UNIVERSITY OF PÉCS

Doctoral School of Biology and Sportbiology

Investigation of Behavioural and Neuropathological Alterations Caused by Repetitive Mild Traumatic Brain Injury in a Rodent Model

PhD Thesis

Tadepalli Sai Ambika

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CONTENTS

ABBREVIATIONS

AD – Alzheimer's disease

APP – Amyloid precursor protein

BBB – Blood brain barrier

BBT – Beam balance test

CTE – Chronic traumatic encephalopathy

DAI – Diffuse axonal injury

DI – Discrimination index

DTI – Diffusion tensor imaging

ELISA – Enzyme linked immunosorbent assay

FA – Fractional anisotropy

GCS – Glasgow coma scale

GFAP – Glial fibrillary acidic protein

MRI – Magnetic resonance imaging

mTBI – Mild traumatic brain injury

MWM – Morris water maze test

NOR – Novel object recognition test

OFT – Open field test

rapTBI – Rapid repetitive mild traumatic brain injury

rmTBI – Repetitive mild traumatic brain injury

sTBI – Severe traumatic brain injury

TBI – Traumatic brain injury

1. INTRODUCTION

The brain is one of the most complex organs of the human body, and contains around one hundred billion neurons and one hundred trillion synapses. It has an intricate network that controls wide array of actions and thoughts, even our behaviour. Due to the plasticity of the human brain, the structure of its synapses and their resulting functions change throughout life (Ho, Lee, and Martin 2011; Holtmaat and Svoboda 2009). Although well-protected by the skull, brain can still sustain damage when subjected to trauma. In some cases the damage can be irreversible, and can cause slight to grave changes in behaviour and ultimately may contribute to the onset of neurocognitive disorders.

Accidental traumatic brain injury (TBI) is the primary cause of death and disability in developed countries, because of its high incidence, morbidity and mortality (Hyder et al. 2007; Langlois, Rutland-Brown, and Wald 2006; O'Dell, Caplan, and Sherer 2006).

Traumatic brain injury is a very heterogeneous disease and affects the most complex organ of the body. It occurs when an external physical force impacts the head, either causing the brain to move within the intact skull or damaging the brain by fracturing the skull (McGinn and Povlishock 2016). Depending on the severity of injury, TBI can have a lasting impact on quality of life for survivors of all ages. Several studies on humans have revealed a wide range of neuropsychological effects following TBI (Baron et al. 2013; Marshall 2000). However, the underlying pathophysiology of TBI, as well the full spectrum of cognitive consequences following an injury are still not well understood. In order to understand the pathophysiology of TBI, appropriate experimental models that accurately represent key clinical and pathological features of different severities of TBI are required, which especially mimic type of brain trauma that is currently observed in repetitive sport injuries.

1.1 Definition and classification of traumatic brain injury

Traumatic brain injury is a form of head trauma which occurs when an external mechanical force hits the head, injuring the brain, possibly leading to temporary or permanent impairment in cognitive, physical, and psychosocial functions. Traumatic brain injury usually results from a violent blow or jolt to the head or body. An object penetrating the skull, such as a bullet or shattered piece of skull, also can cause TBI.

The detailed definition of TBI constantly changes, due to the broad spectrum of severity, pathology, and physiology associated with it. One of the methodological limitations in TBI studies is the inconsistency in the classification and variation in the inclusion criteria. There are different systems for classifying TBI. Based on the level of consciousness and responsiveness following injury, TBI has been classified into mild, moderate and severe injuries, using Glasgow Coma Scale (GCS) score (Sternbach 2000). The Glasgow Coma Scale is a semiquantitative assessment of the conscious state of the patient, and is divided into three components – eye opening, verbal response and motor responses. While GCS scoring for different severities is conflicted, traditionally, Centre for Disease Control (CDC) are considered reliable. Mild TBI can be a simple concussion, and may cause temporary dysfunction of brain cells. Usually the patient recovers on its own, without suffering from any serious damage. The patient has a score of 13-15 on the Glasgow Coma Scale. Moderate TBI can cause loss of consciousness from few minutes to several hours. It causes contusions in the brain tissue and the injured person may experience changes in brain function even for longer durations. Moderate TBI has a score of 9-12 on the Glasgow Coma Scale. Severe TBI can be life-threatening, with a score of 3-8 on the Glasgow Coma Scale. It can result in contusions, torn tissues, post-traumatic seizures, intracranial bleeding and other physical damage to the brain that can result in long-term complications or death (Bener et al. 2010; Heim, Schoettker, and Spahn 2004).

Currently, in conjunction with GCS, other classification systems are also used to assess the type, severity and treatment (Champion et al. 1989; Malec et al. 2007).

Primary and secondary injury: Primary injury is induced immediately following the impact; it usually causes dynamic deformation of the brain tissue, and results in skull lacerations, contusions, and stretch injury to long-tract structures such as axons and blood vessels, and blood-brain barrier damage, Secondary injuries include a very wide array of

mechanisms occurring hours to weeks after the primary insult. The secondary injury is the consequence of a delayed non-mechanical damage which is usually caused by a cascade of oxidative, excitotoxic and inflammatory processes, initiated by the primary injury itself. Axonal damage during the primary injury results in altered electrochemistry of damaged axons. This triggers a release of excitatory neurotransmitters, which engages secondary messenger systems involving calcium ions, and alters transmembrane ion gradients, causing additional neuronal injury. Calcium-mediated proteolysis by calpain and caspases in turn leads to further damage to the axonal cytoskeletal, and eventually neuronal death. Neuroinflammatory processes and microglial activation also contribute to local injury processes, endured long after the initial insult. The most important contributing factors are edema formation due to disruption of blood-brain barrier, increased intracranial pressure, and decreased cerebral blood flow and inflammation-like processes leading to cell death (Borgens and Liu-Snyder 2012; Werner and Engelhard 2007).

Focal and diffuse injuries: Focal injuries refer to injuries localised to a certain area or surface of the brain, and can cause compression of the tissue underneath the cranium at the site of the impact. Focal injury damage constitute subdural and epidural hematomas and contusions. Diffuse injuries, on the other hand, refer to an injury with a globalised effect. It involves dispersed damage to axons, diffuse vascular injury, hypoxic-ischemic injury and brain swelling. The main injury mechanism responsible for diffuse injury is rapid acceleration–deceleration of the head, as seen, for example in high-speed motor-vehicle accidents. Diffuse axonal injury (DAI) is the most common consequence of diffuse TBI. It is typically characterized by axonal damage in multiple regions of the brain parenchyma, often causing impairments in cognitive, autonomic motor, and sensory function owing to disrupted neuronal connectivity. Axonal bulbs, grossly swollen axons due to accumulation of axonal transport proteins, are a pathological hallmark of DAI. Diffuse injuries are also associated with neuronal membrane permeability disruption. This membrane damage, also known as mechanoporation, allows influx of molecules into the plasma membrane, leading to either necrosis or reactive change without cell death.

Closed and penetrating injuries: Closed head injuries occurs when a blunt force impacts the head but the skull is not broken, fractured, or penetrated. It often results in diffuse damage to the brain parenchyma. Penetrating injuries when an object pierces the skull and breaches the dura mater. This may occur when a foreign object (e.g., a bullet) goes through

the skull, enters the brain, and damages specific parts of the brain. Penetrating injuries cause open wounds, which are more likely to cause infection, and aggravate the damage.

To summarise, severity classification of TBI is based on clinical parameters, as well as other factors which may not be related to the injury. This hinders the accurate diagnosis of the severity and the extent of damage caused by the injury. There is a clear need for additional tools such as imaging or biomarker (lab test) methods, not only to identify brain pathologies that require a surgical intervention, but to provide a more accurate diagnosis of the different brain injury subtypes and severities.

1.2 Epidemiology of TBI

Epidemiology of TBI has been extensively studied in developed countries, especially in the USA and the UK. While the incidence of TBI is equally high in developing countries, studies on infectious diseases take centre stage, giving less attention to TBI related research (Perel et al. 2008). In developed countries, TBI caused during vehicular accidents and sports are more common, whereas falls and assault are more likely to cause TBI in developing countries (Bener et al. 2010).

Labelled as the "silent epidemic"— TBI contributes to worldwide death and disability more than any other traumatic insult. In the US, epidemiological monitoring is conducted by the Centre for Disease Control (CDC). According to CDC's data, in 2014, about 2.87 million TBI-related emergency department (ED) visits, hospitalizations, and deaths occurred in the United States (US), including over 837,000 of these health events among children. Motor vehicle crashes were the leading cause of death for individuals aging 15-24, 25-34, and older adults aged ≥75 years. (Gardner and Zafonte 2016). In individuals younger than 45 years, injury is the primary cause of death in the US and other developed countries. The incidence of TBI is rising worldwide, mainly owing to injuries associated with increased use of motor vehicles. The leading causes of TBI are as follows: Falls (28%), motor vehicle crashes (20%), being struck by or against objects (19%) and assaults (11%) (Langlois, Rutland-Brown, and Wald 2006). Severe TBI is known to have high

mortality rates, and in case of survivors, it has a well-established risk for a variety of neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (DeKosky, Ikonomovic, and Gandy 2010; E. Hall and Sundman 2014).

Association between a comorbidity and functional status and risk of death in patients with traumatic brain injury (TBI) has also been an area of interest. Hypertension has been found to be the commonly found pre-existing condition in adults with TBI, and observed to increase the chances of in-hospital mortality according to recent case studies and epidemiological assessments (Sellmann et al. 2012; Thompson, Dikmen, and Temkin 2012; Wu et al. 2011). Therefore it needs to be systematically investigated in preclinical models whether and how pre-existing hypertension could worsen the outcome even in case of a relatively mild, concussive injuries.

1.3 Neurodegenerative and neurocognitive consequences of single and repetitive mild traumatic brain injury

The acute impact of moderate to severe TBI on cognition and behaviour has been well-documented in humans. Cognitive outcome following head-on collision TBI very much resemble the memory deficits reported in patients following frontal lobe damage, e.g., memory loss, impulsivity and emotional instability (Vakil 2005). However, as stated earlier, concussions or mild TBI (mTBI) are much more common and more frequently observed. According to CDC estimates, 1.6-3.8 million sports and recreation related concussions occur each year in the US. The true frequency of concussion is likely to be far greater than registered because concussions are routinely under-recognized, under-reported and typically resolve spontaneously without medical care. Concussions are highly individualised injuries as most people recover relatively quickly and fully. Concussions are a frequent occurrence in contact sports such as football, soccer, rugby and hockey. Two primary complications of concussion are the postconcussion syndrome and second impact syndrome. Postconcussion syndrome (PCS) is the collection of cognitive and behavioural effects of concussion which may persists up to 3 months post-injury (Barlow 2016; Silverberg and Iverson 2011). In one study, functional neuroimaging approach was used

to assess sports-related concussion in which imaging was performed before injury so that neurobehavioural changes resulting from concussion could be better understood. Preseason baseline levels of blood oxygen level–dependent (BOLD) activity were acquired during the performance of a test battery that included mathematical, memory, and sensorimotor coordination tasks. Substantial within-subject increases in the amplitude and extent of the BOLD response were observed during the tasks in injured subjects compared with non-injured controls, suggesting recruitment of additional neural resources in response to moderate processing loads (Jantzen et al. 2004).

Second impact syndrome is a condition in which a second head impact is sustained within a vulnerable period, before the recovery from the initial impact, leading to massive edema, and increased intracranial pressure within minutes of the impact and resulting in brain herniation, followed by coma and death (Cantu 1998; Rabadi and Jordan 2001). Increasing evidence has suggested that athletes may sustain multiple concussions throughout their active career, thus potentially exacerbating their cognitive functions. In a pioneer study conducted by Omalu (Omalu et al. 2005), it was observed that repetitive concussions to American football players can cause cognitive impairment and drastic change in behaviour, and in worst cases, it can lead to *chronic traumatic encephalopathy* (CTE), a neurodegenerative condition causing progressive decline of memory and cognition, as well as aggression, poor impulse control, and even parkinsonism. Repetitive mTBI is also linked to development to *dementia pugilistica*, a form of dementia that is shown to affect athletes who suffer repeated concussions or blows to the head (Ling, Hardy, and Zetterberg 2015). Understanding the long-term sequelae of concussion in humans has been challenging for investigators due to the wide range of the severity of injury, the heterogeneous nature of outcome, and the feasibility of extended follow-up.

In the recent decade, there has been a growing interest to investigate the neuropsychological and pathological effects of repetitive concussion in experimental animal models. Most TBI studies have been primarily conducted in rodents, as they offer the ability to investigate molecular and neurophysiological changes from minutes to weeks following the injury. For example, in a recent experiment, adult mice who received 3 mild impacts with an inter-injury interval of 24h exhibited significant deficits in learning in the Morris water maze (MWM) task at 1 week post-injury, compared to mice who received a single hit (Nichols et al. 2016). In another study, adult mice subjected to 5 hits with a 48h interval had significant deficits in rotarod and Barnes maze 3 weeks post-injury (Mouzon

et al. 2012). Extensive axonal damage caused by repetitive mTBI has also been reported (McAteer et al. 2016; Mouzon et al. 2012; Ojo et al. 2016), as well as neuronal loss in the hippocampus accompanied by cognitive deficits in rodent models of repetitive TBI (Zhao et al. 2012). Heightened tauopathy and glial activation markers such as glial fibrillary acidic protein (GFAP) have also been observed to occur following repetitive mTBI (Rubenstein et al. 2019; Shitaka et al. 2011).

Most of the currently available studies have only investigated acute and sub-acute effects (at 1 to 2 weeks postinjury) of repetitive mTBI (Creeley et al. 2004; Laurer et al. 2001). Since limited number of studies have paid little or no attention to long-lasting functional consequences of head injuries, it is crucial to develop animal models that approximate human repetitive concussion scenarios and investigate sub-acute and chronic behavioural effects of repetitive injuries.

2. AIMS

Therefore, the major aim of the present thesis is to design repetitive mTBI in a rodent model and study the long-term cognitive and neuropathological effects.

Traumatic brain injuries of different severities were investigated first, to assess the long-term effects using a battery of behavioural tests, and molecular biomarkers. Our main goal was to develop an mTBI model which would otherwise not have any observable long-term morphological and cognitive effects, and then use the model as a base to further develop and test two repetitive mild TBI (rmTBI) models. And finally, test cognitive enhancers in an rmTBI model, to reverse/ameliorate cognitive dysfunction.

Specific aims:

1. To develop a mTBI model in adult rats, which would cause only short but not prolonged effects on cognition and memory, by testing TBIs of different severities and survival intervals.
2. To test the sub-acute behavioural effects of mTBI in normotensive and spontaneously hypertensive rats.
3. To develop an rmTBI model in adult rats, and asses the short-term and long-term behavioural and pathological effects. To best mimic the outcome of human repetitive head trauma scenarios, we aim to develop two rmTBI models with short and long inter-injury intervals.
4. To alleviate the cognitive deficits expected in an rmTBI model with memantine, a glutamtatergic NMDAR-receptor antagonist, at sub-acute and chronic stages of TBI.

