Martin MM, Almeida T, Hernandez M, Ramos L, Argueso M, et al. Serum substance P levels are associated with severity and mortality in patients with severe traumatic brain injury. Crit Care. 2015;19:192.

425. Vink R, Gabrielian L, Thornton E. The Role of Substance P in Secondary Pathophysiology after Traumatic Brain Injury. Front Neurol. 2017;8:304.

426. Zacest AC, Vink R, Manavis J, Sarvestani GT, Blumbergs PC. Substance P immunoreactivity increases following human traumatic brain injury. Acta Neurochir Suppl. 2010;106:211-6.

427. Corrigan F, Leonard A, Ghabriel M, Van Den Heuvel C, Vink R. A substance P antagonist improves outcome in female Sprague Dawley rats following diffuse traumatic brain injury. CNS Neurosci Ther. 2012;18(6):513-5.

428. Parenti A, De Logu F, Geppetti P, Benemei S. What is the evidence for the role of TRP channels in inflammatory and immune cells? Br J Pharmacol. 2016;173(6):953-69.

429. Song Y, Bi L, Zhang Z, Huang Z, Hou W, Lu X, et al. Increased levels of calcitonin gene-related peptide in serum accelerate fracture healing following traumatic brain injury. Mol Med Rep. 2012;5(2):432-8.

430. Song Y, Han GX, Chen L, Zhai YZ, Dong J, Chen W, et al. The role of the hippocampus and the function of calcitonin gene-related peptide in the mechanism of traumatic brain injury accelerating fracture-healing. Eur Rev Med Pharmacol Sci. 2017;21(7):1522-31.

431. Taylor BK, Fu W, Kuphal KE, Stiller CO, Winter MK, Chen W, et al. Inflammation enhances Y1 receptor signaling, neuropeptide Y-mediated inhibition of hyperalgesia, and substance P release from primary afferent neurons. Neuroscience. 2014;256:178-94.

432. Lynds R, Lyu C, Lyu GW, Shi XQ, Rosen A, Mustafa K, et al. Neuronal plasticity of trigeminal ganglia in mice following nerve injury. J Pain Res. 2017;10:349-57.

433. Soleimani L, Oquendo MA, Sullivan GM, Mathe AA, Mann JJ. Cerebrospinal fluid neuropeptide Y levels in major depression and reported childhood trauma. Int J Neuropsychopharmacol. 2014;18(1).

434. Ramamoorthy P, Wang Q, Whim MD. Cell type-dependent trafficking of neuropeptide Y-containing dense core granules in CNS neurons. J Neurosci. 2011;31(41):14783-8.

435. Silva AP, Xapelli S, Grouzmann E, Cavadas C. The putative neuroprotective role of neuropeptide Y in the central nervous system. Curr Drug Targets CNS Neurol Disord. 2005;4(4):331-47.

436. Fu W, Wessel CR, Taylor BK. Neuropeptide Y tonically inhibits an NMDARAC1TRPA1/TRPV1 mechanism of the affective dimension of chronic neuropathic pain. Neuropeptides. 2020;80:102024.

437. Reichmann F, Holzer P. Neuropeptide Y: A stressful review. Neuropeptides. 2016;55:99-109.

438. Shende P, Desai D. Physiological and Therapeutic Roles of Neuropeptide Y on Biological Functions. Adv Exp Med Biol. 2020;1237:37-47.

439. Babilon S, Morl K, Beck-Sickinger AG. Towards improved receptor targeting: anterograde transport, internalization and postendocytic trafficking of neuropeptide Y receptors. Biol Chem. 2013;394(8):921-36.

440. Pedragosa-Badia X, Stichel J, Beck-Sickinger AG. Neuropeptide Y receptors: how to get subtype selectivity. Front Endocrinol (Lausanne). 2013;4:5.

441. Loh K, Herzog H, Shi YC. Regulation of energy homeostasis by the NPY system. Trends Endocrinol Metab. 2015;26(3):125-35.

442. Gotzsche CR, Woldbye DP. The role of NPY in learning and memory. Neuropeptides. 2016;55:79-89.

443. Kornhuber J, Zoicas I. Neuropeptide Y prolongs non-social memory in a brain region- and receptor-specific way in male mice. Neuropharmacology. 2020;175:108199.

444. Colmers WF, El Bahh B. Neuropeptide Y and Epilepsy. Epilepsy Curr. 2003;3(2):53-8.

445. Silva AP, Xapelli S, Pinheiro PS, Ferreira R, Lourenco J, Cristovao A, et al. Up-regulation of neuropeptide Y levels and modulation of glutamate release through neuropeptide Y receptors in the hippocampus of kainate-induced epileptic rats. J Neurochem. 2005;93(1):163-70.

446. Mazarati A, Wasterlain CG. Anticonvulsant effects of four neuropeptides in the rat hippocampus during self-sustaining status epilepticus. Neurosci Lett. 2002;331(2):123-7.

447. Vezzani A, Sperk G, Colmers WF. Neuropeptide Y: emerging evidence for a functional role in seizure modulation. Trends Neurosci. 1999;22(1):25-30.

448. Takahashi Y, Tsunashima K, Sadamatsu M, Schwarzer C, Amano S, Ihara N, et al. Altered hippocampal expression of neuropeptide Y, somatostatin, and glutamate decarboxylase in Ihara's epileptic rats and spontaneously epileptic rats. Neurosci Lett. 2000;287(2):105-8.

449. Silva AP, Pinheiro PS, Carvalho AP, Carvalho CM, Jakobsen B, Zimmer J, et al. Activation of neuropeptide Y receptors is neuroprotective against excitotoxicity in organotypic hippocampal slice cultures. FASEB J. 2003;17(9):1118-20.

450. Uckermann OW, A.; Franziska, K.; Wiedemann, P.; Reichenbach, A.; Bringmann, A. . Neuropeptide Y inhibits hypotonic glial cell swelling in the postischemic rat retina via glutamatergic Neuron-to-Glia Signaling. Invest Ophtalmol Vis Sci. 2005;46.

451. Agasse F, Bernardino L, Kristiansen H, Christiansen SH, Ferreira R, Silva B, et al. Neuropeptide Y promotes neurogenesis in murine subventricular zone. Stem Cells. 2008;26(6):1636-45.

452. McIntosh TK, Ferriero D. Changes in neuropeptide Y after experimental traumatic brain injury in the rat. J Cereb Blood Flow Metab. 1992;12(4):697-702.

453. Tauber MG, Ferriero D, Kennedy SL, Sheldon RA, Guerra-Romero L. Brain levels of neuropeptide Y in experimental pneumococcal meningitis. Mol Chem Neuropathol. 1993;18(1-2):15-26.

454. Ekstrand AJ, Cao R, Bjorndahl M, Nystrom S, Jonsson-Rylander AC, Hassani H, et al. Deletion of neuropeptide Y (NPY) 2 receptor in mice results in blockage of NPY-induced angiogenesis and delayed wound healing. Proc Natl Acad Sci U S A. 2003;100(10):6033-8.

455. Decressac M, Prestoz L, Veran J, Cantereau A, Jaber M, Gaillard A. Neuropeptide Y stimulates proliferation, migration and differentiation of neural precursors from the subventricular zone in adult mice. Neurobiol Dis. 2009;34(3):441-9.

456. Geloso MC, Corvino V, Di Maria V, Marchese E, Michetti F. Cellular targets for neuropeptide Y-mediated control of adult neurogenesis. Front Cell Neurosci. 2015;9:85.

457. Spencer B, Potkar R, Metcalf J, Thrin I, Adame A, Rockenstein E, et al. Systemic Central Nervous System (CNS)-targeted Delivery of Neuropeptide Y (NPY) Reduces Neurodegeneration and Increases Neural Precursor Cell Proliferation in a Mouse Model of Alzheimer Disease. J Biol Chem. 2016;291(4):1905-20.

458. Fatoba OK, E.; Saft, C.; Gold, R.; Arning. L.; Elichmann, G., et al. . L22 Intranasal application of NPY and NPY13–36 ameliorate disease pathology in R6/2 mouse model of huntington's disease. J Neurol Neurosurg Psychiatry. 2016:A97-A8.

459. Sorensen AT, Nikitidou L, Ledri M, Lin EJ, During MJ, Kanter-Schlifke I, et al. Hippocampal NPY gene transfer attenuates seizures without affecting epilepsy-induced impairment of LTP. Exp Neurol. 2009;215(2):328-33.

460. Noe F, Vaghi V, Balducci C, Fitzsimons H, Bland R, Zardoni D, et al. Anticonvulsant effects and behavioural outcomes of rAAV serotype 1 vector-mediated neuropeptide Y overexpression in rat hippocampus. Gene Ther. 2010;17(5):643-52.

461. Xapelli S, Silva AP, Ferreira R, Malva JO. Neuropeptide Y can rescue neurons from cell death following the application of an excitotoxic insult with kainate in rat organotypic hippocampal slice cultures. Peptides. 2007;28(2):288-94.

462. Cohen H, Liu T, Kozlovsky N, Kaplan Z, Zohar J, Mathe AA. The neuropeptide Y (NPY)-ergic system is associated with behavioral resilience to stress exposure in an animal model of post-traumatic stress disorder. Neuropsychopharmacology. 2012;37(2):350-63.

463. Wu G, Feder A, Wegener G, Bailey C, Saxena S, Charney D, et al. Central functions of neuropeptide Y in mood and anxiety disorders. Expert Opin Ther Targets. 2011;15(11):1317-31.

464. Christiansen SH, Olesen MV, Gotzsche CR, Woldbye DP. Anxiolytic-like effects after vector-mediated overexpression of neuropeptide Y in the amygdala and hippocampus of mice. Neuropeptides. 2014;48(6):335-44.

465. Yilmazer-Hanke D, O'Loughlin E, McDermott K. Contribution of amygdala pathology to comorbid emotional disturbances in temporal lobe epilepsy. J Neurosci Res. 2016;94(6):486-503.

466. Ip CK, Zhang L, Farzi A, Qi Y, Clarke I, Reed F, et al. Amygdala NPY Circuits Promote the Development of Accelerated Obesity under Chronic Stress Conditions. Cell Metab. 2019;30(1):111-28 e6.

467. Morgan CA, 3rd, Wang S, Southwick SM, Rasmusson A, Hazlett G, Hauger RL, et al. Plasma neuropeptide-Y concentrations in humans exposed to military survival training. Biol Psychiatry. 2000;47(10):902-9.

468. Narvaez M, Borroto-Escuela DO, Santin L, Millon C, Gago B, Flores-Burgess A, et al. A Novel Integrative Mechanism in Anxiolytic Behavior Induced by Galanin 2/Neuropeptide Y Y1 Receptor Interactions on Medial Paracapsular Intercalated Amygdala in Rats. Front Cell Neurosci. 2018;12:119.

469. Sabban EL, Alaluf LG, Serova LI. Potential of neuropeptide Y for preventing or treating post-traumatic stress disorder. Neuropeptides. 2016;56:19-24.

470. Reichmann F, Wegerer V, Jain P, Mayerhofer R, Hassan AM, Frohlich EE, et al. Environmental enrichment induces behavioural disturbances in neuropeptide Y knockout mice. Sci Rep. 2016;6:28182.

471. Baker DG, Bertram TM, Patel PM, Barkauskas DA, Clopton P, Patel S, et al. Characterization of cerebrospinal fluid (CSF) and plasma NPY levels in normal volunteers over a 24-h timeframe. Psychoneuroendocrinology. 2013;38(10):2378-82.

472. Stachura Z, Obuchowicz E, Herman ZS. Neuropeptide Y-like immunoreactivity in lumbar cerebrospinal fluid of patients after severe head trauma. Neuropeptides. 1997;31(1):12-4.

473. HongY ZB, Q. . The clinical significance of plasma NPY and CGRP levels in patients with traumatic brain injury. J Trop Med. 2009(9(9)):1030-4.

474. Kernie SG, Parent JM. Forebrain neurogenesis after focal Ischemic and traumatic brain injury. Neurobiol Dis. 2010;37(2):267-74.

475. Jones KS, Connor B. Intrinsic regulation of adult subventricular zone neural progenitor cells and the effect of brain injury. Am J Stem Cells. 2012;1(1):48-58.

476. Sun Z, Liu S, Kharlamov EA, Miller ER, Kelly KM. Hippocampal neuropeptide Y protein expression following controlled cortical impact and posttraumatic epilepsy. Epilepsy Behav. 2018;87:188-94.

477. Zhu C, Chen Z, Jiang Z. Expression, Distribution and Role of Aquaporin Water Channels in Human and Animal Stomach and Intestines. Int J Mol Sci. 2016;17(9).

478. Liao SG, L.; Lv, L.; Mei, Z. . The regulatory roles of aquaporins in the digestive system. Genes & Diseases. 2020.

479. Duan H, Hao C, Fan Y, Wang H, Liu Y, Hao J, et al. The role of neuropeptide Y and aquaporin 4 in the pathogenesis of intestinal dysfunction caused by traumatic brain injury. J Surg Res. 2013;184(2):1006-12.