3. STUDY 1: BEHAVIOURAL EFFECTS OF TRAUMATIC BRAIN INJURY OF DIFFERENT SEVERITIES

3.1 Introduction

Frequently seen in people engaged in contact sports, mTBIs in the form of a concussion with or without loss of consciousness, pose a serious risk, and accounts for more than 75% of all reported head trauma cases (Cassidy et al. 2004). Mild TBI (mTBI) is most often associated with short-term cognitive dysfunction that tends to resolve within three months of injury (Levin and Robertson 2013; McCrea et al. 2003). Diffuse axonal injury, a process of widespread axonal damage, has become widely accepted as the main pathological substrate of mTBI, and leads to functional and psychological deficits. Patients with mTBI have been observed to have abnormal mean fractional anisotropy values in the corpus callosum (CC) (Inglese et al. 2005). It is not clear whether mTBI causes overt long-term cognitive impairment. Animal models have been used to study the short-term behavioural and pathologic outcome of mTBI (Abdel Baki et al. 2009; Singh et al. 2016; Spain et al. 2010). However, there seems to be inadequate information about the threshold of mild injury in adult rats.

In the following experiment, our aim was to study the behavioural effects of mTBI in adult rats, and evaluate the threshold of injury which will not cause long-term persistent cognitive and structural dysfunction.

3.2 Methods

Subjects

Adult male Wistar rats (Toxi-Coop, Budapest), weighing 400-550g (aged 8-10 months old) were used. Forty-eight rats took part in the behavioural tasks, with 12 rats in each injury/experimental group. Animals were double-housed, under controlled conditions (standard 12 h light cycle from 7 a.m. to 7 p.m., with controlled temperature and humidity). Rats were maintained at 80–85% of their free feeding weight by restricting their laboratory chow supplement. They were fed with 17 g of laboratory chow (ssniff-Spezialdiäten GmbH, Germany) per animal per day. Water was provided ad libitum. Weeks prior to the

behavioural testing, all rats were handled daily. All procedures were approved by the Institutional Animal Use and Care Committee of the University of Pecs and were licensed by the Baranya County Government Office, Hungary (nr: BAI/35/51-107/2016) and carried out in accordance with the ARRIVE guidelines (Drummond, Paterson, and McGrath 2010).

3.3 Experimental Traumatic Brain Injury

All surgical procedures were performed by the Neurotrauma Research Group, at the Szentagothai Research Center. Animals were anaesthetised with isoflurane gas. Anaesthesia was induced for 5 min with 4% isoflurane (Forane, Abbott, Hungary) in 70% N_2O and 30% O_2 in an induction box, and maintained under anaesthesia throughout the injury and surgical procedure. Rats were then ventilated with 1.5% isoflurane in 70% N_2O and 30% O_2 (Inspira ASV, Harvard Apparatus USA). Once the anaesthesia was stabilized, the animals were exposed to an impact acceleration TBI procedure initially described for rats by Foda and Marmarou (1994).

A midline incision was made on the skin to expose the skull. A stainless steel disc (10 mm in diameter and 3 mm thickness) was fixed on the skull in the sagittal midline, centrally between the lambda and bregma landmarks using cyanoacrylate adhesive, in order to reduce the risk of skull fracture. The rat was placed prone on a foam bed under a 2 m high hollow Plexiglass tube with an inner diameter of 10mm, which contained 9 cylindrical brass weights, weighing 50 g each which were attached to each other. The 450 g weight was dropped onto the stainless disc fixed to the rat's skull. Severity of injury was determined as the height from which the weight was dropped (**Table 1**). The rat was then placed back on the stereotaxic frame to remove the disk. The exposed scalp was sutured, and the rat was placed in an empty cage for recovery. Sham animals were prepared for injury in the same fashion, but were not injured.

Experimental groups	Description of injury
Sham	No injury
Mild1	15 cm
Mild2	25 cm
Severe	150 cm

Table 1. *Injury groups were based on the height from which the injury was caused, using the Marmarou model (Foda and Marmarou 1994). Abbreviations: Sham: sham-injured; Mild1: 15cm injury; Mild2: 25cm injury: Seve: 150cm injury. N=12 rats/group.*

Diffusion Tensor Imaging and Image Processing

Changes in the integrity of CC was evaluated using diffusion tensor imaging, at pre- and post-injury time-points, by the NMR Research Group at the Szentagothai Research Center **(See Appendix)**.

Behavioural tests

Beam Balance Test

Fine motor coordination was examined in a beam balance test (BBT), where the rat has to remain upright on a straight, horizontal beam. The beam was made of wood, 1 cm wide and 1 m long. It was placed about 50 cm above the ground, parallel to the floor. Soft bedding material was placed right under it, to cushion the fall. In the test, each animal had 5 trial, 1 habituation trial and 4 test trials. The habituation trial lasted 60 s, during which the rats are pre-trained to the beam, and placed back on it if they slip or fall. In test trials, time spent on the beam by the rat was measured and recorded, until the rat falls or slips. Each test trial had a maximum duration of 60 sec. All animals were tested pre- and post-injury (**Fig. 1**).

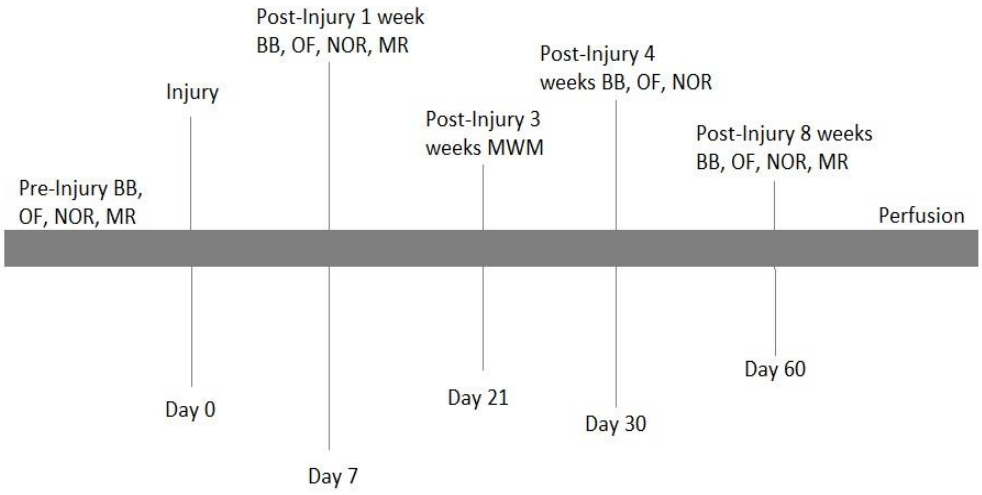

Figure 1: *Overview of experimental schedule. Abbreviations: BB: Beam Balance; OF: Open-Field; NOR: Novel Object Recognition; MWM: Morris Water Maze; MR: Magnetic Resonance-Diffusion Tensor Imaging.*

Open Field Test

Locomotor activity was measured in the open field test (OFT) apparatus (**Fig. 2**). Open field test sessions were run on the day before the test (NOR) sessions in order to habituate rats to the arena. The OFT was performed in an open field box which was made of black-coloured plywood, in size of 57.5x57.5 cm (length x width) surrounded by 39.5 cm high walls. The floor of the arena was divided with light grey painted lines to four by four equal squares. The four squares in the middle of the arena, which were not bordered by walls, were considered together as the centre area of the arena. In each session, rats were allowed to explore the OFT arena for 5 min. After each session, the box was thoroughly cleaned using 20 v/v % ethanol. Line crossings were counted manually. Each session was recorded using a video camcorder (JVC super LoLux color video camera, JVC KENWOOD Corporation, Yokohama, Japan) positioned above the OFT arena, and the Ethovision XT10 tracking software (Noldus, Wageningen, The Netherlands) was used for data acquisition. All animals were tested for baseline measurements (pre-injury) and at post-injury 1, 4 and 8 weeks.

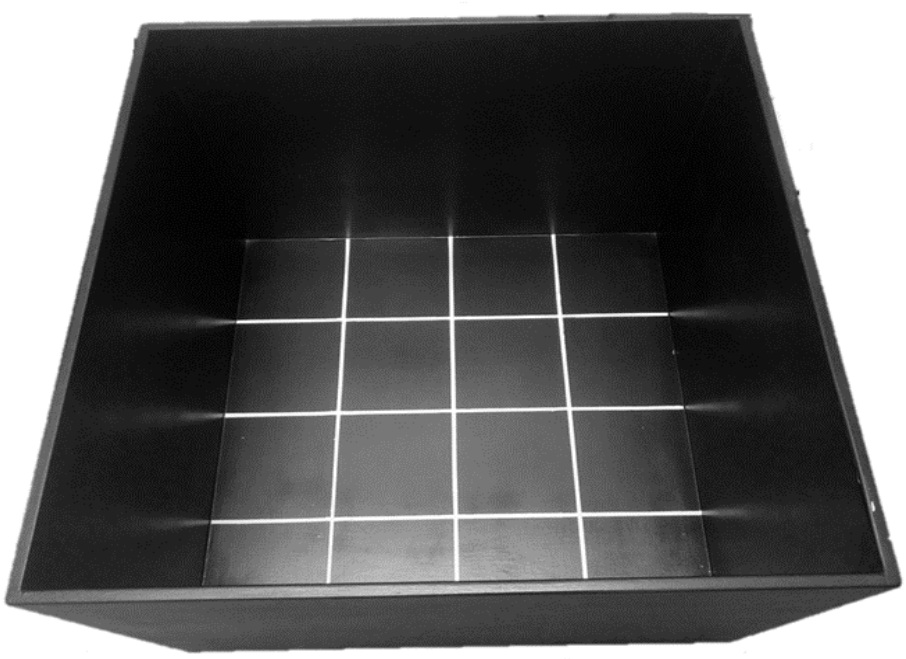

Figure 2: *Rectangular testing apparatus used for open field tests (OFT) and novel object recognition tests (NOR). The apparatus was made of black-painted plywood, in size of 57.5x57.5 cm (length x width) surrounded by 39.5 cm high walls.*

Novel Object Recognition Test

Recognition memory performance of the animals was tested in the novel object recognition test (NOR). The same apparatus (box) was used in the NOR test as in the OFT with the same video tracking system.

The NOR test included 2 trials – one acquisition trial followed by one retention trial after a certain delay (Ennaceur and Delacour 1988). In the first (acquisition) trial, the rats explored 2 identical objects (f + f) placed in the arena for a duration of 3 min. After a 30 min delay, a second (recognition) trial was run with one object identical to the sample and a novel object introduced, which had never been seen by the animal before (f + n). Observation behaviour of the animals in the second trial was also recorded for 3 min. During the delay period, rats were not transferred back to the animal house; they were kept in an empty cage, in a dark room located next to the testing room. In both trials, the time spent with the exploration of one or the other objects was recorded. The animal was considered to explore a given object, when it sniffed the object or put his nose close to it

while facing the object. Four different object-pairs were used: Nescafe-Szappan, Csizsolt-Unicum, Barna-Pohar and Nyuszi-Oszlop (**See Fig. 3**). They were distributed randomly between animals and experimental sessions in a counterbalanced latin-square design (**Table 2**). In the first trial of each NOR test, overall exploratory activity was measured by summing the exploration times for the two objects (SumE1). In the second trial, the time spent with the exploration of the novel (En) and the familiar (Ef) objects were compared by calculating a discrimination index (DI) using the following equation:

$$DI = (En – Ef) / (En + Ef).$$

The DI was a positive number if the novel object was observed for a longer duration, while the DI was negative if the familiar object (f) was observed for longer, and the DI was around zero if the two objects were observed for equally long time. Rats with low exploratory drive in the second trial (i.e., did not observe the two objects together for at least 5 s), or with +1.00 or −1.00 DI were excluded from the analysis.

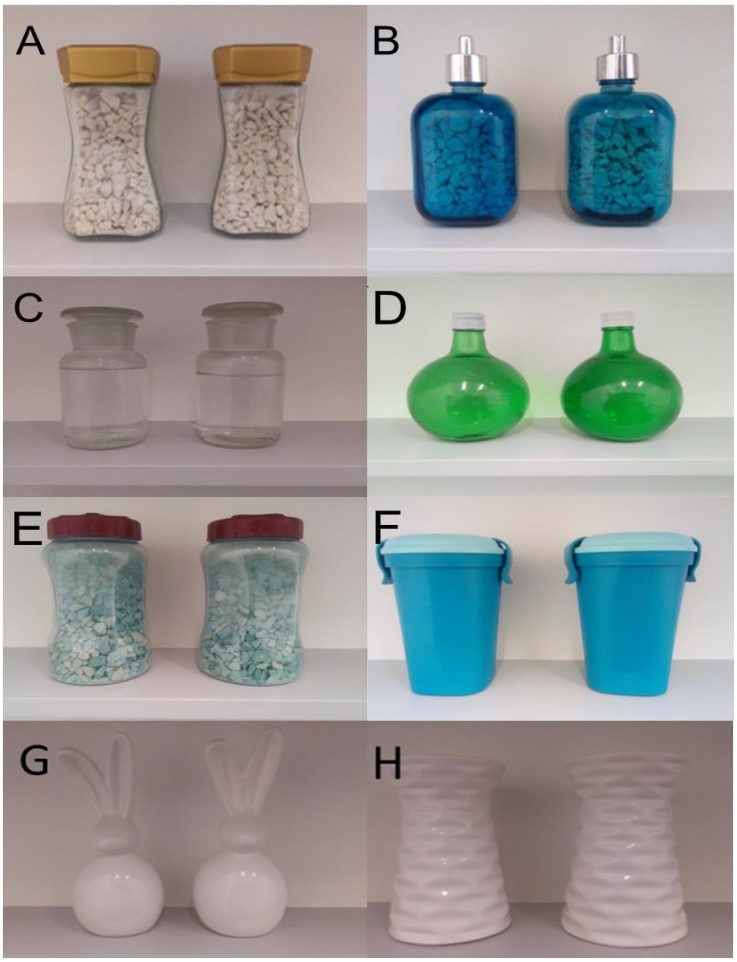

Figure 3: *Object pairs used for the NOR test.* ***A****: Nescafe,* ***B****: Szappan,* ***C****: Csizsolt,* ***D****:Unicum,* ***E****: Barna,* ***F****: Pohar,* ***G****: Nyuszi, and* ***H****: Oszlop.*

Injury Group	Session 1	Session 2	Session 3	Session 4
Sham	Unicum-Csizsolt	Nescafe-Szappan	Pohar-Barna	Nyuszi-Oszlop
Mild1	Pohar-Barna	Unicum-Csizsolt	Nescafe-Szappan	Nyuszi-Oszlop
Mild2	Nescafe-Szappan	Unicum-Csizsolt	Pohar-Barna	Nyuszi-Oszlop
Seve	Nescafe-Szappan	Pohar-Barna	Unicum-Csizsolt	Nyuszi-Oszlop

Table 2: *Latin-square table for the object pairs and NOR sessions. Abbreviations: See Table 1 for injury groups.*

Morris Water Maze Test

Short- and long-term spatial memory of the rats was tested in the Morris water maze (MWM) using a blue, circular pool, 180 cm in diameter and 90 cm in height (Ugo Basile, Gemonio, Italy). Four points around the circumference of the pool were arbitrarily

designated as North, South, East, or West. On this basis, the floor area of the pool was divided into four virtual quadrants (NW, SW, SE, NE). The maze was filled with water up to the height of 30 cm, and the water was made opaque by mixing 200 g milk-powder and 30 ml blue food colouring (E131) in it. The rats were trained in the water maze task in four training sessions on four consecutive days with four trials for each animal on each day. On training days, a hidden platform was placed in the centre of the SW quadrant. In each trial, rats were put into the water facing the wall of the NW quadrant at the beginning of the session, and then in the following trials in a clockwise direction, and were allowed to search for the hidden platform for 120 s. The time elapsed until finding the platform (i.e., sitting on it) was measured as escape latency. If the rat failed to find the platform after 120s, it was gently guided and transferred to the platform by the experimenter, and the cut-off time was recorded as escape latency. The quadrant from where the animal started swimming was changed clockwise in the four consecutive trials on a given day. On the fifth day, the platform was removed from the pool. A single probe trial was performed, and rats were allowed to explore the pool for 120 s. The time spent in the target quadrant was measured during the probe trial as a readout of long-term memory. Experiments were recorded using a Basler GenI acA1300 GigE camera (Basler AG, Ahrensburg, Germany). Data was processed in a PC computer, where Ethovision X10 software (Noldus, Wageningen, Netherlands) was used for recording and data analysis. The rats were tested in the water maze task at post-injury 3 weeks.

Statistical Analysis

All quantitative data are expressed as mean ± standard error of the mean (s.e.m). Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 23 (IBM Corporation, Armonk, NY USA) and MS Excel (Microsoft Corporation, Albuquerque, New Mexico, USA). For BBT and OFT, univariate ANOVA test was applied to compare the injury groups. For the BBT, a repeated measures analysis was applied for a longitudinal comparison of injury groups and the test sessions. In the NOR, Student's t-test was used to analyse the preference for the novel object above the chance level (DI = 0). Morris Water Maze acquisition data were analysed by two-factor mixed-ANOVA (Within-subject factor: DAYS. Between-subject factor: INJURY). A level of $p<0.05$ was considered statistically significant. Where appropriate, data were analysed by the Kruskal-Wallis non-

parametric rank test and pairwise comparisons were made using the Mann-Whitney U test.

3.4 Results

Traumatic brain injury of different severities did not cause any effects on fine motor coordination

In the pre-injury session (**Fig. 4A**), time spent on the beam did not differ between the groups ($F(3, 45) = 0.500$; $p = 0.684$). No immediate or chronic effect of TBI was observed, as seen in the post-injury 1week ($F(3,44) = 0.108$; $p = 0.955$), post-injury 4 week ($F(3,44) = 0.911$; $p = 0.443$), and post-injury 8 week ($F(3, 44) = 0.422$; $p = 0.738$) sessions respectively (**Fig 4B-D**). Repeated measures ANOVA revealed that all the experimental groups were able to improve the duration of time and stayed longer on the beam ($F(3,132) = 12.260$; $p<0.001$). However, no significant difference was observed in the performance of the injury groups ($F(3,44) = 0.429$; $p=0.733$), nor any interaction between the test sessions and the injury groups ($F(9,132) = 0.721$; $p=0.689$).

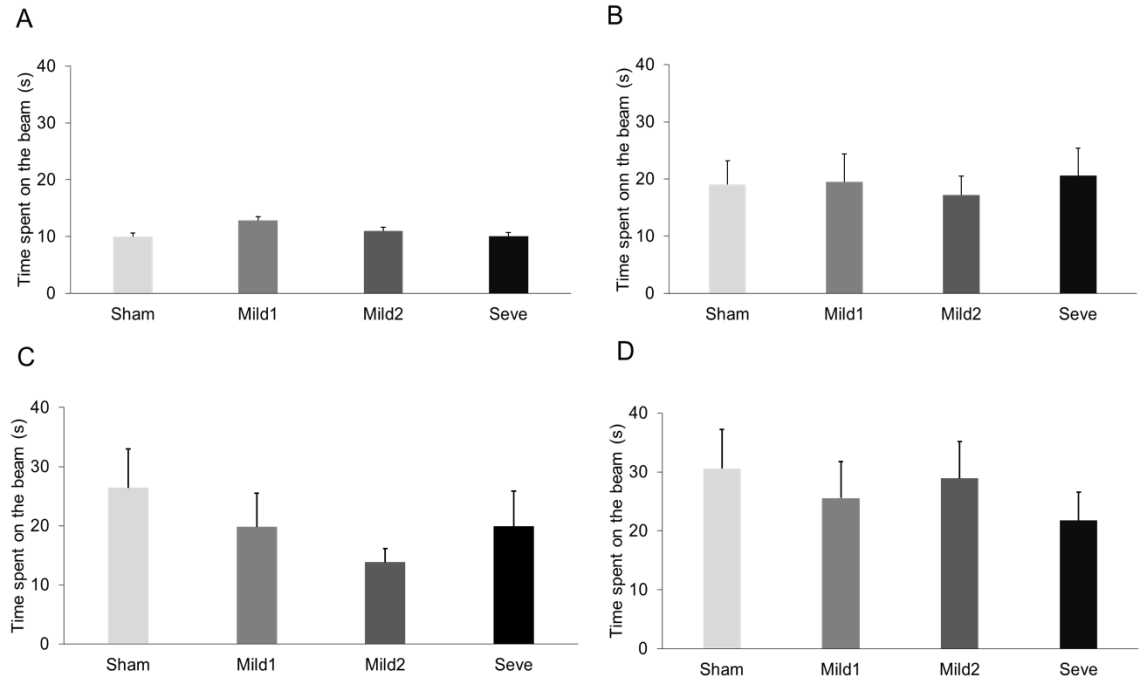

***Figure 4**: Fine motor coordination examined with beam balance test did not indicate any major impairment following TBI. **(A)** Time spent on the beam in the pre-injury session*

*showed similar performance between the groups (p = 0.684; n=12/group). Post-injury 1 week **(B)**, post-injury 4 week **(C)**, and post injury 8 week sessions **(D)** did not reveal any effects of TBI on fine motor skills. Abbreviations: See Table 1 for injury groups.*

Locomotor activity remained unaffected following traumatic brain injury

Locomotor activity was measured by counting line crossings in the OFT apparatus (**Fig. 5**). Animals of all the injury groups exhibited overall good locomotor function in the pre-injury test, with no statistical difference in performance ($F(3,44) = 1.180$; $p=0.328$). All the injury groups performed similarly in both the post-injury 1 week ($F(3,44) =1.074$; $p=0.370$), post-injury 4 weeks ($F(3,44) = 0.307$; $p = 0.820$, and the post-injury 8 weeks tests ($F(3,44) = 0.116$; $p=0.950$), indicating no major impairment in locomotor function as a result of any types of TBI.

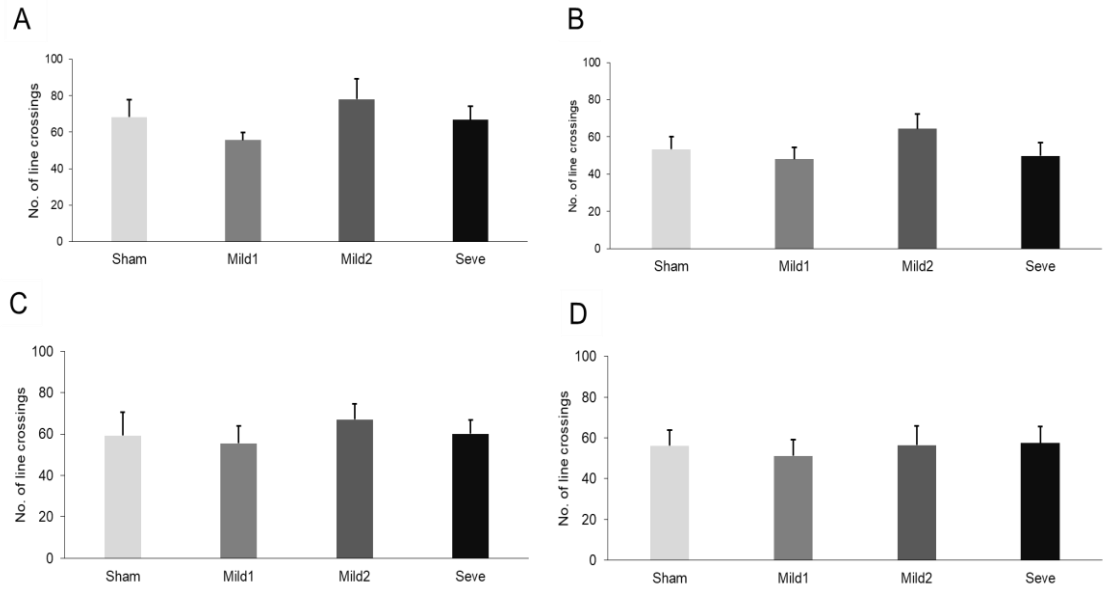

Figure 5: *Locomotor activity measured by counting line crossings in OFT. Pre-injury session **(A)** indicated overall good locomotor function (n = 12/group). Post-injury 1 week session **(B)** indicated a minor, but insignificant, decline in line crossings, indicating habituation, but overall no effect of TBI on locomotor activity (p=0.370). Similarly, post-*

*injury 4 weeks and 8 weeks **(C-D)** indicated that all groups performed similarly. Abbreviations: See Tab. 1 for injury groups.*

Mild2 and Severe, but not Mild1 injuries, caused persistent working memory deficits in the NOR test

In the pre-injury NOR test, all groups were able to discriminate between the familiar and the novel object (**Fig. 6A**). Discrimination index value for each group was above the chance level. For Sham: 0.214 ± 0.052 ($t=4.112$, $df=11$, $p<0.01$); Mild1: 0.255 ± 0.116 ($t=2.198$, $df=11$, $p=0.050$); Mild2: 0.216 ± 0.072 ($t=2.981$, $df=11$, $p<0.05$); Seve: 0.217 ± 0.070 ($t=3.098$, $df=11$, $p=0.01$). All the groups performed similarly in the pre-injury session ($F(3, 44)=0.058$; $p=0.980$).

In the post-injury 1 week NOR session (**Fig. 6B**), only Sham and Mild1 groups were able to discriminate the objects. $F(3,36) = 1.168$, $p=0.336$; Sham: 0.254 ± 0.094 ($t=2.473$, $df=9$, $p<0.05$); Mild1: 0.431 ± 0.091 ($t=4.412$, $df=6$, $p<0.01$); Mild2: 0.132 ± 0.070 ($t=1.892$, $df=11$, $p=0.085$); Seve: 0.109 ± 0.181 ($t=0.604$, $df=10$, $p=0.559$). However in the post-injury 4 weeks NOR session (**Fig. 6C**), only Sham group were able to discriminate between the familiar and the novel object, while Mild1, Mild2 and Seve groups could not recover. $F(3, 30) = 1.228$, $p=0.317$; Sham: 0.193 ± 0.064 ($t=2.982$, $df=7$, $p<0.05$).

In the post-injury 8 weeks session (**Fig. 6D**), Mild1 injury group, along with Sham, was able to recover and was able to perform above chance level ($F(3, 31) = 1.329$, $p=0.282$; Sham: 0.346 ± 0.076 ($t=4.547$, $df=8$, $p<0.01$); Mild1: 0.369 ± 0.150 ($t=2.456$, $df=6$, $p<0.05$).

Mild2 and Seve were unable to discriminate the familiar and novel objects. Mild2: -0.122 ± 0.120 ($t=0.462$, $df=9$, $p=0.655$); Seve: 0.043 ± 0.145 ($t=0.302$, $df=8$, $p=0.770$).

Results suggest that Mild1 group did not suffer from any persistent deficits in NOR test, while Mild2 and Seve groups and were unable to recover from the impairment.

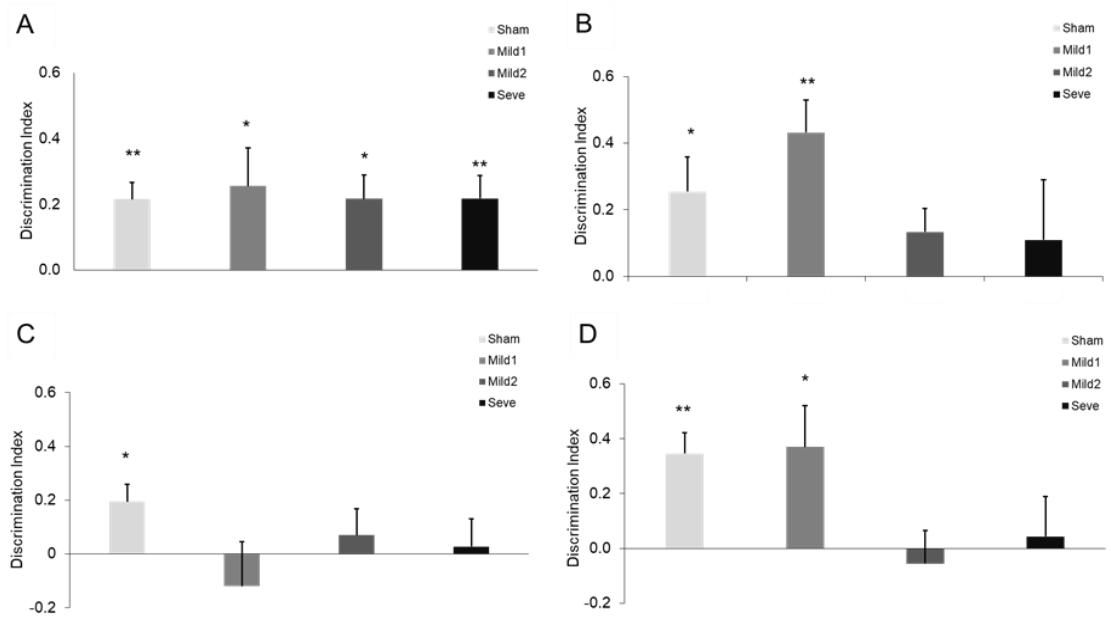

Figure 6*: Recognition memory was tested in NOR test. In pre-injury session* ***(A)****, all groups performed above chance level (F(3, 44)=0.058; p=0.980; Sham: p<0.05, n=12; Mild1: p=0.5, n=12; Mild2: p<0.05, n=12; Seve: p<0.05, n=12). In post-injury 1 week session* ***(B)****, only Sham and Mild1 groups performed above chance level (Sham: p<0.05, n=10; Mild1: p<0.01, n=7).* ***(C)*** *In post-injury 4 weeks session, only the Sham group was able to discriminate between the familiar and the novel object, while other groups were not (Sham: p<0.05, n=8).* ***(D)*** *However, in post-injury 8 weeks session, Mild1 group recovered and was also able to perform above chance level (Sham: p<0.01, n=9; Mild1: p<0.05, n=7). Abbreviations: See Table 1 for injury groups; * one-sample t-test p<0.05; ** one-sample t-test p<0.01.*

Morris water maze test revealed intact spatial memory following mild TBI

For the acquisition phase data, mixed-ANOVA for escape latency indicated that there was no interaction effect between injury groups and training days (F(9, 84)=0.360; p=0.951), and no significant difference between the groups (F(3, 28) = 0.879; p=0.464, n=8/group). However, there was a significant decrease of escape latency in all groups during the training days (F(3, 84)=50.392; p<0.001), suggesting that rats in all injury groups took significantly less time to find the platform on Day 4, compared to Day 1 (**Fig. 7A**).

MWM probe trial results were analysed using Kruskal-Wallis non-parametric rank test and pairwise comparison was made using Mann-Whitney test **(Fig. 7B)**. Time spent in the target quadrant was measured during the probe trial on Day 5. We found marginally significant between-subject effect in the probe trial timing, and Severe group was found to perform the worst, while Sham, Mild1 and Mild2 performed similarly (Kruskal-Wallis: p=0.09; Mann-Whitney: Mild1-Seve, Mild2-Seve p<0.05).