480. Ferreira R, Santos T, Cortes L, Cochaud S, Agasse F, Silva AP, et al. Neuropeptide Y inhibits interleukin-1 beta-induced microglia motility. J Neurochem. 2012;120(1):93-105.

481. Dimitrijevic M, Stanojevic S. The intriguing mission of neuropeptide Y in the immune system. Amino Acids. 2013;45(1):41-53.

482. Ferreira R, Santos T, Viegas M, Cortes L, Bernardino L, Vieira OV, et al. Neuropeptide Y inhibits interleukin-1beta-induced phagocytosis by microglial cells. J Neuroinflammation. 2011;8:169.

483. Ameliorate JL, Ghabriel MN, Vink R. Magnesium enhances the beneficial effects of NK1 antagonist administration on blood-brain barrier permeability and motor outcome after traumatic brain injury. Magnes Res. 2017;30(3):88-97.

484. Jahnen-Dechent W, Ketteler M. Magnesium basics. Clin Kidney J. 2012;5(Suppl 1):i3-i14.

485. de Baaij JH, Hoenderop JG, Bindels RJ. Magnesium in man: implications for health and disease. Physiol Rev. 2015;95(1):1-46.

486. Chen T, Carter BS. Role of magnesium sulfate in aneurysmal subarachnoid hemorrhage management: A meta-analysis of controlled clinical trials. Asian J Neurosurg. 2011;6(1):26-31.

487. Cernak I, Savic VJ, Kotur J, Prokic V, Veljovic M, Grbovic D. Characterization of plasma magnesium concentration and oxidative stress following graded traumatic brain injury in humans. J Neurotrauma. 2000;17(1):53-68.

488. Mendez DR, Corbett R, Macias C, Laptook A. Total and ionized plasma magnesium concentrations in children after traumatic brain injury. Pediatr Res. 2005;57(3):347-52.

489. Passakiotou ML, C.; Kopatzidis, E.; Sounidakis, N.; Asimaki, M.; Gritsi-Gerogianni, N. . Magnesium at admission: is it an outcome marker in the critically ill patient. Crit Care. 2005;9.

490. Turner RC, F.; Vink, R. . Magnesium in acute brain injury. In: Li YVZ, J.H., editor. Metal Ions in Stroke. New York: Springer Publishers.; 2012. p. 445-60.

491. Ozgurtas T, Kahraman S. State of the art of new data on the role of magnesium in brain injury: clinical interest of measurements of total and ionized magnesium. Magnes Res. 2004;17(4):327-34.

492. Donkin JJ, Cernak I, Blumbergs PC, Vink R. A substance P antagonist reduces axonal injury and improves neurologic outcome when administered up to 12 hours after traumatic brain injury. J Neurotrauma. 2011;28(2):217-24.

493. Clerc P, Young CA, Bordt EA, Grigore AM, Fiskum G, Polster BM. Magnesium sulfate protects against the bioenergetic consequences of chronic glutamate receptor stimulation. PLoS One. 2013;8(11):e79982.

494. Li W, Bai YA, Li YJ, Liu KG, Wang MD, Xu GZ, et al. Magnesium sulfate for acute traumatic brain injury. J Craniofac Surg. 2015;26(2):393-8.

495. Liotta EM, Prabhakaran S, Sangha RS, Bush RA, Long AE, Trevick SA, et al. Magnesium, hemostasis, and outcomes in patients with intracerebral hemorrhage. Neurology. 2017;89(8):813-9.

496. Morotti A, Charidimou A, Phuah CL, Jessel MJ, Schwab K, Ayres AM, et al. Association Between Serum Calcium Level and Extent of Bleeding in Patients With Intracerebral Hemorrhage. JAMA Neurol. 2016;73(11):1285-90.

497. Saver JL, Starkman S, Eckstein M, Stratton SJ, Pratt FD, Hamilton S, et al. Prehospital use of magnesium sulfate as neuroprotection in acute stroke. N Engl J Med. 2015;372(6):528-36.

498. Kawata K, Liu CY, Merkel SF, Ramirez SH, Tierney RT, Langford D. Blood biomarkers for brain injury: What are we measuring? Neurosci Biobehav Rev. 2016;68:460-73.

499. Lagerstedt L, Egea-Guerrero JJ, Rodriguez-Rodriguez A, Bustamante A, Montaner J, El Rahal A, et al. Early measurement of interleukin-10 predicts the absence of CT scan lesions in mild traumatic brain injury. PLoS One. 2018;13(2):e0193278.

500. Posti JP, Takala RSK, Lagerstedt L, Dickens AM, Hossain I, Mohammadian M, et al. Correlation of Blood Biomarkers and Biomarker Panels with Traumatic Findings on Computed Tomography after Traumatic Brain Injury. J Neurotrauma. 2019;36(14):2178-89.

501. Papa L, Silvestri S, Brophy GM, Giordano P, Falk JL, Braga CF, et al. GFAP out-performs S100beta in detecting traumatic intracranial lesions on computed tomography in trauma patients with mild traumatic brain injury and those with extracranial lesions. J Neurotrauma. 2014;31(22):1815-22.

502. Nielson JL, Cooper SR, Yue JK, Sorani MD, Inoue T, Yuh EL, et al. Uncovering precision phenotype-biomarker associations in traumatic brain injury using topological data analysis. PLoS One. 2017;12(3):e0169490.

503. Slavoaca D, Muresanu D, Birle C, Rosu OV, Chirila I, Dobra I, et al. Biomarkers in traumatic brain injury: new concepts. Neurol Sci. 2020;41(8):2033-44.

504. Xiong XG, Liang Q, Zhang C, Wang Y, Huang W, Peng W, et al. Serum Proteome Alterations in Patients with Cognitive Impairment after Traumatic Brain Injury Revealed by iTRAQ-Based Quantitative Proteomics. Biomed Res Int. 2017;2017:8572509.

505. He XY, Dan QQ, Wang F, Li YK, Fu SJ, Zhao N, et al. Protein Network Analysis of the Serum and Their Functional Implication in Patients Subjected to Traumatic Brain Injury. Front Neurosci. 2018;12:1049.

506. Park SH, Hwang SK. Prognostic Value of Serum Levels of S100 Calcium-Binding Protein B, Neuron-Specific Enolase, and Interleukin-6 in Pediatric Patients with Traumatic Brain Injury. World Neurosurg. 2018;118:e534-e42.

507. Donato R, Sorci G, Riuzzi F, Arcuri C, Bianchi R, Brozzi F, et al. S100B's double life: intracellular regulator and extracellular signal. Biochim Biophys Acta. 2009;1793(6):1008-22.

508. Gyorgy A, Ling G, Wingo D, Walker J, Tong L, Parks S, et al. Time-dependent changes in serum biomarker levels after blast traumatic brain injury. J Neurotrauma. 2011;28(6):1121-6.

509. Vajtr D, Benada O, Linzer P, Samal F, Springer D, Strejc P, et al. Immunohistochemistry and serum values of S-100B, glial fibrillary acidic protein, and hyperphosphorylated neurofilaments in brain injuries. Soud Lek. 2012;57(1):7-12.

510. Mondello S, Sorinola A, Czeiter E, Vamos Z, Amrein K, Synnot A, et al. Blood-Based Protein Biomarkers for the Management of Traumatic Brain Injuries in Adults Presenting to Emergency Departments with Mild Brain Injury: A Living Systematic Review and Meta-Analysis. J Neurotrauma. 2018.

511. Zhang Z, Ma Z, Zou W, Guo H, Liu M, Ma Y, et al. The Appropriate Marker for Astrocytes: Comparing the Distribution and Expression of Three Astrocytic Markers in Different Mouse Cerebral Regions. Biomed Res Int. 2019;2019:9605265.

512. Hinkle DA, Baldwin SA, Scheff SW, Wise PM. GFAP and S100beta expression in the cortex and hippocampus in response to mild cortical contusion. J Neurotrauma. 1997;14(10):729-38.

513. Benveniste H, Liu X, Koundal S, Sanggaard S, Lee H, Wardlaw J. The Glymphatic System and Waste Clearance with Brain Aging: A Review. Gerontology. 2019;65(2):106-19.

514. Lindblad C, Nelson DW, Zeiler FA, Ercole A, Ghatan PH, von Horn H, et al. Influence of Blood-Brain Barrier Integrity on Brain Protein Biomarker Clearance in Severe Traumatic Brain Injury: A Longitudinal Prospective Study. J Neurotrauma. 2020;37(12):1381-91.

515. Metting Z, Wilczak N, Rodiger LA, Schaaf JM, van der Naalt J. GFAP and S100B in the acute phase of mild traumatic brain injury. Neurology. 2012;78(18):1428-33.

516. Pleines UE, Morganti-Kossmann MC, Rancan M, Joller H, Trentz O, Kossmann T. S-100 beta reflects the extent of injury and outcome, whereas neuronal specific enolase is a better indicator of neuroinflammation in patients with severe traumatic brain injury. J Neurotrauma. 2001;18(5):491-8.

517. Unden J, Astrand R, Waterloo K, Ingebrigtsen T, Bellner J, Reinstrup P, et al. Clinical significance of serum S100B levels in neurointensive care. Neurocrit Care. 2007;6(2):94-9.

518. Savola O, Pyhtinen J, Leino TK, Siitonen S, Niemela O, Hillbom M. Effects of head and extracranial injuries on serum protein S100B levels in trauma patients. J Trauma. 2004;56(6):1229-34; discussion 34.

519. Bouvier D, Fournier M, Dauphin JB, Amat F, Ughetto S, Labbe A, et al. Serum S100B determination in the management of pediatric mild traumatic brain injury. Clin Chem. 2012;58(7):1116-22.

520. Thelin EP, Nelson DW, Bellander BM. Secondary peaks of S100B in serum relate to subsequent radiological pathology in traumatic brain injury. Neurocrit Care. 2014;20(2):217-29.

521. Unden J, Romner B. Can low serum levels of S100B predict normal CT findings after minor head injury in adults?: an evidence-based review and meta-analysis. J Head Trauma Rehabil. 2010;25(4):228-40.

522. Unden J, Ingebrigtsen T, Romner B, Scandinavian Neurotrauma C. Scandinavian guidelines for initial management of minimal, mild and moderate head injuries in adults: an evidence and consensus-based update. BMC Med. 2013;11:50.

523. Stranjalis G, Korfias S, Papapetrou C, Kouyialis A, Boviatsis E, Psachoulia C, et al. Elevated serum S-100B protein as a predictor of failure to short-term return to work or activities after mild head injury. J Neurotrauma. 2004;21(8):1070-5.

524. Gardner RC, Rubenstein R, Wang KKW, Korley FK, Yue JK, Yuh EL, et al. Age-Related Differences in Diagnostic Accuracy of Plasma Glial Fibrillary Acidic Protein and Tau for Identifying Acute Intracranial Trauma on Computed Tomography: A TRACK-TBI Study. J Neurotrauma. 2018;35(20):2341-50.

525. Huebschmann NA, Luoto TM, Karr JE, Berghem K, Blennow K, Zetterberg H, et al. Comparing Glial Fibrillary Acidic Protein (GFAP) in Serum and Plasma Following Mild Traumatic Brain Injury in Older Adults. Front Neurol. 2020;11:1054.

526. Huang XJ, Glushakova O, Mondello S, Van K, Hayes RL, Lyeth BG. Acute Temporal Profiles of Serum Levels of UCH-L1 and GFAP and Relationships to Neuronal and Astroglial Pathology following Traumatic Brain Injury in Rats. J Neurotrauma. 2015;32(16):1179-89.

527. Papa L, Lewis LM, Falk JL, Zhang Z, Silvestri S, Giordano P, et al. Elevated levels of serum glial fibrillary acidic protein breakdown products in mild and moderate traumatic brain injury are associated with intracranial lesions and neurosurgical intervention. Ann Emerg Med. 2012;59(6):471-83.

528. Papa L, Brophy GM, Welch RD, Lewis LM, Braga CF, Tan CN, et al. Time Course and Diagnostic Accuracy of Glial and Neuronal Blood Biomarkers GFAP and UCH-L1 in a Large Cohort of Trauma Patients With and Without Mild Traumatic Brain Injury. JAMA Neurol. 2016;73(5):551-60.

529. Yue JK, Yuh EL, Korley FK, Winkler EA, Sun X, Puffer RC, et al. Association between plasma GFAP concentrations and MRI abnormalities in patients with CT-negative traumatic brain injury in the TRACK-TBI cohort: a prospective multicentre study. Lancet Neurol. 2019;18(10):953-61.

530. Papa L, Zonfrillo MR, Ramirez J, Silvestri S, Giordano P, Braga CF, et al. Performance of Glial Fibrillary Acidic Protein in Detecting Traumatic Intracranial Lesions on Computed Tomography in Children and Youth With Mild Head Trauma. Acad Emerg Med. 2015;22(11):1274-82.

531. Dinarello CA. Overview of the IL-1 family in innate inflammation and acquired immunity. Immunol Rev. 2018;281(1):8-27.

532. Clausen F, Hanell A, Israelsson C, Hedin J, Ebendal T, Mir AK, et al. Neutralization of interleukin-1beta reduces cerebral edema and tissue loss and improves late cognitive outcome following traumatic brain injury in mice. Eur J Neurosci. 2011;34(1):110-23.

533. Ekmark-Lewen S, Flygt J, Fridgeirsdottir GA, Kiwanuka O, Hanell A, Meyerson BJ, et al. Diffuse traumatic axonal injury in mice induces complex behavioural alterations that are normalized by neutralization of interleukin-1beta. Eur J Neurosci. 2016;43(8):1016-33.

534. Holbrook J, Lara-Reyna S, Jarosz-Griffiths H, McDermott M. Tumour necrosis factor signalling in health and disease. F1000Res. 2019;8.

535. Fischer R, Kontermann RE, Pfizenmaier K. Selective Targeting of TNF Receptors as a Novel Therapeutic Approach. Front Cell Dev Biol. 2020;8:401.

536. Faustman D, Davis M. TNF receptor 2 pathway: drug target for autoimmune diseases. Nat Rev Drug Discov. 2010;9(6):482-93.

537. Naude PJ, den Boer JA, Luiten PG, Eisel UL. Tumor necrosis factor receptor cross-talk. FEBS J. 2011;278(6):888-98.

538. Sedger LM, McDermott MF. TNF and TNF-receptors: From mediators of cell death and inflammation to therapeutic giants - past, present and future. Cytokine Growth Factor Rev. 2014;25(4):453-72.

539. Gill J, Motamedi V, Osier N, Dell K, Arcurio L, Carr W, et al. Moderate blast exposure results in increased IL-6 and TNFalpha in peripheral blood. Brain Behav Immun. 2017;65:90-4.

540. Chaban V, Clarke GJB, Skandsen T, Islam R, Einarsen CE, Vik A, et al. Systemic Inflammation Persists the First Year after Mild Traumatic Brain Injury: Results from the Prospective Trondheim Mild Traumatic Brain Injury Study. J Neurotrauma. 2020;37(19):2120-30.

541. Parkin GM, Clarke C, Takagi M, Hearps S, Babl FE, Davis GA, et al. Plasma Tumor Necrosis Factor Alpha Is a Predictor of Persisting Symptoms Post-Concussion in Children. J Neurotrauma. 2019;36(11):1768-75.

542. Dalgard CL, Cole JT, Kean WS, Lucky JJ, Sukumar G, McMullen DC, et al. The cytokine temporal profile in rat cortex after controlled cortical impact. Front Mol Neurosci. 2012;5:6.

543. Kirchhoff C, Buhmann S, Bogner V, Stegmaier J, Leidel BA, Braunstein V, et al. Cerebrospinal IL-10 concentration is elevated in non-survivors as compared to survivors after severe traumatic brain injury. Eur J Med Res. 2008;13(10):464-8.

544. Soares FMdS, N.; Libório Schwarzbold, M.; Paim Diaz, A.; Costa Nunes, J.; Hohl, A., et al. . Interleukin-10 is an independent biomarker of severe traumatic brain injury prognosis. Neuroimmunomodulation. 2012;19:377-85.

545. Alosco ML, Tripodis Y, Fritts NG, Heslegrave A, Baugh CM, Conneely S, et al. Cerebrospinal fluid tau, Abeta, and sTREM2 in Former National Football League Players: Modeling the relationship between repetitive head impacts, microglial activation, and neurodegeneration. Alzheimers Dement. 2018;14(9):1159-70.

546. Benedict C, Blennow K, Zetterberg H, Cedernaes J. Effects of acute sleep loss on diurnal plasma dynamics of CNS health biomarkers in young men. Neurology. 2020;94(11):e1181-e9.

547. Hesse C, Rosengren L, Andreasen N, Davidsson P, Vanderstichele H, Vanmechelen E, et al. Transient increase in total tau but not phospho-tau in human cerebrospinal fluid after acute stroke. Neurosci Lett. 2001;297(3):187-90.

548. Zetterberg H, Blennow K. Fluid markers of traumatic brain injury. Mol Cell Neurosci. 2015;66(Pt B):99-102.

549. Bishop P, Rocca D, Henley JM. Ubiquitin C-terminal hydrolase L1 (UCH-L1): structure, distribution and roles in brain function and dysfunction. Biochem J. 2016;473(16):2453-62.

550. Mondello S, Kobeissy F, Vestri A, Hayes RL, Kochanek PM, Berger RP. Serum Concentrations of Ubiquitin C-Terminal Hydrolase-L1 and Glial Fibrillary Acidic Protein after Pediatric Traumatic Brain Injury. Sci Rep. 2016;6:28203.

551. Takala RS, Posti JP, Runtti H, Newcombe VF, Outtrim J, Katila AJ, et al. Glial Fibrillary Acidic Protein and Ubiquitin C-Terminal Hydrolase-L1 as Outcome Predictors in Traumatic Brain Injury. World Neurosurg. 2016;87:8-20.

552. Cheng F, Yuan Q, Yang J, Wang W, Liu H. The prognostic value of serum neuron-specific enolase in traumatic brain injury: systematic review and meta-analysis. PLoS One. 2014;9(9):e106680.

553. Mercier E, Boutin A, Shemilt M, Lauzier F, Zarychanski R, Fergusson DA, et al.

Predictive value of neuron-specific enolase for prognosis in patients with moderate or severe traumatic brain injury: a systematic review and meta-analysis. CMAJ Open. 2016;4(3):E371-E82.

554. Isgro MA, Bottoni P, Scatena R. Neuron-Specific Enolase as a Biomarker: Biochemical and Clinical Aspects. Adv Exp Med Biol. 2015;867:125-43.

555. Thelin EP, Jeppsson E, Frostell A, Svensson M, Mondello S, Bellander BM, et al. Utility of neuron-specific enolase in traumatic brain injury; relations to S100B levels, outcome, and extracranial injury severity. Crit Care. 2016;20:285.

556. Okuma Y, Liu K, Wake H, Liu R, Nishimura Y, Hui Z, et al. Glycyrrhizin inhibits traumatic brain injury by reducing HMGB1-RAGE interaction. Neuropharmacology. 2014;85:18-26.

557. Fink MP. Bench-to-bedside review: High-mobility group box 1 and critical illness. Crit Care. 2007;11(5):229.

558. Kaplan GB, Vasterling JJ, Vedak PC. Brain-derived neurotrophic factor in traumatic brain injury, post-traumatic stress disorder, and their comorbid conditions: role in pathogenesis and treatment. Behav Pharmacol. 2010;21(5-6):427-37.

559. Wurzelmann M, Romeika J, Sun D. Therapeutic potential of brain-derived neurotrophic factor (BDNF) and a small molecular mimics of BDNF for traumatic brain injury. Neural Regen Res. 2017;12(1):7-12.

560. Neselius S, Brisby H, Theodorsson A, Blennow K, Zetterberg H, Marcusson J. CSF-biomarkers in Olympic boxing: diagnosis and effects of repetitive head trauma. PLoS One. 2012;7(4):e33606.

561. Di Pietro V, Ragusa M, Davies D, Su Z, Hazeldine J, Lazzarino G, et al. MicroRNAs as Novel Biomarkers for the Diagnosis and Prognosis of Mild and Severe Traumatic Brain Injury. J Neurotrauma. 2017;34(11):1948-56.

562. Chesnut R, Aguilera S, Buki A, Bulger E, Citerio G, Cooper DJ, et al. A management algorithm for adult patients with both brain oxygen and intracranial pressure monitoring: the Seattle International Severe Traumatic Brain Injury Consensus Conference (SIBICC). Intensive Care Med. 2020;46(5):919-29.

563. Carney N, Lujan S, Dikmen S, Temkin N, Petroni G, Pridgeon J, et al. Intracranial pressure monitoring in severe traumatic brain injury in latin america: process and methods for a multi-center randomized controlled trial. J Neurotrauma. 2012;29(11):2022-9.

564. Svedung Wettervik T, Howells T, Hillered L, Nilsson P, Engquist H, Lewen A, et al. Mild Hyperventilation in Traumatic Brain Injury-Relation to Cerebral Energy Metabolism, Pressure Autoregulation, and Clinical Outcome. World Neurosurg. 2020;133:e567-e75.

565. Dyhrfort P, Shen Q, Clausen F, Thulin M, Enblad P, Kamali-Moghaddam M, et al. Monitoring of Protein Biomarkers of Inflammation in Human Traumatic Brain Injury Using Microdialysis and Proximity Extension Assay Technology in Neurointensive Care. J Neurotrauma. 2019;36(20):2872-85.

566. Darmanis S, Gallant CJ, Marinescu VD, Niklasson M, Segerman A, Flamourakis G, et al. Simultaneous Multiplexed Measurement of RNA and Proteins in Single Cells. Cell Rep. 2016;14(2):380-9.

567. Hillered L, Dahlin AP, Clausen F, Chu J, Bergquist J, Hjort K, et al. Cerebral microdialysis for protein biomarker monitoring in the neurointensive care setting - a technical approach. Front Neurol. 2014;5:245.

568. Helmy A, Carpenter KL, Menon DK, Pickard JD, Hutchinson PJ. The cytokine response to human traumatic brain injury: temporal profiles and evidence for cerebral parenchymal production. J Cereb Blood Flow Metab. 2011;31(2):658-70.

569. Depreitere B, Guiza F, Van den Berghe G, Schuhmann MU, Maier G, Piper I, et al. Pressure autoregulation monitoring and cerebral perfusion pressure target recommendation in patients with severe traumatic brain injury based on minute-by-minute monitoring data. J Neurosurg. 2014;120(6):1451-7.

570. Czosnyka M, Czosnyka Z, Smielewski P. Pressure reactivity index: journey through the past 20 years. Acta Neurochir (Wien). 2017;159(11):2063-5.

571. Carlson AP, Abbas M, Alunday RL, Qeadan F, Shuttleworth CW. Spreading depolarization in acute brain injury inhibited by ketamine: a prospective, randomized, multiple crossover trial. J Neurosurg. 2018:1-7.

572. Aquino L, Kang CY, Harada MY, Ko A, Do-Nguyen A, Ley EJ, et al. Is Routine Continuous EEG for Traumatic Brain Injury Beneficial? Am Surg. 2017;83(12):1433-7.

573. Baker WB, Balu R, He L, Kavuri VC, Busch DR, Amendolia O, et al. Continuous non-invasive optical monitoring of cerebral blood flow and oxidative metabolism after acute brain injury. J Cereb Blood Flow Metab. 2019;39(8):1469-85.

574. Midulla M, Pescatori L, Chevallier O, Nakai M, Ikoma A, Gehin S, et al. Future of IR: Emerging Techniques, Looking to the Future…and Learning from the Past. J Belg Soc Radiol. 2019;103(1):12.

575. Smith LGF, Milliron E, Ho ML, Hu HH, Rusin J, Leonard J, et al. Advanced neuroimaging in traumatic brain injury: an overview. Neurosurg Focus. 2019;47(6):E17.

576. Lee AL. Advanced Imaging of Traumatic Brain Injury. Korean J Neurotrauma. 2020;16(1):3-17.

577. Sheridan DC, Newgard CD, Selden NR, Jafri MA, Hansen ML. QuickBrain MRI for the detection of acute pediatric traumatic brain injury. J Neurosurg Pediatr. 2017;19(2):259-64.

578. Moen KG, Brezova V, Skandsen T, Haberg AK, Folvik M, Vik A. Traumatic axonal injury: the prognostic value of lesion load in corpus callosum, brain stem, and thalamus in different magnetic resonance imaging sequences. J Neurotrauma. 2014;31(17):1486-96.

579. Douglas DB, Chaudhari R, Zhao JM, Gullo J, Kirkland J, Douglas PK, et al. Perfusion Imaging in Acute Traumatic Brain Injury. Neuroimaging Clin N Am. 2018;28(1):55-65.

580. Wright ME, Wise RG. Can Blood Oxygenation Level Dependent Functional Magnetic Resonance Imaging Be Used Accurately to Compare Older and Younger Populations? A Mini Literature Review. Front Aging Neurosci. 2018;10:371.

581. Edlow BL, Chatelle C, Spencer CA, Chu CJ, Bodien YG, O'Connor KL, et al. Early detection of consciousness in patients with acute severe traumatic brain injury. Brain. 2017;140(9):2399-414.

582. Threlkeld ZD, Bodien YG, Rosenthal ES, Giacino JT, Nieto-Castanon A, Wu O, et al. Functional networks reemerge during recovery of consciousness after acute severe traumatic brain injury. Cortex. 2018;106:299-308.

583. Astrakas LG, Argyropoulou MI. Key concepts in MR spectroscopy and practical approaches to gaining biochemical information in children. Pediatr Radiol. 2016;46(7):941-51.

584. Yoo CH, Baek HM, Song KH, Woo DC, Choe BY. An in vivo proton magnetic resonance spectroscopy study with optimized echo-time technique for concurrent quantification and T2 measurement targeting glutamate in the rat brain. MAGMA. 2020;33(5):735-46.