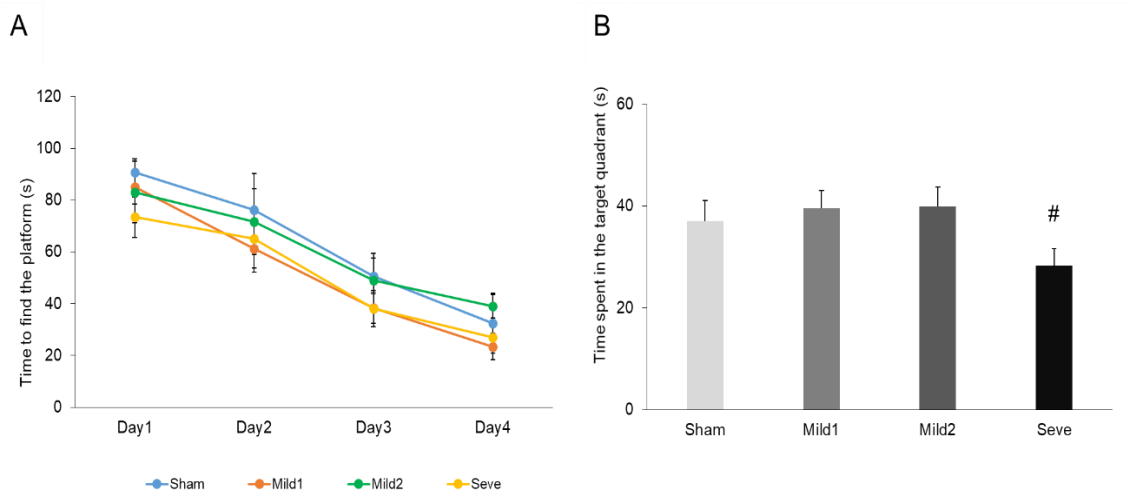

***Figure 7**: Spatial memory was tested using the Morris Water Maze (MWM) test. **(A)** During the acquisition phase, day average results revealed no interaction effect between injury groups and training days (p=0.951), and no significant difference between the groups (p=0.464). **(B)** Analysis of probe trial data revealed that Seve group was significantly worse than Mild1 and Mild2 (p<0.05). Abbreviations: See Table 1 for injury groups. # Tukey's pairwise comparison p<0.05.*

3.5 Discussion

The goal of this study was to investigate an impact-acceleration induced mTBI model in adult rats, which would not cause any persistent cognitive deficits. The primary injury was induced using the Marmarou weight-drop injury model, of different severities, to get a better assessment of the range of cognitive deficits following TBI. The results of this study revealed that animals with mTBI did not suffer from any significant long-term deficits in the behavioural tests, as well as no gross change in the integrity of the brain, especially of

the corpus callosum. In the NOR test, while in the pre-injury and post-injury 1 week sessions the Mild1 group exhibited above chance-level discrimination for novel and familiar objects, at post-injury 4 week session, the group was unable to discriminate significantly between the different objects. In addition, these deficits were found to be transient, as they were not seen in the post-injury 8 weeks session, while the Mild2 and Seve groups did not recover from the impairment following TBI. The sham group consistently performed well, and served as the negative control. In the MWM test, all injury groups were able to learn the task and took significantly less time to find the platform on Day 4 than on Day 1. However, in the probe trial (Day 5), all the groups performed similarly. Lack of deficits seen in the MWM, compared to the NOR test, could indicate that either the TBI had no detrimental effect on spatial memory, or the task was not complex enough for assessment. Moreover, in BBT and OFT, TBI had no significant effect on locomotor activity. Analysis of DTI data revealed no gross mechanical/shear injury to long white matter tracts of the CC following TBI of any severity.

The impact acceleration model of diffuse traumatic brain injury is widely utilized to replicate diffuse TBI without focal lesion to characterize changes that closely parallel abnormalities characteristic of human diffuse TBI. Several TBI studies have been on the fence on the degree of impairment caused by mTBI. Some studies suggest mTBI can cause considerable behavioural, as well as molecular consequences. In human studies, concussions have been shown to cause breakdown of blood-brain barrier (BBB), elevated levels of S100β, and neuroinflammation 6 months post-injury (Michetti et al. 2012; Sahyouni et al. 2017). In a rat model of mild blast TBI, deficits in working memory and neuronal injury were observed within 2 weeks post-injury (Rodriguez et al. 2017).

To summarize, in our study, we developed a 15 cm mTBI model, which causes no persistent cognitive impairment 8 weeks following injury. As per a systemic review of all TBI models in rodents, very few studies have utilised a weight-drop injury model with a projectile drop height of 15 cm (Bodnar et al. 2019). To the best of our knowledge this is the first study to demonstrate in the impact acceleration model of Marmarou in which a mTBI evoked from a height of 15 cm caused no significant long-term neurocognitive alterations. We also found that the 25 cm injury does not appear to be significantly different in the NOR test from the 15 cm injury group. We conclude that the 15 cm injury is suitable to be utilised as a base to develop repetitive mTBI models in rodents in order to replicate and understand the impact and the effects of repetitive concussive injuries in humans.

4. STUDY 2: BEHAVIOURAL EFFECT OF MILD TRAUMATIC BRAIN INJURY IN HYPERTENSIVE RATS

4.1 Introduction

Pre-existing comorbid conditions increase risk of mortality in TBI, most likely by exacerbating secondary injury of brain tissue (Thompson, McCormick, and Kagan 2006).. Induced hypertension, in mouse and rat models, has been shown to cause elevated levels of reactive oxygen species together with blood brain barrier (BBB) dysfunction (Nag, Kapadia, and Stewart 2011; Poulet et al. 2006), eventually altering the brain parenchyma homeostasis, and causing consequential neurodegeneration (Ballabh, Braun, and Nedergaard 2004; Weiss et al. 2009; Yamazaki and Kanekiyo 2017; Zenaro, Piacentino, and Constantin 2017). Spontaneously hypertensive rats (SHR) were developed as a model to study hypertension-related cardiovascular diseases in humans (Trippodo and Frohlich 1981). Hypertensive aged mice were found to exhibit increased permeability of the BBB, which is associated with neuroinflammation and cognitive decline of the animals (Toth et al. 2013). Thus, SHR rats serve as an excellent model to study hypertension as a co-morbidity in TBI.

In the following study we investigated the effects of pre-existing hypertension on cognitive function following mTBI in rats. This study was made in collaboration with the Department of Neurosurgery, University of Pecs.

4.2 Methods

Spontaneously hypertensive rats (SHR, male, 300–350 g, n = 15) and age-matched normotensive Wistar rats (Wistar, male, 300–350 g, n = 15) were purchased from Janvier Labs (Le Genest-Saint-Isle, France) and Toxi-Coop (Budapest, Hungary). Animals were double-housed, under controlled conditions (standard 12 h light cycle from 7 a.m. to 7p.m., with controlled temperature and humidity). Rats were maintained at 80–85% of their free feeding body weight by slightly restricting their laboratory chow supplement. They were fed with 17 g of laboratory chow (ssniff-Spezialdiäten GmbH, Germany) per animal per day. Water was provided ad libitum. Weeks prior to the behavioural testing, all rats were handled daily. All procedures were approved by the Institutional Animal Use and Care

Committee of the University of Pecs Medical School and licensed by the Baranya County Government Office, Hungary (nr: BAI/35/51-107/2016) and carried out in accordance with the ARRIVE guidelines (Drummond, Paterson, and McGrath 2010).

Experimental Traumatic Brain Injury

Mild impact acceleration diffuse brain injury was induced by Marmarou weight drop model, as previously explained. Under isoflurane (2%) anaesthesia, the skull was exposed by a midline incision between the lambda and bregma and a steel disc was fixed with cement on the skull. A 450 g cylindrical weight from 25 cm was dropped to the disc causing mild diffuse traumatic brain injury to the animals. All animals survived the procedures.

Behavioural tests

Open-Field Test

Open field test sessions were run on the day before the main test (NOR) sessions in order to habituate rats to the arena. The OFT was carried out in normotensive Wistar rats and SHRs (n = 15) before and two weeks after mTBI. The OFT was performed as described earlier. During the sessions, the number of line crossings were registered as a measure of locomotor activity and exploration.

Novel Object Recognition Test

Recognition memory performance of the animals was assessed by NOR at pre-injury, and two weeks after mTBI in normotensive Wistar rats and SHRs. The NOR was performed as described earlier. Time spent with the exploration of the novel (En) and the familiar (Ef) objects were compared by calculating a discrimination index (DI) using the following equation: $DI = (En - Ef)/(En + Ef)$. The DI was a positive number if the novel object was observed for a longer time than the familiar object, and the DI was negative if the observation of the familiar object was longer than that of the novel object. DI was around zero if the two objects were observed for equal amount of time. Three object pairs were used: Nescafe-Szappan, Csizsolt-Unicum, and Nyuszi-Oszlop. They were distributed

randomly between animals and experimental sessions in a counterbalanced latin-square design.

Statistical Analysis

All quantitative data are expressed as mean ± standard error of the mean (s.e.m). Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 23 (IBM Corporation, Armonk, NY USA) and MS Excel (Microsoft Corporation, Albuquerque, New Mexico, USA). For OFT, univariate ANOVA test was applied to compare the injury groups. For NOR, student's t-tests were used to analyse the preference for the novel object above the chance level (DI = 0). A level of $p<0.05$ was considered statistically significant. A two-way mixed ANOVA with within-subject factor of pre- and post-injury measurements, and between-subject factor of groups (i.e., Wistar and SHR).

4.3 Results

Normotensive but not spontaneously hypertensive rats showed habituation in OFT test following TBI

In the pre-injury session, both groups showed similar locomotor activity, indicated by number of line crossings (Wistar: 42.200 ± 6.386; SHR: 55.733 ± 4.761).

In post-injury 2 weeks session, Wistar rats had significantly less number of line crossings, which could indicate habituation (Wistar: 19.214 ± 3.993; $p<0.01$), while SHR rats did not show habituation to the OFT apparatus after TBI (SHR: 50.667 ± 5.055) (**Fig. 8A**).

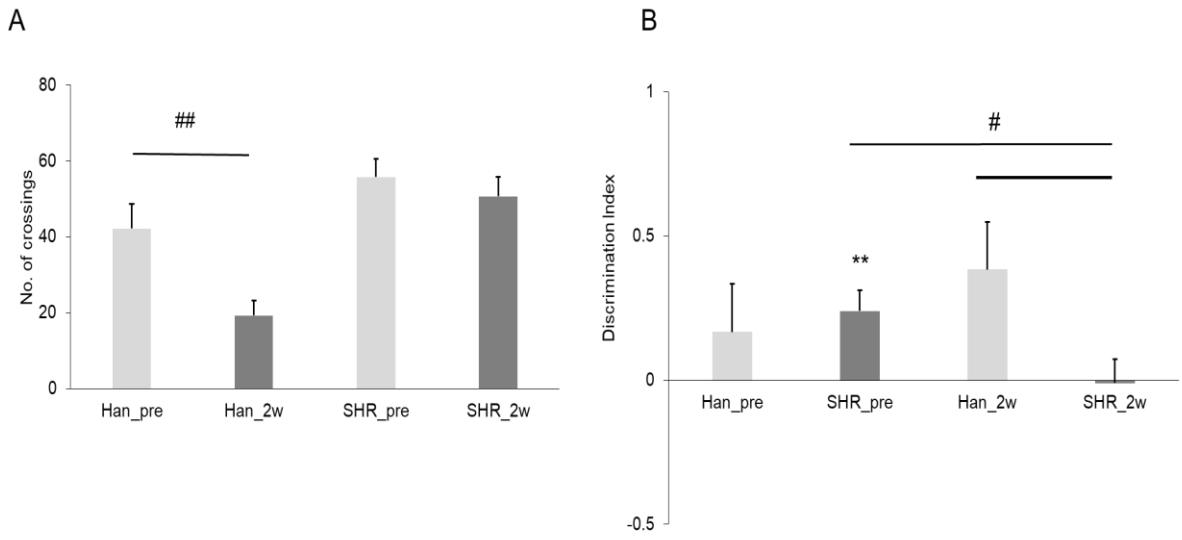

Figure 8*: Locomotor activity (**A**) and working memory performance (**B**) tested in Wistar and SHR rats pre- and post-injury. **(A)** In the OFT, compared to pre-injury session, only Wistar+mTBI(n=14) group showed significantly less number of line crossings, at post-injury 2 weeks (p<0.01). **(B)** In the pre-injury session, SHR rats were able to significantly discriminate the objects (p<0.01, n=11), while the normotensive Wistar rats couldn't (p=0.425, n=5). Wistar+mTBI (n=5) rats performed above chance level in the post-injury 2 weeks session, while SHR+mTBI (n=11) rats were significantly worse than Wistar+mTBI rats and pre-injury SHR rats (p<0.05). Abbreviations: Han: Normotensive Wistar rats, SHR: Spontaneously hypertensive rats, Han_pre: normotensive Wistar rats at pre-injury, SHR_pre: SHR rats at pre-injury, Han_2w: Wistar rats at post-injury 2 weeks, SHR_2w: SHR rats at post-injury 2 weeks. ** one-sample t-test p<0.01; # Tukey's pairwise comparison p<0.05; ## Tukey's pairwise comparison p<0.01.*

SHR rats showed significant working memory deficits following TBI in the NOR test

Intermediate-term declarative memory was tested using the NOR test pre- and post-injury (**Fig 8B**). In the pre-injury session, SHR rats were able to significantly discriminate the objects (0.240 ± 0.071; t=3.372, df=10, p<0.01), while the normotensive Wistar rats couldn't (0.167 ± 0.188; t=0.889, df=4, p=0.424). Two weeks post-injury, normotensive Wistar rats behaved similar to the pre-injury session (0.388 ± 0.165; t=2.310, df=4,

p=0.081). However, mTBI resulted in a significant ($p<0.05$) decrease in the DI of SHR rats indicating impaired memory function (SHR-TBI main effect: $F(1, 22)=5.223$, $p<0.05$)). SHR+mTBI was significantly worse than Wistar+mTBI and SHR rats ($p<0.05$).

4.4 Discussion

The goal of this study was to study hypertension as a co-morbidity for mTBI, and study behavioural effects of mTBI in hypertensive rats. The aim was based on the hypothesis that mTBI in rats with pre-existing hypertension would cause cognitive dysfunction. The injury was induced using the Marmarou weight-drop injury model from a height of 25 cm, to get a better assessment of the range of cognitive deficits following TBI. The OF and NOR results revealed deficits in working memory 2 weeks following mTBI in SHR rats, while normotensive Wistar rats did not suffer from any significant deficits in the behavioural tests.

Hypertension, on its own, causes slow but consistent, long-lasting damage to the brain. Hypertension has been shown to cause production of reactive oxygen species (ROS) (Pinto et al. 2007; Szarka et al. 2017), which leads to increased permeability of blood-brain barrier, microbleeding and neuroinflammation (Raz, Rodrigue, and Acker 2003; Szarka et al. 2017). These hypertension-associated pathologic changes in the brain can cause cognitive dysfunction (Iadecola et al. 2016; Manolio, Olson, and Longstreth 2003). Mild TBI has been observed to cause transient increase in production of ROS, which leads to short-term cognitive difficulties (Choi et al. 2012; Lewén et al. 2001; Marklund et al. 2002). Hypertension exacerbates ROS production, caused by mTBI, and can lead to long-term cognitive dysfunction (Marklund et al. 2002). In the current study, the working memory deficits observed in hypertensive rats following TBI reinforce the hypothesis that hypertension can act as a co-morbidity even in case of mild brain injuries and can amplify the pathologic changes. It is likely that mTBI-induced persistent neuroinflammation in SHR rats caused the BBB damage (Rochfort and Cummins 2015), probably inducing a positive feedback loop, and thus leading to the acceleration of neuroinflammation. These pathologic changes probably contributed to the cognitive impairment observed in the behavioural tests. However, in further experiments, inclusion of a sham-injured group, and

a late follow-up assessment would be necessary to reaffirm and validate the extent and persistence of cognitive impairment observed.

In conclusion, pre-existing hypertension exacerbates the behavioural outcome caused by mTBI. This puts hypertensive patients at a heightened risk of developing neurocognitive disorders such as Alzheimer's (Birkenhäger and Staessen 2004; Iadecola et al. 2016). Overall, in line with presently available epidemiological studies it is very likely, that hypertensive patients with mTBI should be assessed differently compared to normotensive patients and the mechanisms by which hypertension exacerbates the effects of mTBI should be further established in order to selectively target BBB function and achieve neuroprotection in patient populations.

5. STUDY 3: BEHAVIOURAL AND MOLECULAR EFFECT OF REPETITIVE MILD TBI

5.1 Introduction

Mild repetitive brain injuries, either concussive or subconcussive, may increase the risk of developing neurodegenerative disorders, such as dementia, in old age (Stern et al. 2011). Research has shown that following a single incidence of concussion, the brain's auto regulatory mechanisms compensate for the mechanical and physiologic stress. Extracellular potassium concentration can increase massively in the brain after concussion, followed by hypermetabolism lasting up to ten days. This makes the brain more vulnerable to a second impact and leads dysfunction of auto regulation of intracranial and cerebral perfusion pressures (McCrory and Berkovic 1998). Several studies have described functional, as well as pathologic outcomes of repeated mTBI, such as reactive astrogliosis and axonal damage, following injury (DeFord et al. 2002; A. N. B. Hall, Joseph, and Brelsfoard 2016; Ojo et al. 2016; Uryu et al. 2002). Blood or CSF biomarkers can prove to be valuable in diagnosing the extent of cerebral damage following multiple concussive injuries (Diaz-Arrastia et al. 2014). Amyloid precursor protein (APP) is a well-known acute biomarker of impaired axonal transport (Stone et al. 2004), while RMO-14 is a biomarker of neurofilament compaction (Marmarou et al. 2005). Initial effects of trauma cause primary axotomy, which includes shear and stretch injuries to long-tract structures such as axons and blood vessels. Both APP and RMO14 serve as critical biomarkers for studying primary axotomy in single and repetitive mild TBIs. In a mouse model, axonal injury has also been observed in the form of APP and glial fibrillary acidic protein (GFAP) immunoreactive profiles in the corpus callosum 24 hours post-injury (Mouzon et al. 2012). Tau protein phosphorylation and tangle-like pathologies have been observed many months after closed head repetitive TBI in some reports, therefore Tau pathology, as a common feature of several neurodegenerative disorders, has also been implicated in TBI (Kane et al. 2012; McAteer et al. 2016). Similarly, S100β, a calcium binding protein, has been noted as a biomarker in assessment of outcome in patients with TBI (Goyal et al. 2012; Thelin, Nelson, and Bellander 2017).

Most of the currently available studies have investigated acute and sub-acute effects of repetitive mTBI, revealing impaired spatial learning and memory (Creeley et al. 2004;

Laurer et al. 2001). Unfortunately, little is known about the cumulative effect of multiple episodes of mTBI with different inter-injury intervals and their long-term effects.

In our following experiment, we developed two different repetitive mTBI models in rats, on the basis of the time interval between the successive injuries, and compared the effects of repetitive mTBI on cognition. In order to determine the temporal window of vulnerability of the brain to secondary injury (Longhi et al. 2005), we designed repetitive mTBI models with short and long inter-injury intervals. The goal of our study was two-fold: 1) to determine the temporal window of vulnerability of the brain to a second impact, and 2) to assess the effect of repetitive mTBI on behavioural and molecular outcomes. Furthermore, we also investigated blood levels of pTau, S100β and GFAP proteins, and the occurrence of classical immunohistochemical markers of axonal injury after TBI (APP, RMO-14).

5.2 Methods

Subjects

Adult male Long Evans rats (Charles River Laboratories, Germany, aged 8-10 months at the beginning of the study) weighing 400-500 g were used. Seventy rats were used in the long-term behavioural testing, while an additional fifteen rats were used for post-injury 24-h immunohistochemistry. Animals were pair-housed, and were kept under controlled environmental conditions (standard 12 h light cycle from 7 a.m. to 7 p.m., with controlled ambient temperature and humidity). Rats were maintained at 80–85% of their free feeding weight by restricting their laboratory chow supplement. Typically, they were fed with 17 g of standard laboratory chow (ssniff-Spezialdiäten GmbH, Germany) per animal per day. Normal tap water was provided ad libitum. Weeks prior to the behavioural testing, all rats were regularly handled for proper acclimatization to the lab environment and experimenters. All procedures were approved by the Institutional Animal Use and Care Committee of the University of Pecs and were licensed by the Baranya County Government Office, Hungary (nr: BAI/35/51-107/2016) and carried out in accordance with the ARRIVE guidelines.

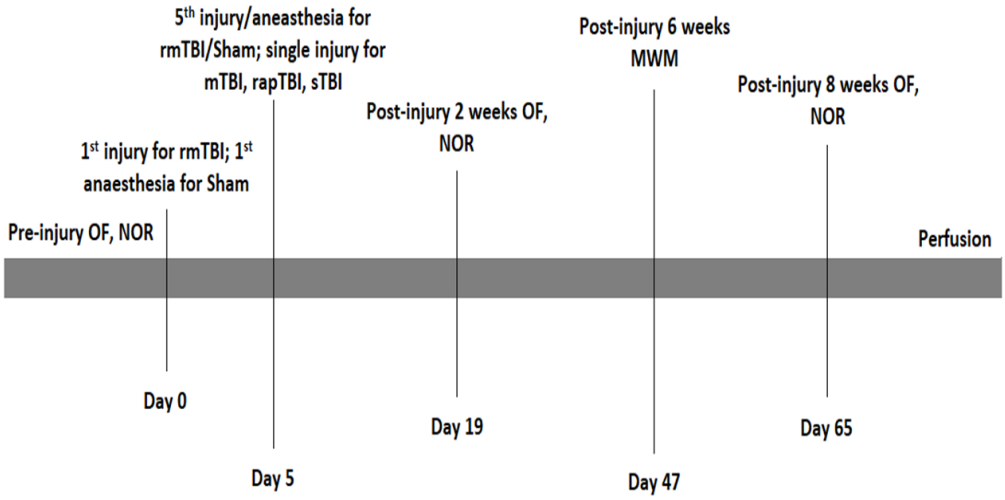

Figure 9*: Overview of the experimental schedule. Abbreviations: Sham: sham-injured; mTBI: mild TBI; rmTBI: repetitive mild TBI; rapTBI: rapid repetitive TBI; sTBI: severe TBI; OF: open field; NOR: novel object recognition; MWM: morris water maze.*

Experimental Traumatic Brain Injury

Animals were anaesthetised with isoflurane gas. Anaesthesia was induced for 5 min with 4% isoflurane (Forane, Abbott, Hungary) in 70% N_2O and 30% O_2 in an induction box, and rats were maintained under anaesthesia throughout the injury and surgical procedure. Rats were ventilated with 1.5% isoflurane in 70% N_2O and 30% O_2 (Inspira ASV, Harvard Apparatus USA). Once the anaesthesia was stabilized, the animals were exposed to an impact acceleration method of TBI initially described for rats by Foda and Marmarou (Foda and Marmarou 1994).

A midline incision was made to expose the skull from the bregma to the lambda craniometric points. A stainless steel disc (10 mm in diameter and 3 mm thickness) was fixed on the skull centrally between the lambda and bregma craniometric points using

cyanoacrylate adhesive, in order to reduce the risk of skull fracture. The rat was placed prone on a foam bed under a 2 m high, hollow Plexiglass tube with an inner diameter of 10 mm, which contained 9 cylindrical brass weights (weighing 50 g each) that were attached to each other. The total 450g weight was dropped onto the stainless disc fixed to the skull. Severity of injury was determined as the height from which the weight was dropped. The rmTBI animals were operated and anesthetized to receive one 15cm injury on each day, for five days, whereas the rapTBI animals received all the five injuries on the same day, under a single, continuous administration of anaesthesia. Sham animals were prepared for injury in the same fashion, but were not injured (**Fig. 9; Tab. 3**).

Groups	**Injury**
Sham	5 anesthesia, 24 hours apart; no injury
Single mTBI (mTBI)	15 cm; 1 hit
Repetitve mTBI (rmTBI)	15 cm; 5 hits, 24 hours apart
Rapid repetitve TBI (rapTBI)	15 cm; 5 hits, 5 minutes apart
Severe (sTBI)	1 hit; 150 cm

Table 3: *Summary of experimental groups. Abbreviations: Sham: sham-injured; mTBI: mild TBI; rmTBI: repetitive mild TBI; rapTBI: rapid repetitive TBI; sTBI: severe TBI.*

Behavioural tests

Open-Field Test

Locomotor activity was measured in the open field test (OFT) apparatus. Open field test was performed as described earlier. All animals were tested for baseline measurements (pre-injury) and at post-injury 2 weeks and 8 weeks (**Fig. 9**).

Novel Object Recognition Test

Recognition memory performance of the animals was tested in the NOR test. The same apparatus (box) was used in the NOR test as in the OFT with the same video tracking system.

The NOR test was performed as described earlier. Rats with low exploratory drive in the second trial (i.e., did not observe the two objects together for at least 5 s), or with +1.00 or −1.00 DI were excluded from the analysis. All animals were tested for baseline measurements (pre-injury) and at post-injury 2 weeks and 8 weeks.

Morris Water Maze Test

Long-term spatial memory of the rats was tested in the Morris water maze (MWM). The MWM test was performed as described earlier. The rats were tested in the water maze task at post-injury 6-7 weeks (**Fig. 9**). The overall task was divided into two weeks due to the large sample size.

Analysis of molecular markers of TBI

Enzyme-Linked Immuno Sorbent Assay

Eight weeks post-injury, venous blood samples were obtained from all of the rats through cardiac puncture. Samples were drawn into 10ml serum separator tubes and centrifuged at 2500 rpm for 15 min after collection. The serum was then stored at −70 °C until analysis. Commercially available sandwich ELISA kits (Elabscience®, USA) were used to measure concentration of serum pTau protein (cat. no. E-EL-R1090), GFAP (cat. no. E-EL-R1428) and S100β protein (cat. no. E-EL-R0868). 100 µl of serum samples were added to each well on the ELISA plate, and allowed to incubate for 90 min at 37 ºC, followed by incubation with 100 µl of biotinylated detection antibody. The plates were then washed three times with buffer and 100 µl of horseradish peroxidase-conjugate was added, followed by incubation for 30 min at room temperature. Finally, plates were washed three times with buffer and developed with 90 µl of substrate reagent for 15 min. The reaction was stopped with 50 µl of stop solution and samples were read at 450 nm with a multimodel, high-performance CLARIOStar microplate reader (BMG Labtech GmbH, Ortenberg, Germany).

Immunohistochemistry

Twenty-four hours after the TBI, 3 rats from each experimental group were euthanized with an overdose of sodium pentobarbital and were transcardially perfused with 4% paraformaldehyde containing fixative solution. On the next day, brains were removed and immersed in the same fixative overnight (16–18 h). A midline, 5mm-wide block of the brainstem was removed using a sagittal brain blocking device (Acrylic Brain Matrix for Rat, World Precision Instruments, Sarasota, FL) to include the region extending from the interpeduncular fossa to the second cervical segment. All blocks were sectioned sagittaly with a Vibratome Series 1500 Tissue Sectioning System (Technical Products International Inc., St. Louis, MO) at a thickness of 40 μm and collected in PBS. Sections were collected in a serial fashion then processed for immunohistochemical localization of damaged axonal profiles via the detection of the amyloid precursor protein (APP).

Sections were washed three times for 10 min with PBS, then treated with 0.3% hydrogen peroxide (H2O2) in PBS for 30 min to suppress endogenous peroxidase activity followed by washing three times in PBS. The sections were then exposed to controlled-temperature microwave antigen-retrieval in citrate buffer (pH 6.0, 0.1M) with PELCo BioWave 34700-230 (Ted Pella Inc., Redding, CA, USA). After three quick rinses in PBS, sections were immersed for 60 min in 10% bovine serum albumin (BSA) diluted in PBS containing 0.2% Triton X-100. The next step was the incubation of the sections overnight at 4 oC in rabbit anti-APP antibody (cat. no. 51-2700, Invitrogen, Thermo Fisher Scientific, Waltham, MA) diluted with 1% BSA/PBS at 1:1000 and then washed with PBS three times for 10 min. Thereafter, the sections were subjected to the staining protocol of the Vectastain Universal Elite ABC Kit (PK-6200, Vector Laboratories, Inc., Burlingame, CA). Finally, the end product of the immunohistochemical reaction was visualized with diaminobenzidine (DAB): sections were rinsed for 5 min in a 0.67 g/l DAB and 0.3 g/l H2O2 containing PBS solution. After subsequent washing in PBS 2 times for 10 min, the sections were mounted and cleared for routine light microscopic examination.

Statistical Analysis

All quantitative data are expressed as mean ± standard error of the mean (s.e.m). Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 23 (IBM Corporation, Armonk, NY USA) and MS Excel (Microsoft Corporation, Albuquerque, New Mexico, USA). For analysing performance in the OFT, NOR and MWM probe-trial, one-way ANOVA test was applied to compare the performance of the injury groups, followed by Tukey's post-hoc test. In the NOR, Student's t-test was used to analyse the preference for the novel object above the chance level (DI = 0). Morris Water Maze acquisition data were analysed by two-factor mixed-ANOVA (Within-subject factor: DAYS. Between-subject factor: INJURY). Protein concentrations in different experimental groups measured with ELISA were compared using Kruskal-Wallis non-parametric rank test and Dunn's post-hoc test. A level of $p<0.05$ was considered statistically significant in all analyses.

5.3 Results

Repetitive mild TBI has no long-lasting effects on locomotor activity

Locomotor activity was measured by counting line crossings in the OFT apparatus (**Fig. 10A**). Animals of all the injury groups exhibited overall good locomotor function in the pre-injury test, with no statistical difference in performance ($F(4,80)=0.417$; $p=0.417$). All the injury groups performed similarly in both the post-injury 2 weeks ($F(4,65)=0.835$; $p=0.508$) and the post-injury 8 weeks tests ($F(4,65)=0.138$; $p=0.967$), indicating no major impairment in locomotor function as a result of any types of TBI.