585. Aaen GS HB, Sheridan C, Colbert C, McKenney M, Kido D et al. Magnetic resonance spectroscopy predicts outcomes for children with nonaccidental trauma. Pediatrics. 2010;125:295-303.

586. Holshouser B, Pivonka-Jones J, Nichols JG, Oyoyo U, Tong K, Ghosh N, et al. Longitudinal Metabolite Changes after Traumatic Brain Injury: A Prospective Pediatric Magnetic Resonance Spectroscopic Imaging Study. J Neurotrauma. 2019;36(8):1352-60.

587. Stein DG, Sayeed I. Bridging the translational divide: Emerging strategies in pharmacological approaches to traumatic brain injury. Neuropharmacology. 2019;145(Pt B):131-2.

588. Fontana AC. Current approaches to enhance glutamate transporter function and expression. J Neurochem. 2015;134(6):982-1007.

589. Lyeth B. Application of Novel Therapeutic Agents for CNS Injury: NAAG Peptidase Inhibitors. In: Kobeissy FH, editor. Brain Neurotrauma: Molecular, Neuropsychological, and Rehabilitation Aspects. New York: CRC Press/Taylor & Francis Publishers.; 2015. p. 109-15.

590. Edwards P, Arango M, Balica L, Cottingham R, El-Sayed H, Farrell B, et al. Final results of MRC CRASH, a randomised placebo-controlled trial of intravenous corticosteroid in adults with head injury-outcomes at 6 months. Lancet. 2005;365(9475):1957-9.

591. Yunoki M, Kawauchi M, Ukita N, Noguchi Y, Nishio S, Ono Y, et al. Effects of lecithinized superoxide dismutase on traumatic brain injury in rats. J Neurotrauma. 1997;14(10):739-46.

592. Karlsson M, Pukenas B, Chawla S, Ehinger JK, Plyler R, Stolow M, et al. Neuroprotective Effects of Cyclosporine in a Porcine Pre-Clinical Trial of Focal Traumatic Brain Injury. J Neurotrauma. 2018.

593. Davis SM, Lees KR, Albers GW, Diener HC, Markabi S, Karlsson G, et al. Selfotel in acute ischemic stroke : possible neurotoxic effects of an NMDA antagonist. Stroke. 2000;31(2):347-54.

594. Kawoos U, Abutarboush R, Zarriello S, Qadri A, Ahlers ST, McCarron RM, et al. N-acetylcysteine Amide Ameliorates Blast-Induced Changes in Blood-Brain Barrier Integrity in Rats. Front Neurol. 2019;10:650.

595. Brabazon F, Wilson CM, Jaiswal S, Reed J, Frey WHN, Byrnes KR. Intranasal insulin treatment of an experimental model of moderate traumatic brain injury. J Cereb Blood Flow Metab. 2017;37(9):3203-18.

596. Marmarou A, Nichols J, Burgess J, Newell D, Troha J, Burnham D, et al. Effects of the bradykinin antagonist Bradycor (deltibant, CP-1027) in severe traumatic brain injury: results of a multi-center, randomized, placebo-controlled trial. American Brain Injury Consortium Study Group. J Neurotrauma. 1999;16(6):431-44.

597. Darlington CL. Dexanabinol: a novel cannabinoid with neuroprotective properties. IDrugs. 2003;6(10):976-9.

598. Zhou Z, Chen H, Zhang K, Yang H, Liu J, Huang Q. Protective effect of nerve growth factor on neurons after traumatic brain injury. J Basic Clin Physiol Pharmacol. 2003;14(3):217-24.

599. Howard RB, Sayeed I, Stein DG. Suboptimal Dosing Parameters as Possible Factors in the Negative Phase III Clinical Trials of Progesterone for Traumatic Brain Injury. J Neurotrauma. 2017;34(11):1915-8.

600. Pan ZY, Zhao YH, Huang WH, Xiao ZZ, Li ZQ. Effect of progesterone administration

on the prognosis of patients with severe traumatic brain injury: a meta-analysis of randomized clinical trials. Drug Des Devel Ther. 2019;13:265-73.

601. Bailey ZS, Nilson E, Bates JA, Oyalowo A, Hockey KS, Sajja V, et al. Cerium Oxide Nanoparticles Improve Outcome after In Vitro and In Vivo Mild Traumatic Brain Injury. J Neurotrauma. 2020;37(12):1452-62.

602. Takahashi T, Marushima A, Nagasaki Y, Hirayama A, Muroi A, Puentes S, et al. Novel neuroprotection using antioxidant nanoparticles in a mouse model of head trauma. J Trauma Acute Care Surg. 2020;88(5):677-85.

603. Hammond FM, Sherer M, Malec JF, Zafonte RD, Dikmen S, Bogner J, et al. Amantadine Did Not Positively Impact Cognition in Chronic Traumatic Brain Injury: A Multi-Site, Randomized, Controlled Trial. J Neurotrauma. 2018;35(19):2298-305.

604. Schneider EB, Efron DT, MacKenzie EJ, Rivara FP, Nathens AB, Jurkovich GJ. Premorbid statin use is associated with improved survival and functional outcomes in older head-injured individuals. J Trauma. 2011;71(4):815-9.

605. Temkin NR, Anderson GD, Winn HR, Ellenbogen RG, Britz GW, Schuster J, et al. Magnesium sulfate for neuroprotection after traumatic brain injury: a randomised controlled trial. Lancet Neurol. 2007;6(1):29-38.

606. Siren AL, Fasshauer T, Bartels C, Ehrenreich H. Therapeutic potential of erythropoietin and its structural or functional variants in the nervous system. Neurotherapeutics. 2009;6(1):108-27.

607. Hajmohammadi M, Khaksari M, Soltani Z, Shahrokhi N, Najafipour H, Abbasi R. The Effect of Candesartan Alone and Its Combination With Estrogen on Post-traumatic Brain Injury Outcomes in Female Rats. Front Neurosci. 2019;13:1043.

608. Vigil FA, Bozdemir E, Bugay V, Chun SH, Hobbs M, Sanchez I, et al. Prevention of brain damage after traumatic brain injury by pharmacological enhancement of KCNQ (Kv7, "M-type") K(+) currents in neurons. J Cereb Blood Flow Metab. 2020;40(6):1256-73.

609. Robinson BD, Isbell CL, Anasooya Shaji C, Kurek S, Jr., Regner JL, Tharakan B. Quetiapine protects the blood-brain barrier in traumatic brain injury. J Trauma Acute Care Surg. 2018;85(5):968-76.

610. Carlson SW, Yan H, Dixon CE. Lithium increases hippocampal SNARE protein abundance after traumatic brain injury. Exp Neurol. 2017;289:55-63.

611. Chung JY, Krapp N, Wu L, Lule S, McAllister LM, Edmiston WJ, 3rd, et al. Interleukin-1 Receptor 1 Deletion in Focal and Diffuse Experimental Traumatic Brain Injury in Mice. J Neurotrauma. 2019;36(2):370-9.

612. Khuman J, Meehan WP, 3rd, Zhu X, Qiu J, Hoffmann U, Zhang J, et al. Tumor necrosis factor alpha and Fas receptor contribute to cognitive deficits independent of cell death after concussive traumatic brain injury in mice. J Cereb Blood Flow Metab. 2011;31(2):778-89.

613. Alves OL, Doyle AJ, Clausen T, Gilman C, Bullock R. Evaluation of topiramate neuroprotective effect in severe TBI using microdialysis. Ann N Y Acad Sci. 2003;993:25-34; discussion 48-53.

614. Malek AJ, Robinson BD, Hitt AR, Shaver CN, Tharakan B, Isbell CL. Doxycycline improves traumatic brain injury outcomes in a murine survival model. J Trauma Acute Care Surg. 2020;89(3):435-40.

615. Jayakumar AR, Tong XY, Ruiz-Cordero R, Bregy A, Bethea JR, Bramlett HM, et al. Activation of NF-kappaB mediates astrocyte swelling and brain edema in traumatic brain injury. J Neurotrauma. 2014;31(14):1249-57.

616. Stankowska DL, Mueller BH, 2nd, Oku H, Ikeda T, Dibas A. Neuroprotective effects of inhibitors of Acid-Sensing ion channels (ASICs) in optic nerve crush model in rodents. Curr Eye Res. 2018;43(1):84-95.

617. Sen AP, Gulati A. Use of magnesium in traumatic brain injury. Neurotherapeutics. 2010;7(1):91-9.

618. Vink R, Donkin JJ, Cruz MI, Nimmo AJ, Cernak I. A substance P antagonist increases brain intracellular free magnesium concentration after diffuse traumatic brain injury in rats. J Am Coll Nutr. 2004;23(5):538S-40S.

619. Vidal-Jorge M, Sanchez-Guerrero A, Mur-Bonet G, Castro L, Radoi A, Riveiro M, et al. Does Normobaric Hyperoxia Cause Oxidative Stress in the Injured Brain? A Microdialysis Study Using 8-Iso-Prostaglandin F2alpha as a Biomarker. J Neurotrauma. 2017;34(19):2731-42.

620. Veenith TV, Carter EL, Geeraerts T, Grossac J, Newcombe VF, Outtrim J, et al. Pathophysiologic Mechanisms of Cerebral Ischemia and Diffusion Hypoxia in Traumatic Brain Injury. JAMA Neurol. 2016;73(5):542-50.

621. Taher A, Pilehvari Z, Poorolajal J, Aghajanloo M. Effects of Normobaric Hyperoxia in Traumatic Brain Injury: A Randomized Controlled Clinical Trial. Trauma Mon. 2016;21(1):e26772.

622. Claassen J. Brain Oxygen Monitoring and the Potential for Precision Medicine for Acutely Brain-Injured Patients. Neurocrit Care. 2019;31(2):247-8.

623. Feng JZ, Wang WY, Zeng J, Zhou ZY, Peng J, Yang H, et al. Optimization of brain metabolism using metabolic-targeted therapeutic hypothermia can reduce mortality from traumatic brain injury. J Trauma Acute Care Surg. 2017;83(2):296-304.

624. Olah E, Poto L, Hegyi P, Szabo I, Hartmann P, Solymar M, et al. Therapeutic Whole-Body Hypothermia Reduces Death in Severe Traumatic Brain Injury if the Cooling Index Is Sufficiently High: Meta-Analyses of the Effect of Single Cooling Parameters and Their Integrated Measure. J Neurotrauma. 2018;35(20):2407-17.

625. Yokobori S, Gajavelli S, Mondello S, Mo-Seaney J, Bramlett HM, Dietrich WD, et al. Neuroprotective effect of preoperatively induced mild hypothermia as determined by biomarkers and histopathological estimation in a rat subdural haematoma decompression model. J Neurosurg. 2013;118(2):370-80.

626. Suehiro E, Koizumi H, Fujisawa H, Fujita M, Kaneko T, Oda Y, et al. Diverse effects of hypothermia therapy in patients with severe traumatic brain injury based on the computed tomography classification of the traumatic coma data bank. J Neurotrauma. 2015;32(5):353-8.

627. Dietrich WD, Bramlett HM. The evidence for hypothermia as a neuroprotectant in traumatic brain injury. Neurotherapeutics. 2010;7(1):43-50.

628. Sinclair HL, Andrews PJ. Bench-to-bedside review: Hypothermia in traumatic brain injury. Crit Care. 2010;14(1):204.

629. Chen H, Wu F, Yang P, Shao J, Chen Q, Zheng R. A meta-analysis of the effects of therapeutic hypothermia in adult patients with traumatic brain injury. Crit Care. 2019;23(1):396.

630. Burgess S, Abu-Laban RB, Slavik RS, Vu EN, Zed PJ. A Systematic Review of Randomized Controlled Trials Comparing Hypertonic Sodium Solutions and Mannitol for Traumatic Brain Injury: Implications for Emergency Department Management. Ann Pharmacother. 2016;50(4):291-300.

631. Mojtahedzadeh M, Ahmadi A, Mahmoodpoor A, Beigmohammadi MT, Abdollahi M, Khazaeipour Z, et al. Hypertonic saline solution reduces the oxidative stress responses in traumatic brain injury patients. J Res Med Sci. 2014;19(9):867-74.

632. Witherspoon B, Ashby NE. The Use of Mannitol and Hypertonic Saline Therapies in Patients with Elevated Intracranial Pressure: A Review of the Evidence. Nurs Clin North Am. 2017;52(2):249-60.

633. Godoy DA, Seifi A, Garza D, Lubillo-Montenegro S, Murillo-Cabezas F. Hyperventilation Therapy for Control of Posttraumatic Intracranial Hypertension. Front Neurol. 2017;8:250.

634. Brandi G, Stocchetti N, Pagnamenta A, Stretti F, Steiger P, Klinzing S. Cerebral metabolism is not affected by moderate hyperventilation in patients with traumatic brain injury. Crit Care. 2019;23(1):45.