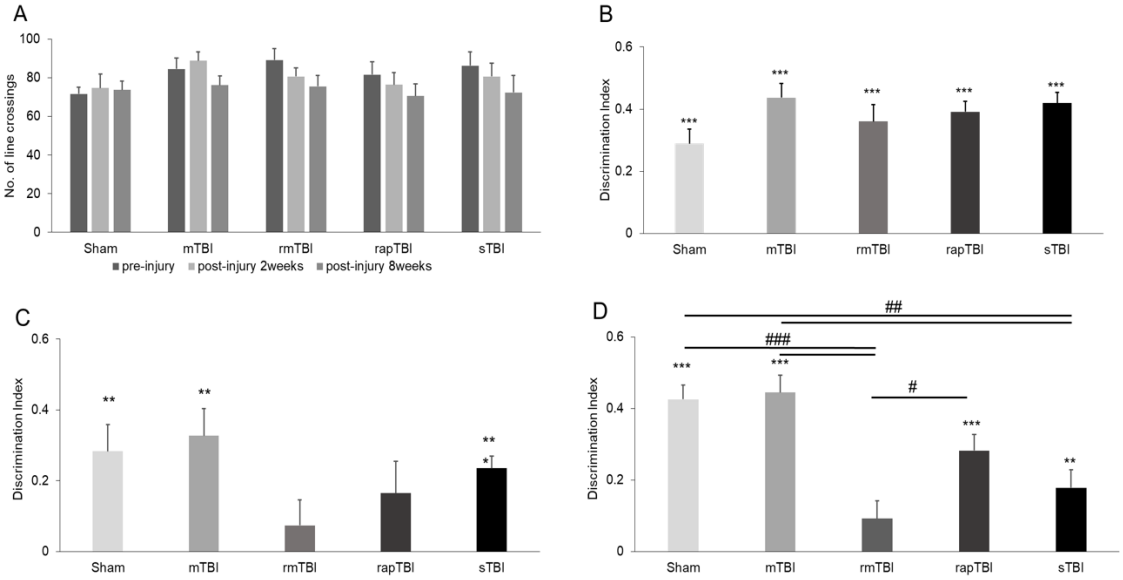

Figure 10*: Effects of different kinds of TBI on the behavioural performance of rats in the OFT and in the NOR task.* ***(A)*** *Locomotor activity was measured by counting line crossings in the open field test (Sham and sTBI: n=13/group, mTBI, rmTBI, rapTBI: n=14/group). No gross locomotor deficits were observed in any experimental groups in any measurement points.* ***(B)*** *In the pre-injury NOR test, all groups performed similarly, and were able to discriminate between familiar and novel objects (p=0.190; n=14/group).* ***(C)*** *In the post-injury 2 weeks NOR test, both repetitive injury groups, rmTBI (n=11) and rapTBI (n=12), were unable to discriminate between the familiar and the novel objects, while other groups performed normally.* ***(D)*** *In the post-injury 8 weeks NOR test, only rmTBI (n=12) failed to discriminate between the novel and the familiar object (p=0.09), and performed significantly worse compared to Sham (n=13; Sham vs. rmTBI: p<0.001), mTBI (n=13; mTBI vs. rmTBI: p<0.001) and rapTBI (n=13; rapTBI vs. rmTBI: p<0.05) groups. Abbreviations: see Table 3 for injury groups., * = one-sample t-test p<0.05; ** = one-sample t-test p<0.01; *** = one-sample t-test p<0.001; # = Tukey's pairwise comparison p<0.05; ## = Tukey's pairwise comparison p<0.01; ### = Tukey's pairwise comparison p<0.001.*

Repetitive mild TBI caused persistent long-term impairment in the NOR test

In the pre-injury NOR test, all groups were able to discriminate between the familiar and the novel object (**Fig. 10B**). Discrimination index value for each group was above the chance level. For Sham: 0.289 ± 0.046 ($t=6.039$, $df=13$, $p<0.001$); mTBI: 0.437 ± 0.045 ($t=9.226$, $df=13$, $p<0.001$); rmTBI: 0.360 ± 0.053 ($t=6.085$, $df=13$, $p<0.001$); rapTBI: 0.391 ± 0.033 ($t=10.981$, $df=13$, $p<0.001$); sTBI: 0.419 ± 0.033 ($t=11.225$, $df=13$, $p<0.001$). All the groups performed similarly in the pre-injury session ($F(4, 66)=1.580$; $p=0.190$).

In the post-injury 2 weeks NOR test (**Fig. 10C**), both repetitive injury groups were unable to discriminate between the familiar and the novel objects (rmTBI: 0.073 ± 0.072, $t=0.896$, $df=10$, $p=0.396$, and rapTBI: 0.165 ± 0.09, $t=1.640$, $df=11$, $p=0.129$), while other groups performed normally (Sham: 0.283 ± 0.075, $t=3.456$, $df=10$, $p<0.01$; mTBI: 0.326 ± 0.076, $t=4.108$, $df=12$, $p<0.01$; sTBI: 0.235 ± 0.034, $t=6.032$, $df=10$, $p<0.001$). Analysis of variance statistics did not show a main effect of TBI in this time window 2 weeks after the trauma ($F(4, 53)=1.556$, $p=0.200$).

In the post-injury 8 weeks NOR test (**Fig. 10D**), rmTBI group still failed to discriminate between the novel and the familiar object (0.092 ± 0.049, $t=1.857$, $df=11$, $p=0.09$), while other mild injury groups performed significantly above the chance level (mTBI: 0.444 ± 0.048, $t=9.082$, $df=12$, $p<0.001$; rapTBI: 0.282 ± 0.045, $t=6.257$, $df=13$, $p<0.001$). Repetitive mild TBI group also performed worse in comparison with the Sham, the mTBI and even the rapTBI groups ($F(4, 59) = 10.385$, $p<0.001$; Sham vs. rmTBI: $p<0.001$; mTBI vs. rmTBI: $p<0.001$; rapTBI vs. rmTBI: $p<0.05$). Although sTBI group discriminated between the novel and familiar objects (0.178 ± 0.050, $t=3.559$, $df=11$, $p<0.01$), they performed significantly worse than the Sham and the mTBI groups (Sham vs. sTBI: $p<0.01$; mTBI vs. sTBI: $p<0.01$). Results indicate that rmTBI group suffered from significant deficits in memory retention and recall, compared to the Sham and mTBI groups, while rapTBI group recovered.

Rapid repetitive mild TBI caused deficits in recall of spatial learning in the MWM

For the acquisition phase data, mixed-ANOVA for escape latency indicated that there was no interaction effect between injury groups and training days ($F(12, 192)=0.610$; $p=0.832$) (**Fig. 11A**). Also, tracking of swimming path length did not show any interaction between the injury groups and the training days ($F(12, 195)=0.470$; $p=0.931$), suggesting that the injury did not alter swimming strategy (**Fig. 11B**). However, there was a significant decrease of escape latency in all groups during the training days ($F(3, 192)=29.668$; $p<0.05$), suggesting that rats in all injury groups took significantly less time to find the platform on Day 4, compared to Day 1. Assessment of reference memory in the MWM probe trial was measured in terms of time spent in the target quadrant during the probe trial on Day 5 (Fig. 11C). Compared to Sham and single mTBI groups, only rapTBI group performed significantly worse ($F(4,65)=4.111$; $p<0.01$; Sham vs. rapTBI: 45.273 s ± 2.261 vs. 34.516 s ± 1.907, $p<0.05$; mTBI vs. rapTBI: 44.489 s ± 2.535 vs. 34.516 s ± 1.907, $p<0.05$). Single mTBI group performed similar to the Sham group, indicating no effect of single mTBI on spatial learning and memory (Sham vs. mTBI: 45.273 s ± 2.261 vs. 44.489 s ± 2.535, $p=0.99$). Surprisingly, sTBI group did not perform worse than Sham group (Sham vs. sTBI: 45.273 s ± 2.261 vs. 37.746 s ± 2.974, $p=0.176$).

Based on the probe trial results, only the rapTBI group suffered from deficits in the retention of long-term spatial memory in the MWM.

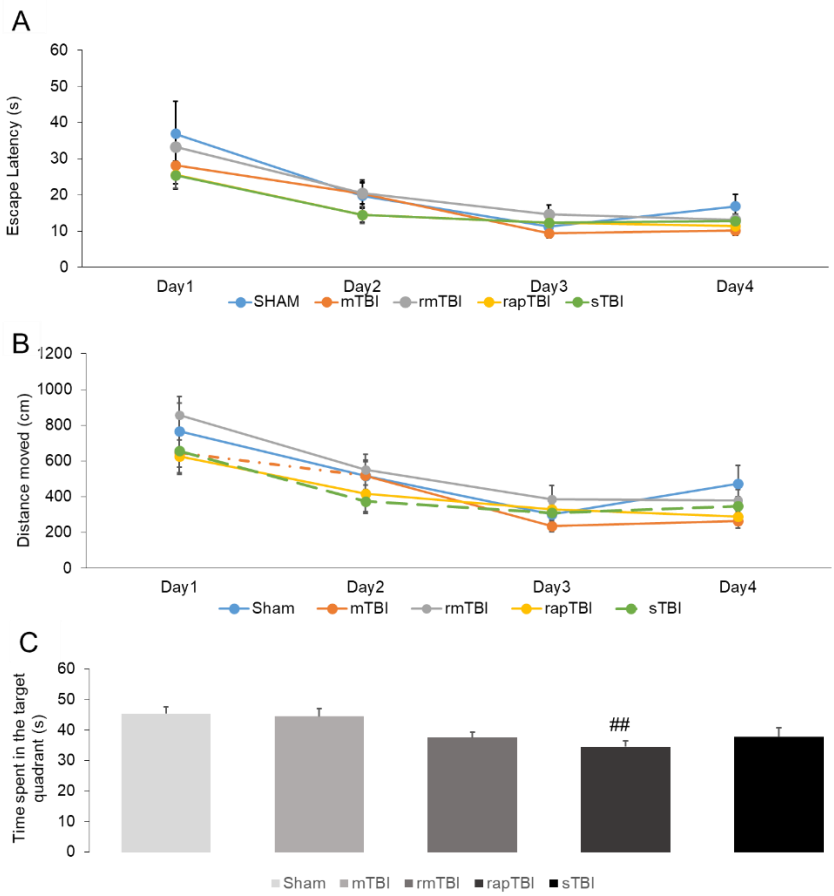

***Figure 11**: **(A)** Spatial learning was tested using the acquisition phase of the Morris water maze (MWM). Repeated measures ANOVA for escape latency indicated no interaction between injury groups and experimental sessions ($F(12,192) = 0.610$; $p = 0.832$). **(B)** Swimming path length did not show any interaction between the injury groups and the training days ($F(12,195) = 0.470$; $p = 0.931$) **(C)** In the MWM probe trial, compared to Sham and single mTBI groups, only rapTBI was significantly worse ($F(4,65) = 4.111$; $p<0.01$. Abbreviations: See Table 3 for injury groups. ## = Tukey's pairwise comparison $p<0.01$.*

Elevated blood GFAP levels were observed in the severe injury group

Two months following injury, sTBI had significantly higher serum GFAP levels (**Fig. 12A**), compared to Sham (Kruskal-Wallis $\chi2=9.775$, df=4, $p<0.05$; Sham vs. sTBI: 0.741±0.213 ng/ml vs. 2.062±0.261 ng/ml; $p<0.05$), mTBI (mTBI vs. sTBI: 1.115±0.259 ng/ml vs. 2.062±0.261 ng/ml; $p<0.05$) and rapTBI (rapTBI vs. sTBI: 1.070±0.255 ng/ml

vs. 2.062±0.261 ng/ml; p<0.05). Average serum levels of GFAP in rmTBI was between the average levels observed in Sham and sTBI groups, showing a level non-significantly higher than in Sham animals but also non-significantly lower than in the sTBI group (rmTBI: 1.477±0.317 ng/ml; rmTBI vs. sTBI: p=0.135; rmTBI vs. Sham: p=0.171).

No significant difference was observed in serum pTau levels between any injury groups (Kruskal-Wallis $\chi 2=3.006$, df=4, p=0.557) (**Fig. 12B**). Similarly, serum S100β levels were not found to be significantly different in all the injury groups (Kruskal-Wallis $\chi 2=5.379$, df=4, p=0.251) (**Fig. 12C**).

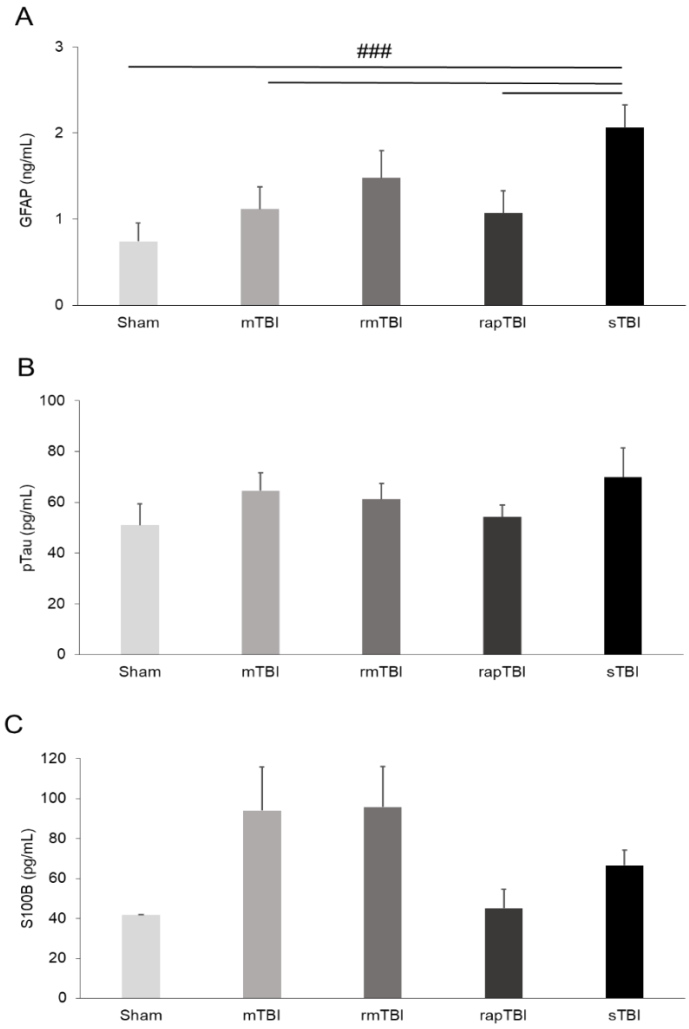

***Figure 12**: Elevated blood biomarkers were tested at 8 weeks following the injury with Sandwich-ELISA. **(A)** sTBI had significantly higher serum GFAP levels, compared to Sham (p<0.05; Sham vs. sTBI: p<0.05), mTBI (mTBI vs. sTBI: p<0.05), and rapTBI (rapTBI vs. sTBI: p<0.05). **(B)** For pTau, no significant difference was between injury group in serum levels (Kruskal-Wallis chi-squared p=0.557). **(C)** Similarly, S100β levels*

were not found to be significantly different in all the injury groups (Kruskal-Wallis chi-squared p = 0.251). Abbreviations: See Table 3 for injury groups. ### = Tukey's pairwise comparison p<0.001.

Histological markers of axonal injury are present in sTBI but not in other TBI groups

To evaluate the axonal injury as a result of TBI of different severities, immunohistochemically labelled sections of the brainstem at the pontomedullary junction were examined under light microscope. Qualitative examination of brain slices revealed a few, scattered APP and RMO-14 immunopositive profiles only in the sTBI group, while other groups did not show any immunopositivity. Since only the sTBI injury group exhibited sparse APP positive and RMO-14 positive profiles, the histological markers were not quantified. Representative images of the immunohistochemical examination were shown on **Fig. 13**.

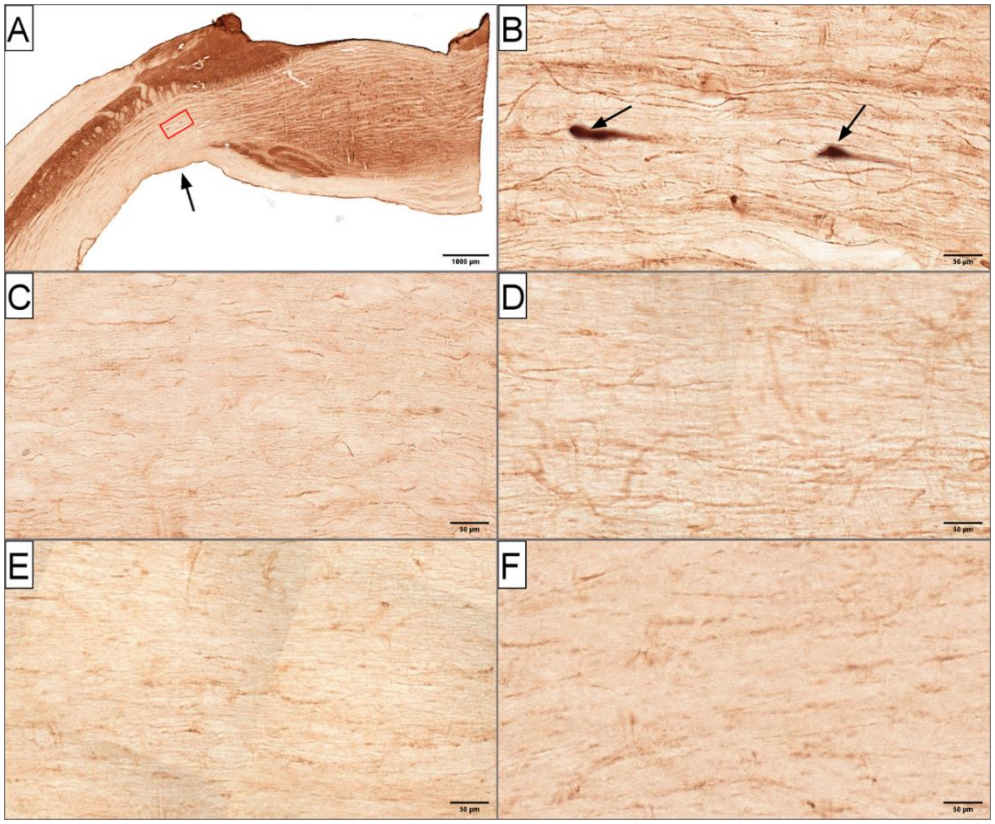

***Figure 13**: Representative photomicrographs of sagittal sections of the brainstem at the pontomedullary junction (red box) stained with amyloid precursor protein (APP)*

*immunohistochemistry **(A)**, at post injury 24 h. A few, scattered immunoreactive axonal profiles (indicated with arrowheads) were observed only in the sTBI group **(B)**, while Sham **(C)**, mTBI **(D)**, rmTBI **(E)** and rapTBI **(F)** groups did not show any APP profiles (Courtesy of the Neurotrauma Research Group). Abbreviations: See Table 3 for injury groups.*

5.4 Discussion

The current study characterizes a model of repetitive mild TBI that replicates key functional and histological features of clinical injury. To the best of our knowledge this is the first study to demonstrate in the impact acceleration model of Marmarou that a repeated mild TBI evoked from a height of 15 cm should lead to irreversible neurocognitive alterations. This finding was not accompanied by significant increase of the number of APP or RMO-14 immunoreactive axonal profiles at 24 h post-injury, indicating that axonal injury in the brainstem is not a major player in such alterations at the acute time-point or, alternatively, participate in the pathology via different mechanisms. However, 8 weeks after sTBI, the glial marker GFAP that is primarily considered an acute indicator of TBI was associated with neurocognitive impairment implicating ongoing/late onset glial pathology to the observed changes. Not surprisingly, the acute glial/BBB marker S100β did not display significant alterations at 8 weeks after TBI.

In earlier studies, chronic cognitive deficits and memory impairment have been observed in human TBI, as well as in experimental TBI models (Baron et al. 2013; Ling, Hardy, and Zetterberg 2015; Washington et al 2012). In case of a single mild TBI, memory impairment can be transient in nature. However, repetitive mild TBI could have long-lasting or irreversible effects (Stern et al. 2011). In our study, we found acute cognitive effects in both repetitive injury groups, while Sham and single mTBI groups did not show any deficits in intermediate-term object recognition memory in the NOR test or in spatial long-term memory in the MWM. However, object recognition memory deficits were the greatest in the rmTBI group, compared to other groups at post-injury 8 weeks NOR test, while the rapTBI group recovered. In contrast, long-term spatial memory deficits in the MWM probe-trial were more prominent in the rapTBI group at post-injury 6-7 weeks than in the rmTBI group. These findings suggest that repetitive mild TBI has a chronic effect

on cognitive functions regardless of the time interval between successive injuries, and mimics the functional deficits seen in humans with multiple concussive episodes. On the other hand, cognitive symptoms differed in terms of severity and affected memory domains depending on the time interval between the occurrence of repetitive TBI events. Note that the OFT did not show any effect on basic locomotor function following any severity of injury. Thus, we can conclude that the measures of cognitive performance were not confounded by any non-specific motor effects caused by TBI.

Based on the ELISA results, serum GFAP levels were significantly higher in sTBI group, compared to Sham, mTBI and rapTBI groups. Increased expression of GFAP is a marker of astrocyte activation (Brenner 2014; Eng and Ghirnikar 1994). GFAP plays a critical role in inhibiting inflammatory response after the injury effectively limiting neuronal damage (Lei et al. 2015; Vos et al. 2010), and it is a well-known acute biomarker of TBI. In a rat model of repetitive mild TBI, GFAP in the form of reactive gliosis was found in the cortex on the injured side 3 months following injury (Brooks et al. 2017). We also found increased level of GFAP in the blood of the severely injured rats (sTBI), while the blood level of GFAP in rats subjected to rmTBI was between the level of the Sham and sTBI groups. This indicates that GFAP levels better correspond to the severity of the injury (Nylén et al. 2007) than to the functional outcomes. Interestingly, elevated GFAP in sTBI did not coincide with significant memory loss in the post-injury 8 weeks NOR test. It is plausible that increased GFAP level represented activated repair mechanisms following sTBI, while rmTBI induced much less extent of astrocyte activation even though they exhibited significant cognitive impairment. Interestingly, pTau was not found to be significantly higher in injured groups compared to Sham two months after TBI. Phosphorylated-tau protein, which has already been well characterised in Alzheimer's disease and in other tauopathies (Buée et al. 2000; Spillantini and Goedert 2013), has also been implicated in the pathology of TBI, and elevated levels of pTau are now recognized as both acute and chronic biomarkers (Rubenstein et al. 2017; Tsitsopoulos and Marklund 2013). Formation of tau oligomers have been observed in the brain of rats at 4 h and 24 h following TBI (Hawkins et al. 2013). While most studies reported elevated pTau protein in the cortex several weeks after the injury (Cheng et al. 2014; Hawkins et al. 2013; Rubenstein et al. 2019), only one study found that transgenic mice with human tau show white matter degeneration and impaired visuospatial learning after repetitive mild TBI with only transient tau pathology in the cerebral cortex (Mouzon et al. 2018). While the

pathobiological role of tau in repetitive mild TBI remains a subject of debates, it is likely that accumulation of tau is more pronounced at the site of injury. Contusions at the site of impact causing cytoskeletal disruption could cause transient tau accumulation. S100β, a calcium-binding protein found primarily on astrocytes and Schwann cells, is used as a parameter of acute glial activation (Kleindienst et al. 2007; Vos et al. 2010). From our findings, S100β levels in serum at post-injury 8 weeks was almost negligible in all injury groups. S100β protein has been found to be a sensitive biomarker, and its concentration in serum and cerebrospinal fluid (CSF) immediately after TBI has been correlated with severity and outcome of the injury (Blyth et al. 2009; Goyal et al. 2012; Kleindienst et al. 2007). This possibly explains the insignificant serum levels of S100β 8 weeks after TBI in our study, and it seems that S100β is no longer expressed in long-terms after TBI even if functional deficits are still present.

Twenty-four hours after a single severe TBI, APP and RMO-14 immunoreactive profiles were observed in the pontomedullary junction of rats, indicating axonal injury. Compared to sTBI, other injury groups did not show explicit immunoreactive profiles, indicating minimal or no axonal damage. Amyloid precursor protein is a well-known acute biomarker of impaired axonal transport (Stone et al. 2004), while RMO-14 is a biomarker of neurofilament compaction (Marmarou et al. 2005). Initial effects of trauma cause primary axotomy, which includes shear and stretch injuries to long-tract structures. Distortion of the axonal cytoskeleton causes impaired axonal transport and neurofilament compaction (Gaetz 2004; Johnson, Stewart, and Smith 2013; Stone et al. 2004). Both APP and RMO-14 serve as critical biomarkers for studying primary axotomy in single and repetitive mild TBI. Based on our findings, rats with single or repetitive mild injury did not have extensive primary axotomy in the brainstem, which also explains the lack of motor deficits in the OFT.

In conclusion, we developed two efficient scenarios of the impact-acceleration repetitive mild TBI model with either short (rapTBI) or long (rmTBI) inter-injury intervals. The inter-injury interval played a crucial role in determining the extent and duration of cognitive impairment following injury. Compared to the rapTBI injury, the rmTBI scenario, with 24 h inter-injury interval, displayed long-term cognitive deficits without histological consequences. This difference between the two repetitive models could be attributed to the temporal window of vulnerability of the brain to a second impact, allowing rats with rapTBI to recover faster, compared to rmTBI rats.

Our study reaffirms that repetitive concussive injuries with longer inter-injury interval causes persistent neurobehavioral alterations. These results are broadly consistent with findings in human studies, where repeated concussions increase the risk of developing chronic traumatic encephalopathy, causing cognitive difficulties, including short-term memory problems and executive dysfunction (Saigal and Berger 2014; Stern et al. 2011). Moving forward, based on the current findings, we believe that rmTBI model is suitable to assess novel therapeutic strategies for the management of short- and also long-term consequences of repetitive TBI.

6. STUDY 4: PHARMACOLOGICAL AMELIORATION OF COGNITIVE DEFICITS CAUSED BY REPETITIVE MILD TRAUMATIC BRAIN INJURY

6.1 Introduction

Lately, there has been a growing interest in understanding the pathophysiology of repetitive mild TBI (rmTBI). As found in several studies, rmTBI is associated with persistent long-term memory impairment, emotional instability, speech irregularities and subtle changes in motor coordination (Corsellis, Bruton, and Freeman-Browne 1973; Goldfinger et al. 2018; McKee et al. 2013).

These studies have highlighted the importance of preclinical evaluation of potential therapies for TBI in animal models that mimic the human disorder as a prelude to the translation of these into clinical trials. Preclinical TBI studies indicate that glutamate-mediated excitotoxicity plays an early and critical role in the cascade of secondary injury events following TBI (Katayama et al. 1990; Palmer et al. 1993; Takahashi, Manaka, and Sano 1981; Yi and Hazell 2006). Immediately after the mechanical injury to the brain, there is disruption of neuronal membranes and axonal stretching. In addition, nonspecific depolarization leads to an early, indiscriminate release of the excitatory neurotransmitter, glutamate. Excitotoxicity results from the over activity of glutamate on NMDA receptors, which causes calcium ions overload, thus triggering multiple cell death signalling pathways (Parsons et al. 2013; Yi and Hazell 2006).

However, only few studies targeting glutamatergic neurotoxicity, specifically mediated by the antagonism of the N-methyl-D-aspartate receptor (NMDAR), have shown to be successful (Mei et al. 2018; Yurkewicz et al. 2005).

Our aim was to test whether NMDAR antagonist, memantine, could improve posttraumatic behavioural outcomes, in our rmTBI model. We used 3 doses of memantine, 0.1 mg/kg, 0.3 mg/kg and 1.0 mg/kg, in an rmTBI model at sub-acute and chronic stages, for evaluation in MWM and NOR.

6.2 Methods

Subjects

Fourty six adult male Long Evans rats (Janvier Labs, France, aged 4 months at the beginning of the study) weighing 300-400 g were used. Animals were pair-housed, and were kept under controlled conditions (standard 12 h light cycle from 7 a.m. to 7 p.m., with controlled temperature and humidity). Rats were maintained at 80–85% of their free feeding weight by restricting their laboratory chow supplement. Typically, they were fed with 17 g of laboratory chow (ssniff-Spezialdiäten GmbH, Germany) per animal per day. Water was provided ad libitum. Weeks prior to the behavioural testing, all rats were regularly handled for proper acclimatization to the lab environment and experimenters. During the experiments, every effort was made to minimize distress of animals. All experimental procedures were approved by the Animal Welfare Committee of the University of Pécs. All procedures were approved by the Institutional Animal Use and Care Committee of the University of Pecs and were licensed by the Baranya County Government Office, Hungary (nr: BAI/35/51-107/2016) and carried out in accordance with the ARRIVE guidelines.

Experimental Traumatic Brain Injury

All surgical procedures were performed by Neurotrauma research group, at University of Pecs. Surgery procedures and induction of injury were performed as previously described. Sham animals were prepared for injury in the same fashion, but were not injured.

Validation of neuroprotective effects of memantine

Memantine, a non-competitive antagonist of n-methyl d-aspartate (NMDA) receptor (Parsons, Danysz, and Quack 1999; Reisberg et al. 2003) was dissolved in physiological saline to a final injection volume of 1 ml/kg. Memantine was administered subcutaneously 40 min before the behavioural experiments.

Behavioural tests

Novel Object Recognition Test

The NOR test was performed as described earlier. Five different object-pairs were used, 1 pair of objects in pre-injury, and 4 pairs of objects in post-injury assessment. They were distributed randomly between animals and experimental sessions in a counterbalanced latin-square design.

The DI was a positive number if the novel object was observed for a longer duration, while the DI was negative if the familiar object was observed for longer, and the DI was around zero if the two objects were observed for equally long time. Rats with low exploratory drive in the trial #2(i.e., did not observe the two objects together for at least 5 s), or with +1.00 or −1.00 DI were excluded from the analysis.

Rats were sorted into either Sham, rmTBI-control or treatment groups based on their DI in the pre-injury test, to make sure all the groups have similar DI.

The NOR tests were performed at 6-9 weeks post-injury time-points. Rats were injected subcutaneously with either vehicle or memantine, 40 min before the acquisition trial. Treatments were administered in a within-subject design, such that, all subjects received vehicle and all doses of memantine in different NOR sessions **(See Table 4).**

Treatment group ID	Description of treatment
Sham Injured (Sham)	*No injury* (only anesthesia) *n=10*
rmTBI Control (rmTBI-control)	5 hits from the height of 15cm – at 24h inter-injury interval; *not treated* *n=10*
rmTBI - Mematine within-subject	5 hits from the height of 15cm – at 24h inter-injury interval; *treated with mematine doses 0.1, 0.3 and 1.0 mg/kg* *n=10*
rmTBI-VEH	5 hits from the height of 15cm – at 24h inter-injury interval; *treated with vehicle in the within-subject design* *n=10*

Table 4: *Summary of experimental groups and treatments for NOR session. Abbreviations: Sham: sham injured; rmTBI-control: rmTBI untreated group; rmTBI-Mem: rmTBI treated with memantine; rmTBI-VEH: rmTBI treated with vehicle.*

Morris Water Maze Test

Long-term spatial memory of the rats was tested in the Morris water maze (MWM), at post-injury 3 weeks time-point, Treatment in MWM was administered in a between-subject design, to avoid habituation to the task apparatus in repeated sessions.. The rats were injected subcutaneously with either vehicle or memantine 40 min before the 1st trial on each acquisition day. No treatment was administered on the probe trial day **(See Table 5)**.