635. Velle F, Lewen A, Howells T, Enblad P, Nilsson P. Intracranial pressure-based barbiturate coma treatment in children with refractory intracranial hypertension due to traumatic brain injury. J Neurosurg Pediatr. 2019:1-9.

636. Giammattei L, Messerer M, Cherian I, Starnoni D, Maduri R, Kasper EM, et al. Current Perspectives in the Surgical Treatment of Severe Traumatic Brain Injury. World Neurosurg. 2018;116:322-8.

637. Kolias AG, Viaroli E, Rubiano AM, Adams H, Khan T, Gupta D, et al. The current status of decompressive craniectomy in traumatic brain injury. Curr Trauma Rep. 2018;4(4):326-32.

638. Fatima N, Al Rumaihi G, Shuaib A, Saqqur M. The Role of Decompressive Craniectomy in Traumatic Brain Injury: A Systematic Review and Meta-analysis. Asian J Neurosurg. 2019;14(2):371-81.

639. Shohami E, Shapira Y, Cotev S. Experimental closed head injury in rats: prostaglandin production in a noninjured zone. Neurosurgery. 1988;22(5):859-63.

640. Flierl MA, Stahel PF, Beauchamp KM, Morgan SJ, Smith WR, Shohami E. Mouse closed head injury model induced by a weight-drop device. Nat Protoc. 2009;4(9):1328-37.

641. Umschweif G, Alexandrovich AG, Trembovler V, Horowitz M, Shohami E. The role and dynamics of beta-catenin in precondition induced neuroprotection after traumatic brain injury. PLoS One. 2013;8(10):e76129.

642. Serova LI, Laukova M, Alaluf LG, Pucillo L, Sabban EL. Intranasal neuropeptide Y reverses anxiety and depressive-like behavior impaired by single prolonged stress PTSD model. Eur Neuropsychopharmacol. 2014;24(1):142-7.

643. Laukova M, Alaluf LG, Serova LI, Arango V, Sabban EL. Early intervention with intranasal NPY prevents single prolonged stress-triggered impairments in hypothalamus and ventral hippocampus in male rats. Endocrinology. 2014;155(10):3920-33.

644. Sabban EL, Laukova M, Alaluf LG, Olsson E, Serova LI. Locus coeruleus response to single-prolonged stress and early intervention with intranasal neuropeptide Y. J Neurochem. 2015;135(5):975-86.

645. Leitao RA, Sereno J, Castelhano JM, Goncalves SI, Coelho-Santos V, Fontes-Ribeiro C, et al. Aquaporin-4 as a New Target against Methamphetamine-Induced Brain Alterations: Focus on the Neurogliovascular Unit and Motivational Behavior. Mol Neurobiol. 2018;55(3):2056-69.

646. Coelho-Santos V, Leitao RA, Cardoso FL, Palmela I, Rito M, Barbosa M, et al. The TNF-alpha/NF-kappaB signaling pathway has a key role in methamphetamine-induced blood-brain barrier dysfunction. J Cereb Blood Flow Metab. 2015;35(8):1260-71.

647. Di Benedetto B, Malik VA, Begum S, Jablonowski L, Gomez-Gonzalez GB, Neumann ID, et al. Fluoxetine Requires the Endfeet Protein Aquaporin-4 to Enhance Plasticity of Astrocyte Processes. Front Cell Neurosci. 2016;10:8.

648. de Aquino CC, Leitao RA, Oliveira Alves LA, Coelho-Santos V, Guerrant RL, Ribeiro CF, et al. Effect of Hypoproteic and High-Fat Diets on Hippocampal Blood-Brain Barrier Permeability and Oxidative Stress. Front Nutr. 2018;5:131.

649. McComb S, Chan PK, Guinot A, Hartmannsdottir H, Jenni S, Dobay MP, et al. Efficient apoptosis requires feedback amplification of upstream apoptotic signals by effector caspase-3 or -7. Sci Adv. 2019;5(7):eaau9433.

650. Bonfoco E, Krainc D, Ankarcrona M, Nicotera P, Lipton SA. Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. Proc Natl Acad Sci U S A. 1995;92(16):7162-6.

651. Witgen BM, Lifshitz J, Grady MS. Inbred mouse strains as a tool to analyze hippocampal neuronal loss after brain injury: a stereological study. J Neurotrauma. 2006;23(9):1320-9.

652. Kim DH, Ko IG, Kim BK, Kim TW, Kim SE, Shin MS, et al. Treadmill exercise inhibits traumatic brain injury-induced hippocampal apoptosis. Physiol Behav. 2010;101(5):660-5.

653. Van Opdenbosch N, Lamkanfi M. Caspases in Cell Death, Inflammation, and Disease. Immunity. 2019;50(6):1352-64.

654. Yuan J, Najafov A, Py BF. Roles of Caspases in Necrotic Cell Death. Cell. 2016; 167(7):1693-704.

655. Jiang CT, Wu WF, Deng YH, Ge JW. Modulators of microglia activation and polarization in ischemic stroke (Review). Mol Med Rep. 2020;21(5):2006-18.

656. Ou K, Copland DA, Theodoropoulou S, Mertsch S, Li Y, Liu J, et al. Treatment of diabetic retinopathy through neuropeptide Y-mediated enhancement of neurovascular microenvironment. J Cell Mol Med. 2020;24(7):3958-70.

657. Smialowska M, Domin H, Zieba B, Kozniewska E, Michalik R, Piotrowski P, et al. Neuroprotective effects of neuropeptide Y-Y2 and Y5 receptor agonists in vitro and in vivo. Neuropeptides. 2009;43(3):235-49.

658. Boldrini M, Fulmore CA, Tartt AN, Simeon LR, Pavlova I, Poposka V, et al. Human Hippocampal Neurogenesis Persists throughout Aging. Cell Stem Cell. 2018;22(4):589-99 e5.

659. Corvino V, Marchese E, Podda MV, Lattanzi W, Giannetti S, Di Maria V, et al. The neurogenic effects of exogenous neuropeptide Y: early molecular events and long-lasting effects in the hippocampus of trimethyltin-treated rats. PLoS One. 2014;9(2):e88294.

660. Daugherty AM, Bender AR, Raz N, Ofen N. Age differences in hippocampal subfield volumes from childhood to late adulthood. Hippocampus. 2016;26(2):220-8.

661. Smith C, Gentleman SM, Leclercq PD, Murray LS, Griffin WS, Graham DI, et al. The neuroinflammatory response in humans after traumatic brain injury. Neuropathol Appl Neurobiol. 2013;39(6):654-66.

662. Marin-Teva JL, Cuadros MA, Martin-Oliva D, Navascues J. Microglia and neuronal cell death. Neuron Glia Biol. 2011;7(1):25-40.

663. Li T, Zhang S. Microgliosis in the Injured Brain: Infiltrating Cells and Reactive Microglia Both Play a Role. Neuroscientist. 2016;22(2):165-70.

664. Kumar A, Loane DJ. Neuroinflammation after traumatic brain injury: opportunities for therapeutic intervention. Brain Behav Immun. 2012;26(8):1191-201.

665. Scott G, Hellyer PJ, Ramlackhansingh AF, Brooks DJ, Matthews PM, Sharp DJ. Thalamic inflammation after brain trauma is associated with thalamo-cortical white matter damage. J Neuroinflammation. 2015;12:224.

666. Mannix RC, Whalen MJ. Traumatic brain injury, microglia, and Beta amyloid. Int J Alzheimers Dis. 2012;2012:608732.

667. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nat Rev Immunol. 2008;8(12):958-69.

668. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity. 2014;41(1):14-20.

669. Novak ML, Koh TJ. Phenotypic transitions of macrophages orchestrate tissue repair. Am J Pathol. 2013;183(5):1352-63.

670. Taylor SE, Morganti-Kossmann C, Lifshitz J, Ziebell JM. Rod microglia: a morphological definition. PLoS One. 2014;9(5):e97096.

671. Streit WJ, Xue QS, Tischer J, Bechmann I. Microglial pathology. Acta Neuropathol Commun. 2014;2:142.

672. Swanson MEV, Murray HC, Ryan B, Faull RLM, Dragunow M, Curtis MA. Quantitative immunohistochemical analysis of myeloid cell marker expression in human cortex captures microglia heterogeneity with anatomical context. Sci Rep. 2020;10(1):11693.

673. DiBona VL, Zhu W, Shah MK, Rafalia A, Ben Cheikh H, Crockett DP, et al. Loss of Par1b/MARK2 primes microglia during brain development and enhances their sensitivity to injury. J Neuroinflammation. 2019;16(1):11.

674. Madathil SK, Wilfred BS, Urankar SE, Yang W, Leung LY, Gilsdorf JS, et al. Early Microglial Activation Following Closed-Head Concussive Injury Is Dominated by Pro-Inflammatory M-1 Type. Front Neurol. 2018;9:964.

675. Ziebell JM, Taylor SE, Cao T, Harrison JL, Lifshitz J. Rod microglia: elongation, alignment, and coupling to form trains across the somatosensory cortex after experimental diffuse brain injury. J Neuroinflammation. 2012;9:247.

676. Semple BD, Bye N, Rancan M, Ziebell JM, Morganti-Kossmann MC. Role of CCL2 (MCP-1) in traumatic brain injury (TBI): evidence from severe TBI patients and CCL2-/- mice. J Cereb Blood Flow Metab. 2010;30(4):769-82.

677. Hellewell SC, Yan EB, Agyapomaa DA, Bye N, Morganti-Kossmann MC. Post-traumatic hypoxia exacerbates brain tissue damage: analysis of axonal injury and glial responses. J Neurotrauma. 2010;27(11):1997-2010.

678. Byrnes KR, Loane DJ, Stoica BA, Zhang J, Faden AI. Delayed mGluR5 activation limits neuroinflammation and neurodegeneration after traumatic brain injury. J Neuroinflammation. 2012;9:43.

679. Katsumoto A, Lu H, Miranda AS, Ransohoff RM. Ontogeny and functions of central nervous system macrophages. J Immunol. 2014;193(6):2615-21.

680. Oberheim NA, Takano T, Han X, He W, Lin JH, Wang F, et al. Uniquely hominid features of adult human astrocytes. J Neurosci. 2009;29(10):3276-87.

681. Rosa JMF-A, V.; Navarrete, M.; Palomino-Antolin, A.; Fernandez-Lopez, E.; Narros-Fernandez, P., et al. . Microglia-to-astrocyte communication modulates synaptic and cerebrovascular functions following traumatic brain injury. BioRxiv. 2020;2020.03.01.972158.

682. Anzabi M, Ardalan M, Iversen NK, Rafati AH, Hansen B, Ostergaard L. Hippocampal Atrophy Following Subarachnoid Hemorrhage Correlates with Disruption of Astrocyte Morphology and Capillary Coverage by AQP4. Front Cell Neurosci. 2018;12:19.

683. Ardalan M, Rafati AH, Nyengaard JR, Wegener G. Rapid antidepressant effect of ketamine correlates with astroglial plasticity in the hippocampus. Br J Pharmacol. 2017;174(6):483-92.

684. Mestre H, Kostrikov S, Mehta RI, Nedergaard M. Perivascular spaces, glymphatic dysfunction, and small vessel disease. Clin Sci (Lond). 2017;131(17):2257-74.

685. Iliff JJ, Wang M, Liao Y, Plogg BA, Peng W, Gundersen GA, et al. A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid beta. Sci Transl Med. 2012;4(147):147ra11.

686. Cullen DK, Vernekar VN, LaPlaca MC. Trauma-induced plasmalemma disruptions in three-dimensional neural cultures are dependent on strain modality and rate. J Neurotrauma. 2011;28(11):2219-33.

687. Floyd CL, Gorin FA, Lyeth BG. Mechanical strain injury increases intracellular sodium and reverses Na+/Ca2+ exchange in cortical astrocytes. Glia. 2005;51(1):35-46.

688. Walko TD, 3rd, Bola RA, Hong JD, Au AK, Bell MJ, Kochanek PM, et al. Cerebrospinal fluid mitochondrial DNA: a novel DAMP in pediatric traumatic brain injury. Shock. 2014;41(6):499-503.

689. Kim JV, Dustin ML. Innate response to focal necrotic injury inside the blood-brain barrier. J Immunol. 2006;177(8):5269-77.

690. Brambilla R, Hurtado A, Persaud T, Esham K, Pearse DD, Oudega M, et al. Transgenic inhibition of astroglial NF-kappa B leads to increased axonal sparing and sprouting following spinal cord injury. J Neurochem. 2009;110(2):765-78.

691. Lee SC, Liu W, Dickson DW, Brosnan CF, Berman JW. Cytokine production by human fetal microglia and astrocytes. Differential induction by lipopolysaccharide and IL-1 beta. J Immunol. 1993;150(7):2659-67.

692. Clarke LE, Liddelow SA, Chakraborty C, Munch AE, Heiman M, Barres BA. Normal aging induces A1-like astrocyte reactivity. Proc Natl Acad Sci U S A. 2018;115(8):E1896-E905.

693. St-Pierre JA, Nouel D, Dumont Y, Beaudet A, Quirion R. Sub-population of cultured hippocampal astrocytes expresses neuropeptide Y Y(1) receptors. Glia. 2000;30(1):82-91.

694. Ramamoorthy P, Whim MD. Trafficking and fusion of neuropeptide Y-containing dense-core granules in astrocytes. J Neurosci. 2008;28(51):13815-27.

695. Morganti JM, Riparip LK, Chou A, Liu S, Gupta N, Rosi S. Age exacerbates the CCR2/5-mediated neuroinflammatory response to traumatic brain injury. J Neuroinflammation. 2016;13(1):80.

696. Chou A, Krukowski K, Morganti JM, Riparip LK, Rosi S. Persistent Infiltration and Impaired Response of Peripherally-Derived Monocytes after Traumatic Brain Injury in the Aged Brain. Int J Mol Sci. 2018;19(6).

697. Boisvert MM, Erikson GA, Shokhirev MN, Allen NJ. The Aging Astrocyte Transcriptome from Multiple Regions of the Mouse Brain. Cell Rep. 2018;22(1):269-85.

698. Di Pietro V, Yakoub KM, Caruso G, Lazzarino G, Signoretti S, Barbey AK, et al. Antioxidant Therapies in Traumatic Brain Injury. Antioxidants (Basel). 2020;9(3).

699. Sun Y, Bai L, Niu X, Wang Z, Yin B, Bai G, et al. Elevated Serum Levels of Inflammation-Related Cytokines in Mild Traumatic Brain Injury Are Associated With Cognitive Performance. Front Neurol. 2019;10:1120.

700. Bachstetter AD, Webster SJ, Goulding DS, Morton JE, Watterson DM, Van Eldik LJ. Attenuation of traumatic brain injury-induced cognitive impairment in mice by targeting increased cytokine levels with a small molecule experimental therapeutic. J Neuroinflammation. 2015;12:69.

701. Woodcock T, Morganti-Kossmann MC. The role of markers of inflammation in traumatic brain injury. Front Neurol. 2013;4:18.

702. Alderson P, Roberts I. Corticosteroids for acute traumatic brain injury. Cochrane Database Syst Rev. 2005(1):CD000196.

703. Yu I, Inaji M, Maeda J, Okauchi T, Nariai T, Ohno K, et al. Glial cell-mediated deterioration and repair of the nervous system after traumatic brain injury in a rat model as assessed by positron emission tomography. J Neurotrauma. 2010;27(8):1463-75.

704. Xiong Y, Mahmood A, Chopp M. Current understanding of neuroinflammation after traumatic brain injury and cell-based therapeutic opportunities. Chin J Traumatol. 2018;21(3):137-51.

705. Hanna K, Hamidi M, Vartanyan P, Henry M, Castanon L, Tang A, et al. Non-neurologic organ dysfunction plays a major role in predicting outcomes in pediatric traumatic brain injury. J Pediatr Surg. 2020;55(8):1590-5.

706. Byun K, Bayarsaikhan E, Kim D, Son M, Hong J, Jeong GB, et al. Activated microglial cells synthesize and secrete AGE-albumin. Anat Cell Biol. 2012;45(1):47-52.

707. Park JH, Park JA, Ahn JH, Kim YH, Kang IJ, Won MH, et al. Transient cerebral ischemia induces albumin expression in microglia only in the CA1 region of the gerbil hippocampus. Mol Med Rep. 2017;16(1):661-5.

708. Yang Y, Salayandia VM, Thompson JF, Yang LY, Estrada EY, Yang Y. Attenuation of acute stroke injury in rat brain by minocycline promotes blood-brain barrier remodeling and alternative microglia/macrophage activation during recovery. J Neuroinflammation. 2015;12:26.

709. Noll T, Hempel A, Piper HM. Neuropeptide Y reduces macromolecule permeability of coronary endothelial monolayers. Am J Physiol. 1996;271(5 Pt 2):H1878-83.

710. Nan YS, Feng GG, Hotta Y, Nishiwaki K, Shimada Y, Ishikawa A, et al. Neuropeptide Y enhances permeability across a rat aortic endothelial cell monolayer. Am J Physiol Heart Circ Physiol. 2004;286(3):H1027-33.

711. Li J, Du Y, Jiang Z, Tian Y, Qiu N, Wang Y, et al. Y1 receptor ligand-based nanomicelle as a novel nanoprobe for glioma-targeted imaging and therapy. Nanoscale. 2018;10(13):5845-51.

712. Kochanek PM, Jackson TC, Jha RM, Clark RSB, Okonkwo DO, Bayir H, et al. Paths to Successful Translation of New Therapies for Severe Traumatic Brain Injury in the Golden Age of Traumatic Brain Injury Research: A Pittsburgh Vision. J Neurotrauma. 2020;37(22):2353-71.

713. Graham DI, McIntosh TK, Maxwell WL, Nicoll JA. Recent advances in neurotrauma. J Neuropathol Exp Neurol. 2000;59(8):641-51.

714. Thompson HJ, Lifshitz J, Marklund N, Grady MS, Graham DI, Hovda DA, et al. Lateral fluid percussion brain injury: a 15-year review and evaluation. J Neurotrauma. 2005;22(1):42-75.

715. Khalin I, Jamari NL, Razak NB, Hasain ZB, Nor MA, Zainudin MH, et al. A mouse model of weight-drop closed head injury: emphasis on cognitive and neurological deficiency. Neural Regen Res. 2016;11(4):630-5.

716. Bree D, Stratton J, Levy D. Increased severity of closed head injury or repetitive subconcussive head impacts enhances post-traumatic headache-like behaviors in a rat model. Cephalalgia. 2020;40(11):1224-39.

717. Albert-Weissenberger C, Siren AL. Experimental traumatic brain injury. Exp Transl Stroke Med. 2010;2(1):16.

718. Johnson VE, Meaney DF, Cullen DK, Smith DH. Animal models of traumatic brain injury. Handb Clin Neurol. 2015;127:115-28.

719. Feldman AM. Bench-to-Bedside; Clinical and Translational Research; Personalized Medicine; Precision Medicine-What's in a Name? Clin Transl Sci. 2015;8(3):171-3.

720. Stein DG. Embracing failure: What the Phase III progesterone studies can teach about TBI clinical trials. Brain Inj. 2015;29(11):1259-72.

721. Risdall JE, Menon DK. Traumatic brain injury. Philos Trans R Soc Lond B Biol Sci. 2011;366(1562):241-50.

722. Semple BD, Dixit S, Shultz SR, Boon WC, O'Brien TJ. Sex-dependent changes in neuronal morphology and psychosocial behaviors after pediatric brain injury. Behav Brain Res. 2017;319:48-62.

723. Doran SJ, Ritzel RM, Glaser EP, Henry RJ, Faden AI, Loane DJ. Sex Differences in Acute Neuroinflammation after Experimental Traumatic Brain Injury Are Mediated by Infiltrating Myeloid Cells. J Neurotrauma. 2019;36(7):1040-53.

724. Bhatt R, Bhatt S, Rameshwar P, Siegel A. Amygdaloid kindled seizures induce weight gain that reflects left hemisphere dominance in rats. Physiol Behav. 2004;82(2-3):581-7.

725. Hum KM, Megna S, Burnham WM. Lack of laterality in the effects of right and left amygdala kindling on weight gain in female rats. Epilepsy Res. 2009;87(1):40-6.

726. Zhang H, Shen Y, Wang W, Gao H. Rat model of focal cerebral ischemia in the dominant hemisphere. Int J Clin Exp Med. 2015;8(1):504-11.

727. Goldstein KR, Bhatt R, Barton BE, Zalcman SS, Rameshwar P, Siegel A. Effects of hemispheric lateralization and site specificity on immune alterations induced by kindled temporal lobe seizures. Brain Behav Immun. 2002;16(6):706-19.

728. Aungst SL, Kabadi SV, Thompson SM, Stoica BA, Faden AI. Repeated mild traumatic brain injury causes chronic neuroinflammation, changes in hippocampal synaptic plasticity, and associated cognitive deficits. J Cereb Blood Flow Metab. 2014;34(7):1223-32.

729. Zanier ER, Lee SM, Vespa PM, Giza CC, Hovda DA. Increased hippocampal CA3 vulnerability to low-level kainic acid following lateral fluid percussion injury. J Neurotrauma. 2003;20(5):409-20.

730. Dalton MA, McCormick C, Maguire EA. Differences in functional connectivity along the anterior-posterior axis of human hippocampal subfields. Neuroimage. 2019;192:38-51.

731. Lei Y, Yaroslavsky I, Tejani-Butt SM. Strain differences in the distribution of N-methyl-d-aspartate and gamma (gamma)-aminobutyric acid-A receptors in rat brain. Life Sci. 2009;85(23-26):794-9.

732. Reid WM, Rolfe A, Register D, Levasseur JE, Churn SB, Sun D. Strain-related differences after experimental traumatic brain injury in rats. J Neurotrauma. 2010;27(7):1243-53.

733. Tan AA, Quigley A, Smith DC, Hoane MR. Strain differences in response to traumatic brain injury in Long-Evans compared to Sprague-Dawley rats. J Neurotrauma. 2009;26(4):539-48.

734. Jiang M, Sun L, Feng DX, Yu ZQ, Gao R, Sun YZ, et al. Neuroprotection provided by isoflurane pre-conditioning and post-conditioning. Med Gas Res. 2017;7(1):48-55.

735. Clausen F, Hansson HA, Raud J, Marklund N. Intranasal Administration of the Antisecretory Peptide AF-16 Reduces Edema and Improves Cognitive Function Following Diffuse Traumatic Brain Injury in the Rat. Front Neurol. 2017;8:39.

736. Al-Olama M, Lange S, Lonnroth I, Gatzinsky K, Jennische E. Uptake of the antisecretory factor peptide AF-16 in rat blood and cerebrospinal fluid and effects on elevated intracranial pressure. Acta Neurochir (Wien). 2015;157(1):129-37.

737. Zhang JY, Lee JH, Gu X, Wei ZZ, Harris MJ, Yu SP, et al. Intranasally Delivered Wnt3a Improves Functional Recovery after Traumatic Brain Injury by Modulating Autophagic, Apoptotic, and Regenerative Pathways in the Mouse Brain. J Neurotrauma. 2018;35(5):802-13.

738. Lee HJ, Ryu JS, Vig PJ. Current strategies for therapeutic drug delivery after traumatic CNS injury. Ther Deliv. 2019;10(4):251-63.

739. Guennoun R, Frechou M, Gaignard P, Liere P, Slama A, Schumacher M, et al. Intranasal administration of progesterone: A potential efficient route of delivery for cerebroprotection after acute brain injuries. Neuropharmacology. 2019;145(Pt B):283-91.

740. Renner DB, Svitak AL, Gallus NJ, Ericson ME, Frey WH, 2nd, Hanson LR. Intranasal delivery of insulin via the olfactory nerve pathway. J Pharm Pharmacol. 2012;64(12):1709-14.

741. Rapoport A, Winner P. Nasal delivery of antimigraine drugs: clinical rationale and evidence base. Headache. 2006;46 Suppl 4:S192-201.

742. Camp RS, L.; Stier, C.; Sabban, E. . Effects of Intranasal NPY on Cardiovascular Parameters and Activity in SPS Model of PTSD: Telemetric Studies. FASEBJ. 2018;31(1).

743. Craft S, Baker LD, Montine TJ, Minoshima S, Watson GS, Claxton A, et al. Intranasal insulin therapy for Alzheimer disease and amnestic mild cognitive impairment: a pilot clinical trial. Arch Neurol. 2012;69(1):29-38.

744. Lochhead JJ, Wolak DJ, Pizzo ME, Thorne RG. Rapid transport within cerebral perivascular spaces underlies widespread tracer distribution in the brain after intranasal administration. J Cereb Blood Flow Metab. 2015;35(3):371-81.

745. Nwokafor C, Serova LI, Sabban EL. Preclinical findings on the potential of intranasal neuropeptide Y for treating hyperarousal features of PTSD. Ann N Y Acad Sci. 2019;1455(1):149-59.

746. Westin UE, Bostrom E, Grasjo J, Hammarlund-Udenaes M, Bjork E. Direct nose-to-brain transfer of morphine after nasal administration to rats. Pharm Res. 2006; 23(3):565-72.

747. Wu H, Hu K, Jiang X. From nose to brain: understanding transport capacity and transport rate of drugs. Expert Opin Drug Deliv. 2008;5(10):1159-68.

748. Nonaka N, Farr SA, Kageyama H, Shioda S, Banks WA. Delivery of galanin-like peptide to the brain: targeting with intranasal delivery and cyclodextrins. J Pharmacol Exp Ther. 2008;325(2):513-9.

749. Khan M, Dhammu TS, Matsuda F, Annamalai B, Dhindsa TS, Singh I, et al. Targeting the nNOS/peroxynitrite/calpain system to confer neuroprotection and aid functional recovery in a mouse model of TBI. Brain Res. 2016;1630:159-70.

750. Javia A, Thakkar H. Intranasal delivery of tapentadol hydrochloride-loaded chitosan nanoparticles: formulation, characterisation and its in vivo evaluation. J Microencapsul. 2017;34(7):644-58.

751. Djupesland PG, Messina JC, Mahmoud RA. The nasal approach to delivering treatment for brain diseases: an anatomic, physiologic, and delivery technology overview. Ther Deliv. 2014;5(6):709-33.

752. Jiang Y, Li Y, Liu X. Intranasal delivery: circumventing the iron curtain to treat neurological disorders. Expert Opin Drug Deliv. 2015;12(11):1717-25.

753. Tayebati SK, Nwankwo IE, Amenta F. Intranasal drug delivery to the central nervous system: present status and future outlook. Curr Pharm Des. 2013;19(3):510-26.

754. Stoica BA, Loane DJ, Zhao Z, Kabadi SV, Hanscom M, Byrnes KR, et al. PARP-1 inhibition attenuates neuronal loss, microglia activation and neurological deficits after traumatic brain injury. J Neurotrauma. 2014;31(8):758-72.

755. Lv Q, Lan W, Sun W, Ye R, Fan X, Ma M, et al. Intranasal nerve growth factor attenuates tau phosphorylation in brain after traumatic brain injury in rats. J Neurol Sci. 2014;345(1-2):48-55.

756. Lacroix JS, Ricchetti AP, Morel D, Mossimann B, Waeber B, Grouzmann E. Intranasal administration of neuropeptide Y in man: systemic absorption and functional effects. Br J Pharmacol. 1996;118(8):2079-84.

757. Szekely M, Petervari E, Pakai E, Hummel Z, Szelenyi Z. Acute, subacute and chronic effects of central neuropeptide Y on energy balance in rats. Neuropeptides. 2005;39(2):103-15.

758. Islam SU, Shehzad A, Ahmed MB, Lee YS. Intranasal Delivery of Nanoformulations: A Potential Way of Treatment for Neurological Disorders. Molecules. 2020;25(8).

759. Veronesi MC, Alhamami M, Miedema SB, Yun Y, Ruiz-Cardozo M, Vannier MW. Imaging of intranasal drug delivery to the brain. Am J Nucl Med Mol Imaging. 2020;10(1):1-31.

760. See GA, F.; Dahlizar, S.; Okada, A.; Fadli, M.; Hijikuro, I., et al. . Enhanced nose-to-brain delivery of tranilast using liquid crystal formulations. J Control Release. 2020;325:1-9.

761. Sakane T, Akizuki M, Yamashita S, Sezaki H, Nadai T. Direct drug transport from the rat nasal cavity to the cerebrospinal fluid: the relation to the dissociation of the drug. J Pharm Pharmacol. 1994;46(5):378-9.

762. Charlton ST, Davis SS, Illum L. Evaluation of effect of ephedrine on the transport of drugs from the nasal cavity to the systemic circulation and the central nervous system. J Drug Target. 2007;15(5):370-7.

763. van den Berg MP, Romeijn SG, Verhoef JC, Merkus FW. Serial cerebrospinal fluid sampling in a rat model to study drug uptake from the nasal cavity. J Neurosci Methods. 2002;116(1):99-107.

764. Giunchedi P, Gavini E, Bonferoni MC. Nose-to-Brain Delivery. Pharmaceutics. 2020;12(2).

765. Dalpiaz A, Gavini E, Colombo G, Russo P, Bortolotti F, Ferraro L, et al. Brain uptake of an anti-ischemic agent by nasal administration of microparticles. J Pharm Sci. 2008;97(11):4889-903.

766. Gao X, Chen J, Tao W, Zhu J, Zhang Q, Chen H, et al. UEA I-bearing nanoparticles for brain delivery following intranasal administration. Int J Pharm. 2007;340(1-2):207-15.

767. Born J, Lange T, Kern W, McGregor GP, Bickel U, Fehm HL. Sniffing neuropeptides: a transnasal approach to the human brain. Nat Neurosci. 2002;5(6):514-6.

768. Jones NC, Prior MJ, Burden-Teh E, Marsden CA, Morris PG, Murphy S. Antagonism of the interleukin-1 receptor following traumatic brain injury in the mouse reduces the number of nitric oxide synthase-2-positive cells and improves anatomical and functional outcomes. Eur J Neurosci. 2005;22(1):72-8.

769. Burmeister AR, Johnson MB, Chauhan VS, Moerdyk-Schauwecker MJ, Young AD, Cooley ID, et al. Human microglia and astrocytes constitutively express the neurokinin-1 receptor and functionally respond to substance P. J Neuroinflammation. 2017;14(1):245.

770. Perez-Polo JR, Rea HC, Johnson KM, Parsley MA, Unabia GC, Xu G, et al. Inflammatory consequences in a rodent model of mild traumatic brain injury. J Neurotrauma. 2013;30(9):727-40.

771. McGonigle P, Ruggeri B. Animal models of human disease: challenges in enabling translation. Biochem Pharmacol. 2014;87(1):162-71.

772. Jickling GC, Sharp FR. Improving the translation of animal ischemic stroke studies to humans. Metab Brain Dis. 2015;30(2):461-7.

773. Oberheim NA, Wang X, Goldman S, Nedergaard M. Astrocytic complexity distinguishes the human brain. Trends Neurosci. 2006;29(10):547-53.

774. Zhang Y, Sloan SA, Clarke LE, Caneda C, Plaza CA, Blumenthal PD, et al. Purification and Characterization of Progenitor and Mature Human Astrocytes Reveals Transcriptional and Functional Differences with Mouse. Neuron. 2016;89(1):37-53.

775. Morales DM, Marklund N, Lebold D, Thompson HJ, Pitkanen A, Maxwell WL, et al. Experimental models of traumatic brain injury: do we really need to build a better mousetrap? Neuroscience. 2005;136(4):971-89.

776. Marklund N, Hillered L. Animal modelling of traumatic brain injury in preclinical drug development: where do we go from here? Br J Pharmacol. 2011;164(4):1207-29.

777. Montenigro PH, Alosco ML, Martin BM, Daneshvar DH, Mez J, Chaisson CE, et al. Cumulative Head Impact Exposure Predicts Later-Life Depression, Apathy, Executive Dysfunction, and Cognitive Impairment in Former High School and College Football Players. J Neurotrauma. 2017;34(2):328-40.

778. Hibbard MR, Ashman TA, Spielman LA, Chun D, Charatz HJ, Melvin S. Relationship between depression and psychosocial functioning after traumatic brain injury. Arch Phys Med Rehabil. 2004;85(4 Suppl 2):S43-53.

779. Ashman TA, Gordon WA, Cantor JB, Hibbard MR. Neurobehavioral consequences of traumatic brain injury. Mt Sinai J Med. 2006;73(7):999-1005.

780. Malkesman O, Tucker LB, Ozl J, McCabe JT. Traumatic brain injury - modeling neuropsychiatric symptoms in rodents. Front Neurol. 2013;4:157.

781. Wright DW, Yeatts SD, Silbergleit R, Palesch YY, Hertzberg VS, Frankel M, et al. Very early administration of progesterone for acute traumatic brain injury. N Engl J Med. 2014;371(26):2457-66.

782. Lind NM, Moustgaard A, Jelsing J, Vajta G, Cumming P, Hansen AK. The use of pigs in neuroscience: modeling brain disorders. Neurosci Biobehav Rev. 2007;31(5):728-51.

783. Hawrylycz M, Miller JA, Menon V, Feng D, Dolbeare T, Guillozet-Bongaarts AL, et al. Canonical genetic signatures of the adult human brain. Nat Neurosci. 2015;18(12):1832-44.

784. Margulies S, Hicks R, Combination Therapies for Traumatic Brain Injury Workshop L. Combination therapies for traumatic brain injury: prospective considerations. J Neurotrauma. 2009;26(6):925-39.

785. Minkkinen M, Iverson GL, Kotilainen AK, Pauniaho SL, Mattila VM, Lehtimaki T, et al. Prospective Validation of the Scandinavian Guidelines for Initial Management of Minimal, Mild, and Moderate Head Injuries in Adults. J Neurotrauma. 2019;36(20):2904-12.

786. Santos ME, De Sousa L, Castro-Caldas A. [Epidemiology of craniocerebral trauma in Portugal]. Acta Med Port. 2003;16(2):71-6.

787. Hansen BA, Bruserud O. Hypomagnesemia in critically ill patients. J Intensive Care. 2018;6:21.

788. Lim LJH, Ho RCM, Ho CSH. Dangers of Mixed Martial Arts in the Development of Chronic Traumatic Encephalopathy. Int J Environ Res Public Health. 2019;16(2).

789. Ercole A, Thelin EP, Holst A, Bellander BM, Nelson DW. Kinetic modelling of serum S100b after traumatic brain injury. BMC Neurol. 2016;16:93.

790. Martin RM, Wright MJ, Lutkenhoff ES, Ellingson BM, Van Horn JD, Tubi M, et al. Traumatic hemorrhagic brain injury: impact of location and resorption on cognitive outcome. J Neurosurg. 2017;126(3):796-804.

791. Thorsell A, Mathe AA. Neuropeptide Y in Alcohol Addiction and Affective Disorders. Front Endocrinol (Lausanne). 2017;8:178.

792. Sabban FV, R.; Turner, R.J. . Inflammation in acute CNS injury: A focus on the role of substance P. Br J Pharmacol. 2016;173:703-15.

793. Chiodera P, Volpi R, Pilla S, Cataldo S, Coiro V. Decline in circulating neuropeptide Y levels in normal elderly human subjects. Eur J Endocrinol. 2000;143(5):715-6.

794. Nelson TS, Fu W, Donahue RR, Corder GF, Hokfelt T, Wiley RG, et al. Facilitation of neuropathic pain by the NPY Y1 receptor-expressing subpopulation of excitatory interneurons in the dorsal horn. Sci Rep. 2019;9(1):7248.

795. Sabban EL, Serova LI. Potential of Intranasal Neuropeptide Y (NPY) and/or Melanocortin 4 Receptor (MC4R) Antagonists for Preventing or Treating PTSD. Mil Med. 2018;183(suppl_1):408-12.

796. El-Salhy M, Hausken T. The role of the neuropeptide Y (NPY) family in the pathophysiology of inflammatory bowel disease (IBD). Neuropeptides. 2016;55:137-44.

797. Holzer P, Reichmann F, Farzi A. Neuropeptide Y, peptide YY and pancreatic polypeptide in the gut-brain axis. Neuropeptides. 2012;46(6):261-74.

798. Wintermark M, Sanelli PC, Anzai Y, Tsiouris AJ, Whitlow CT, American College of Radiology Head Injury I. Imaging evidence and recommendations for traumatic brain injury: advanced neuro- and neurovascular imaging techniques. AJNR Am J Neuroradiol. 2015;36(2):E1-E11.

799. Cook NL, Vink R, Donkin JJ, van den Heuvel C. Validation of reference genes for normalization of real-time quantitative RT-PCR data in traumatic brain injury. J Neurosci Res. 2009;87(1):34-41.

800. Unden J, Bellner J, Astrand R, Romner B. Serum S100B levels in patients with epidural haematomas. Br J Neurosurg. 2005;19(1):43-5.

801. Raabe A, Grolms C, Keller M, Dohnert J, Sorge O, Seifert V. Correlation of computed tomography findings and serum brain damage markers following severe head injury. Acta Neurochir (Wien). 1998;140(8):787-91; discussion 91-2.

802. Haimoto H, Hosoda S, Kato K. Differential distribution of immunoreactive S100-alpha and S100-beta proteins in normal nonnervous human tissues. Lab Invest. 1987;57(5):489-98.

803. Pfortmueller CA, Drexel C, Krahenmann-Muller S, Leichtle AB, Fiedler GM, Lindner G, et al. S-100 B Concentrations Are a Predictor of Decreased Survival in Patients with Major Trauma, Independently of Head Injury. PLoS One. 2016;11(3):e0152822.

804. da Rocha AB, Schneider RF, de Freitas GR, Andre C, Grivicich I, Zanoni C, et al. Role of serum S100B as a predictive marker of fatal outcome following isolated severe head injury or multitrauma in males. Clin Chem Lab Med. 2006;44(10):1234-42.

805. Nayak R, Attry S, Ghosh SN. Serum Magnesium as a Marker of Neurological Outcome in Severe Traumatic Brain Injury Patients. Asian J Neurosurg. 2018;13(3):685-8.

806. van den Heuvel C, Vink R. The role of magnesium in traumatic brain injury. Clin Calcium. 2004;14(8):9-14.

807. Shindo Y, Yamanaka R, Hotta K, Oka K. Inhibition of Mg(2+) Extrusion Attenuates Glutamate Excitotoxicity in Cultured Rat Hippocampal Neurons. Nutrients. 2020;12(9).

808. Imer M, Omay B, Uzunkol A, Erdem T, Sabanci PA, Karasu A, et al. Effect of magnesium, MK-801 and combination of magnesium and MK-801 on blood-brain barrier permeability and brain edema after experimental traumatic diffuse brain injury. Neurol Res. 2009;31(9):977-81.

809. Vink R, McIntosh TK, Demediuk P, Weiner MW, Faden AI. Decline in intracellular free Mg2+ is associated with irreversible tissue injury after brain trauma. J Biol Chem. 1988;263(2):757-61.

810. Iseri LT, French JH. Magnesium: nature's physiologic calcium blocker. Am Heart J. 1984;108(1):188-93.

811. McKee JA, Brewer RP, Macy GE, Borel CO, Reynolds JD, Warner DS. Magnesium neuroprotection is limited in humans with acute brain injury. Neurocrit Care. 2005;2(3):342-51.

812. Severino P, Netti L, Mariani MV, Maraone A, D'Amato A, Scarpati R, et al. Prevention of Cardiovascular Disease: Screening for Magnesium Deficiency. Cardiol Res Pract. 2019;2019:4874921.

813. Vatsalya V, Gala KS, Mishra M, Schwandt ML, Umhau J, Cave MC, et al. Lower Serum Magnesium Concentrations are associated With Specific Heavy Drinking Markers, Pro-Inflammatory Response and Early-Stage Alcohol-associated Liver Injury section sign. Alcohol Alcohol. 2020;55(2):164-70.

814. Moore HB, Tessmer MT, Moore EE, Sperry JL, Cohen MJ, Chapman MP, et al. Forgot calcium? Admission ionized-calcium in two civilian randomized controlled trials of prehospital plasma for traumatic hemorrhagic shock. J Trauma Acute Care Surg. 2020;88(5):588-96.

815. Ditzel RM, Jr., Anderson JL, Eisenhart WJ, Rankin CJ, DeFeo DR, Oak S, et al. A review of transfusion- and trauma-induced hypocalcemia: Is it time to change the lethal triad to the lethal diamond? J Trauma Acute Care Surg. 2020;88(3):434-9.

816. Naghibi T, Mohajeri M, Dobakhti F. Inflammation and Outcome in Traumatic Brain Injury: Does Gender Effect on Survival and Prognosis? J Clin Diagn Res. 2017;11(2):PC06-PC9.

817. Alves JL, Rato J, Silva V. Why Does Brain Trauma Research Fail? World Neurosurg. 2019;130:115-21.

818. Kurland D, Hong C, Aarabi B, Gerzanich V, Simard JM. Hemorrhagic progression of a contusion after traumatic brain injury: a review. J Neurotrauma. 2012;29(1):19-31.

819. Charry JD, Falla JD, Ochoa JD, Pinzon MA, Tejada JH, Henriquez MJ, et al. External Validation of the Rotterdam Computed Tomography Score in the Prediction of Mortality in Severe Traumatic Brain Injury. J Neurosci Rural Pract. 2017;8(Suppl 1):S23-S6.

820. Mahadewa TGB, Golden N, Saputra A, Ryalino C. Modified Revised Trauma-Marshall score as a proposed tool in predicting the outcome of moderate and severe traumatic brain injury. Open Access Emerg Med. 2018;10:135-9.

821. Schneiderman H. Glasgow Coma Creep: Problems of Recognition and Communication. Am J Med. 2015;128(12):1270-1.

822. Jalali R, Rezaei M. A comparison of the glasgow coma scale score with full outline of unresponsiveness scale to predict patients' traumatic brain injury outcomes in intensive care units. Crit Care Res Pract. 2014;2014:289803.

823. Rubin ML, Yamal JM, Chan W, Robertson CS. Prognosis of Six-Month Glasgow Outcome Scale in Severe Traumatic Brain Injury Using Hospital Admission Characteris-

tics, Injury Severity Characteristics, and Physiological Monitoring during the First Day Post-Injury. J Neurotrauma. 2019;36(16):2417-22.

824. Bouvier D, Castellani C, Fournier M, Dauphin JB, Ughetto S, Breton M, et al. Reference ranges for serum S100B protein during the first three years of life. Clin Biochem. 2011;44(10-11):927-9.

825. Campbell DE, Raftery N, Tustin R, 3rd, Tustin NB, Desilvio ML, Cnaan A, et al. Measurement of plasma-derived substance P: biological, methodological, and statistical considerations. Clin Vaccine Immunol. 2006;13(11):1197-203.

826. Goyal A, Failla MD, Niyonkuru C, Amin K, Fabio A, Berger RP, et al. S100b as a prognostic biomarker in outcome prediction for patients with severe traumatic brain injury. J Neurotrauma. 2013;30(11):946-57.

827. Skillback T, Delsing L, Synnergren J, Mattsson N, Janelidze S, Nagga K, et al. CSF/serum albumin ratio in dementias: a cross-sectional study on 1861 patients. Neurobiol Aging. 2017;59:1-9.

828. Marchi N, Fazio V, Cucullo L, Kight K, Masaryk T, Barnett G, et al. Serum transthyretin monomer as a possible marker of blood-to-CSF barrier disruption. J Neurosci. 2003;23(5):1949-55.

829. Kleindienst A, Meissner S, Eyupoglu IY, Parsch H, Schmidt C, Buchfelder M. Dynamics of S100B release into serum and cerebrospinal fluid following acute brain injury. Acta Neurochir Suppl. 2010;106:247-50.

830. Bellander BM, Olafsson IH, Ghatan PH, Bro Skejo HP, Hansson LO, Wanecek M, et al. Secondary insults following traumatic brain injury enhance complement activation in the human brain and release of the tissue damage marker S100B. Acta Neurochir (Wien). 2011;153(1):90-100.

831. Plog BA, Nedergaard M. The Glymphatic System in Central Nervous System Health and Disease: Past, Present, and Future. Annu Rev Pathol. 2018;13:379-94.

832. Iliff JJ, Chen MJ, Plog BA, Zeppenfeld DM, Soltero M, Yang L, et al. Impairment of glymphatic pathway function promotes tau pathology after traumatic brain injury. J Neurosci. 2014;34(49):16180-93.

833. Rasmussen MK, Mestre H, Nedergaard M. The glymphatic pathway in neurological disorders. Lancet Neurol. 2018;17(11):1016-24.

834. Dadas A, Washington J, Diaz-Arrastia R, Janigro D. Biomarkers in traumatic brain injury (TBI): a review. Neuropsychiatr Dis Treat. 2018;14:2989-3000.

835. David A, Mari C, Vignaud F, Masson D, Planche L, Bord E, et al. Evaluation of S100B blood level as a biomarker to avoid computed tomography in patients with mild head trauma under antithrombotic medication. Diagn Interv Imaging. 2017;98(7-8):551-6.

836. Bolker JA. Selection of Models: Evolution and the Choice of Species for Translational Research. Brain Behav Evol. 2019;93(2-3):82-91.

837. Lee DK, In J, Lee S. Standard deviation and standard error of the mean. Korean J Anesthesiol. 2015;68(3):220-3.

838. Barde MP, Barde PJ. What to use to express the variability of data: Standard deviation or standard error of mean? Perspect Clin Res. 2012;3(3):113-6.

839. O'Leary R A, Nichol AD. Pathophysiology of severe traumatic brain injury. J Neurosurg Sci. 2018;62(5):542-8.

840. Ruprecht R, Scheurer E, Lenz C. Systematic review on the characterization of chronic traumatic encephalopathy by MRI and MRS. J Magn Reson Imaging. 2019;49(1):212-28.

841. Habtemariam S. The brain-derived neurotrophic factor in neuronal plasticity and neuroregeneration: new pharmacological concepts for old and new drugs. Neural Regen Res. 2018;13(6):983-4.

842. Shrirao AB, Kung FH, Omelchenko A, Schloss RS, Boustany NN, Zahn JD, et al. Microfluidic platforms for the study of neuronal injury in vitro. Biotechnol Bioeng. 2018;115(4):815-30.

843. Skolnick BE, Maas AI, Narayan RK, van der Hoop RG, MacAllister T, Ward JD, et al. A clinical trial of progesterone for severe traumatic brain injury. N Engl J Med. 2014;371(26):2467-76.

844. Dekmak A, Mantash S, Shaito A, Toutonji A, Ramadan N, Ghazale H, et al. Stem cells and combination therapy for the treatment of traumatic brain injury. Behav Brain Res. 2018;340:49-62.

845. Yokobori S, Hosein K, Burks S, Sharma I, Gajavelli S, Bullock R. Biomarkers for the clinical differential diagnosis in traumatic brain injury--a systematic review. CNS Neurosci Ther. 2013;19(8):556-65.

846. Carteron L, Bouzat P, Oddo M. Cerebral Microdialysis Monitoring to Improve Individualized Neurointensive Care Therapy: An Update of Recent Clinical Data. Front Neurol. 2017;8:601.

847. Retzios AD. Why do so many clinical trials fail? 2009 [Available from: http://adrclinresearch.com/Issues_in_Clinical_Research_links/Why%20Pivotal%20Clinical%20Trials%20Fail%20-%20Part%201_v12L_a.pdf.

848. McConeghy KW, Hatton J, Hughes L, Cook AM. A review of neuroprotection pharmacology and therapies in patients with acute traumatic brain injury. CNS Drugs. 2012;26(7):613-36.

849. Schwamm LH. Progesterone for traumatic brain injury--resisting the sirens' song. N Engl J Med. 2014;371(26):2522-3.

850. Bonds BW, Dhanda A, Wade C, Massetti J, Diaz C, Stein DM. Prognostication of Mortality and Long term Functional Outcomes Following Traumatic Brain Injury: Can We Do Better? J Neurotrauma. 2015.

851. Menon DK, Maas AI. Traumatic brain injury in 2014. Progress, failures and new approaches for TBI research. Nat Rev Neurol. 2015;11(2):71-2.

852. Lu J, Gary KW, Neimeier JP, Ward J, Lapane KL. Randomized controlled trials in adult traumatic brain injury. Brain Inj. 2012;26(13-14):1523-48.

853. Walker WC, Stromberg KA, Marwitz JH, Sima AP, Agyemang AA, Graham KM, et al. Predicting Long-Term Global Outcome after Traumatic Brain Injury: Development of a Practical Prognostic Tool Using the Traumatic Brain Injury Model Systems National Database. J Neurotrauma. 2018;35(14):1587-95.

854. Nourallah B, Zeiler FA, Calviello L, Smielewski P, Czosnyka M, Menon DK. Critical thresholds for intracranial pressure vary over time in non-craniectomised traumatic brain injury patients. Acta Neurochir (Wien). 2018;160(7):1315-24.

855. Kumaria A. In vitro models as a platform to investigate traumatic brain injury. Altern Lab Anim. 2017;45(4):201-11.

856. Kreeger K. From bench to bedside. Nature. 2003;424(6952):1090-1.

857. Petraglia AL, Dashnaw ML, Turner RC, Bailes JE. Models of mild traumatic brain injury: translation of physiological and anatomic injury. Neurosurgery. 2014;75 Suppl 4:S34-49.

858. Alali AS, Vavrek D, Barber J, Dikmen S, Nathens AB, Temkin NR. Comparative study of outcome measures and analysis methods for traumatic brain injury trials. J Neurotrauma. 2015;32(8):581-9.

859. Harrison DA, Prabhu G, Grieve R, Harvey SE, Sadique MZ, Gomes M, et al. Risk Adjustment In Neurocritical care (RAIN)--prospective validation of risk prediction models for adult patients with acute traumatic brain injury to use to evaluate the optimum location and comparative costs of neurocritical care: a cohort study. Health Technol Assess. 2013;17(23):vii-viii, 1-350.

860. Maas AIR, Menon DK, Adelson PD, Andelic N, Bell MJ, Belli A, et al. Traumatic brain injury: integrated approaches to improve prevention, clinical care, and research. Lancet Neurol. 2017;16(12):987-1048.

861. Ahirrao M, Shrotriya S. In vitro and in vivo evaluation of cubosomal in situ nasal gel containing resveratrol for brain targeting. Drug Dev Ind Pharm. 2017;43(10):1686-93.

862. Bodanapally UK, Sours C, Zhuo J, Shanmuganathan K. Imaging of Traumatic Brain Injury. Radiol Clin North Am. 2015;53(4):695-715, viii.

863. Smitherman E, Hernandez A, Stavinoha PL, Huang R, Kernie SG, Diaz-Arrastia R, et al. Predicting Outcome after Pediatric Traumatic Brain Injury by Early Magnetic Resonance Imaging Lesion Location and Volume. J Neurotrauma. 2016;33(1):35-48.

864. Gavett BE, Stern RA, McKee AC. Chronic traumatic encephalopathy: a potential late effect of sport-related concussive and subconcussive head trauma. Clin Sports Med. 2011;30(1):179-88, xi.

865. Asplund CA, Best TM. Brain damage in American Football. BMJ. 2015;350:h1381.

866. Theadom A, Mahon S, Hume P, Starkey N, Barker-Collo S, Jones K, et al. Incidence of Sports-Related Traumatic Brain Injury of All Severities: A Systematic Review. Neuroepidemiology. 2020;54(2):192-9.

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NEUROPEPTIDE RESPONSE IN TRAUMATIC BRAIN INJURY

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