Treatment group ID	Description of treatment
Sham	*No treatment* *n=11*
rmTBI-control	*No treatment* *n=9*
rmTBI+Mem0.1	*Treated with memantine dose 0.1mg/kg* *n=9*
rmTBI+Mem0.3	*Treated with memantine dose 0.3mg/kg* *n=8*
rmTBI+Mem1.0	*Treated with memantine dose 1.0mg/kg* *n=9*

Table 5: *Summary of experimental groups and treatments for MWM session. Abbreviations: Sham: sham injured; rmTBI-control: rmTBI untreated group; rmTBI-Mem0.1: rmTBI treated with memantine 0.1mg/kg; rmTBI-Mem0.3: rmTBI treated with memantine 0.3mg/kg; rmTBI-Mem1.0: rmTBI treated with memantine 1.0mg/kg*

Statistical Analysis

All quantitative data are expressed as mean ± standard error of the mean (s.e.m). Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 23 (IBM Corporation, Armonk, NY USA) and MS Excel (Microsoft Corporation, Albuquerque, New Mexico, USA). For analysing performance in the NOR and MWM probe-trial, one-way ANOVA test was applied to compare the injury groups, followed by Tukey's post-hoc comparison test. In the NOR, Student's t-test was used to analyse the preference for the novel object above the chance level (DI = 0). Morris Water Maze acquisition data were analysed by two-factor mixed-ANOVA (Within-subject factor: DAYS. Between-subject factor: TREATMENTS).

6.3 Results

Highest dose of memantine significantly improved performance in the NOR test

For the comparison between Sham and rmTBI-control group (**Fig. 14A**), only Sham group could discriminate between the novel and the familiar objects, while rmTBI-control failed to do so. Sham: 0.263 ± 0.070, t=1.513, df=5, $p<0.05$; rmTBI-control: 0.088 ± 0.163, t=0.541, df=6, p=0.608.

In the post-injury within-memantine NOR sessions (**Fig.14B**), only Mem1.0 treatment improved the performance of the rats to discriminate between familiar and novel objects (VEH: 0.132 ± 0.121, t=1.089, df=8, p=0.308; Mem0.1: 0.232 ± 0.140, t=1.649, df=8, p=0.138; Mem0.3: 0.117 ± 0.095, t=1.234, df=8, p=0.252; Mem1.0: 0.364 ± 0.099, t=3.652, df=9, $p<0.01$). ANOVA statistics did not show a main effect of the memantine treatment ($F(3,37)=1.012$, p=0.400).

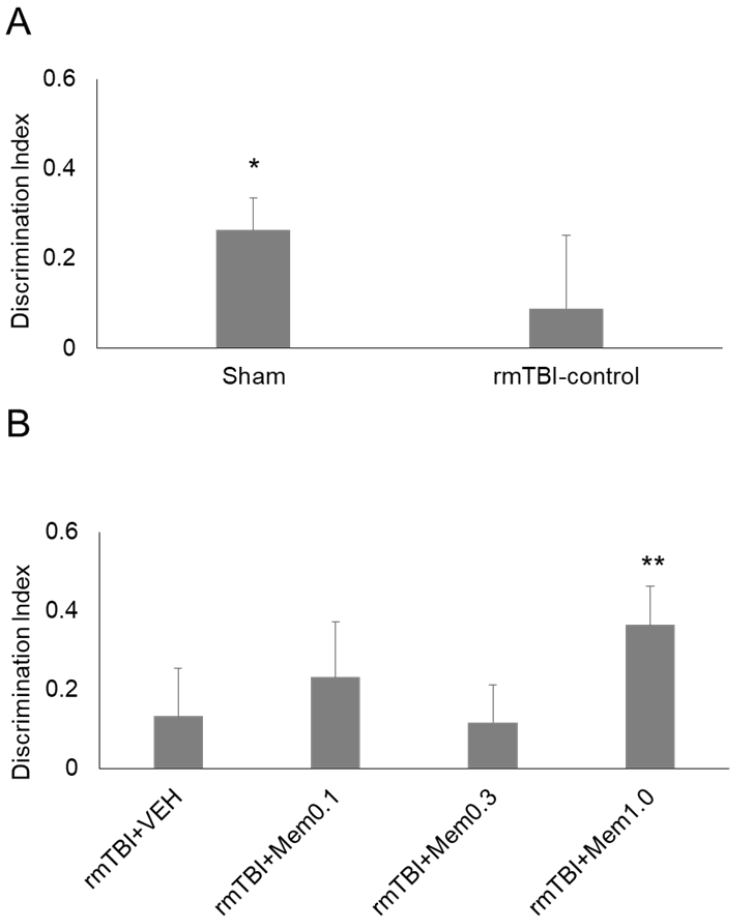

Figure 14: *Highest doses of memantine improves performance in the novel object recognition (NOR) test post-injury.* ***(A)*** *rmTBI-control group could not discriminate between familiar and novel objects, while Sham group could (Sham: n=6, p<0.05; rmTBI-control: n=7, p=0.608).* ***(B)*** *In the within-memantine session, only the highest memantine dose (rmTBI+Mem1.0) could improve the performance of the rmTBI injured rats to discriminate between the familiar and novel objects (rmTBI+Mem1.0: n=10, p<0.01). However, there was no main effect (p=0.400). Abbreviations: see* ***Table 4*** *for injury groups. * one-sample t-test p<0.05; ** one-sample t-test p<0.01.*

Treatment with memantine did not have beneficial effects on spatial performance in MWM

For the acquisition phase data, mixed-ANOVA for escape latency indicated that there was no interaction effect between treatment with memantine and training days (F(12,120)=1.616, p=0.096) (**Fig. 15A**).

Also, tracking of swimming path length did not show any interaction between the treatment and the training days (F(12,120)=1.069, p=0.393), suggesting that the treatment

did not alter or improve swimming strategy (**Fig. 15B**). However, there was a significant decrease of escape latency in all groups during the training days ($F(3,120)=53.172$, $p<0.001$), suggesting that rats in all treatment groups took significantly less time to find the platform on Day 4, compared to Day 1.

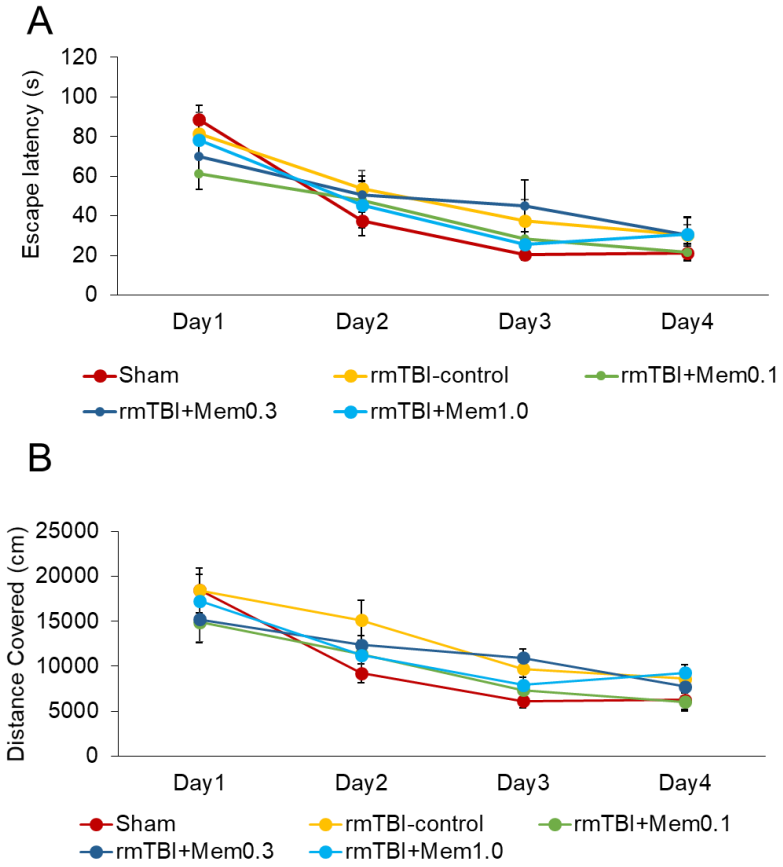

Figure 15**: No significant effect of repetitive mild TBI or memantine treatment was observed on reference memory in the Morris Water Maze (MWM). **(A)** Repeated measures ANOVA for escape latency indicated no interaction between the acquisition phase and treatment groups ($p=0.096$; Considerable drop in the escape latency on Day 4 in all treatment groups $p<0.001$). **(B)** Similarly, swimming path length also did not show any interaction between the treatment groups and the training days ($p=0.393$). For abbreviations see **Table 5.

Assessment of reference memory in the MWM probe trial was measured in terms of time spent in the target quadrant during the probe trial on Day 5 (**Fig. 16**). Treatment did not had any significant effect on the reference memory in the MWM, as the time spent in the target quadrant were not statistically different ($F_{4, 41} = 1.078$, $p=0.381$). Sham: 39.0 s ± 4.7, rmTBI-control: 32.2 s ± 3.7, Mem0.1: 40.0 s ± 4.8, Mem0.3: 32.3 s ± 1.9, and Mem1.0: 39.0 s ± 4.7.

Based on the probe trial results, treatment with memantine did not significantly improve reference memory in recalling the location of the target quadrant in the TBI groups compared to the rmTBI-control group.

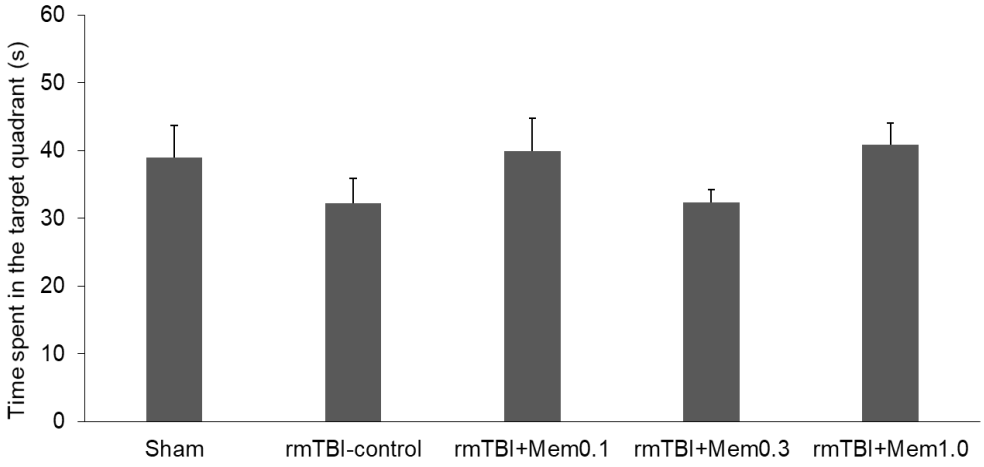

Figure 16*: MWM probe trial results did not indicate any significant difference in the time spent in the target quadrant between the treatments and control groups (p=0.381). For abbreviations see* ***Fig 14****.*

6.4 Discussion

From the results, we found that high dose of memantine was successful in attenuating working memory deficits in NOR test. Memantine at 1.0mg/kg dose was able to improve the discrimination index above the chance level, while vehicle treatment could not discriminate significantly. However, treatment with memantine did not significantly

improve spatial working memory and retention on the MWM test, compared to sham-injured and rmTBI-control (rmTBI-control) groups. Similarly, memantine treatment did not significantly reduce the path length to find the platform during the acquisition phase, compared to the rmTBI-control group. Moreover, as control rats did not show significant impairment, either in the acquisition phase (Day1-4) or in the probe trial (Day 5), it is also possible that the rmTBI-induced learning and memory dysfunction was not evident at the post-injury 3-week time-point.

To our knowledge, this is the first study to evaluate the potential protective effects of an NMDAR antagonist (memantine) in rmTBI at sub-acute (3 weeks post-injury) and chronic phases (6-9 weeks post-injury) in the MWM and NOR test, respectively.

The pathophysiology of TBI is not completely understood, however, post-traumatic neurochemical dysfunction of the glutamatergic, dopaminergic, adrenergic, serotonergic, and cholinergic systems are among the postulated contributors to the symptoms and morphological alterations. One of the major hypotheses underlying therapeutic strategies for the treatment of traumatic brain injury has been that brain trauma results in the excessive release of the excitatory neurotransmitter glutamate, which initiates a complex process of cell injury which if uninterrupted will result in calcium ions influx and consequent cell death (Bullock et al. 1998). One of the main roles of NMDA receptor antagonists is to reduce Ca^{2+} influx by modulating the cellular gates (Marshall 2000). Results from preclinical models of single-instance severe TBI indicate that targeting glutamate-inducedtoxicity may be beneficial during the transient and short-lived posttraumatic NMDAR hyperactivation (Biegon et al. 2004). Small clinical case series and a retrospective case study of the NMDAR antagonist amantadine suggest that NMDAR blockade with twice daily administration of amantadine at 200 mg/kg dose, improves cognitive outcomes, such as verbal memory and reaction time, in patients with concussive injury, and its clinical trials for single mild TBI instances are ongoing (Reddy et al. 2013). Memantine is an FDA approved treatment for moderate to severe AD (Parsons, Danysz, and Quack 1999; Reisberg et al. 2003) and it could prove to be beneficial in attenuating rmTBI induced functional and behavioural symptoms. Traxoprodil, a substituted 4-phenylpiperidine, which antagonizes the NMDA receptor at an allosteric regulatory site, was also found to be effective in treating severe TBI, when administered 2h post-injury (Yurkewicz et al. 2005).

In addition, other potential therapeutic targets need to be investigated. Currently, no FDA-approved pharmacological therapies are available to ameliorate cognitive deficits and impairment observed in the chronic phase of TBI (Wheaton, Mathias, and Vink 2011). Taking into account the occurrence and frequency of rmTBI in contact sports and in military personnel, the development of an efficacious therapeutic to target these neuro-behavioural alterations to improve learning and memory is the need of the hour. Numerous preclinical studies have established that in the days to weeks after TBI, there is a decrease in cholinergic signalling (Arciniegas 2011; Kelso and Oestreich 2012; Shin and Dixon 2015). There is reduced high-affinity choline uptake (Dixon et al. 1994), and transient depression of cholinesterase activity in the hippocampus (Valiyaveettil et al. 2012). At the receptor level, there is a loss of up to 50% of α7 nAChRs after controlled cortical impact (Hoffmeister et al. 2011; Verbois, Scheff, and Pauly 2003). Although cholinergic signalling is decreased chronically after TBI, it is not completely absent. This suggests that therapeutic compounds acting on the remaining endogenous cholinergic activity may also be efficacious. Few preliminary studies have established that cholinergic agonists can attenuate memory impairments following TBI (Dixon, Ma, and Marion 1997; Guseva et al. 2008; Verbois et al. 2003). Recently, PHA-543613, an alpha7 nicotinic acetylcholine receptor agonist has also been found to exhibit neuroprotection, through both neuronal survival and microglial activation, in an in vivo neuroinflammatory excitotoxic rat model (Foucault-Fruchard et al. 2017). Taken together, over the past decade, numerous neuroprotective agents with varying mechanisms of action have been evaluated for the treatment of head injury, but none thus far have convincingly demonstrated efficacy in the overall population (Maas 2000; Marshall 2000). Nevertheless, in light of these potential issues, developing a potent and efficient therapy to treat the neurobehavioural alterations following TBI is still a new and challenging area.

In conclusion, here we have established that treatment with memantine could significantly alleviate cognitive deficits at chronic stages of rmTBI. This study suggests that NMDAR antagonist therapy after rmTBI may be beneficial in treating chronic post-injury dysfunction, potentially attenuating NMDAR mediated excitotoxicity, and directly addresses NMDAR therapeutic targets after rmTBI, which could be relevant to athletes with multiple concussive episodes.

7. CONCLUSIONS

From the present series of experiments, the following novel findings and conclusions can be drawn:

A traumatic brain injury inflicted from the height of 15 cm causes sub-acute effects but no sign of any persistent structural and functional alterations were seen in the brain. In addition, we also found that the 25 cm injury did not appear to be significantly different in the NOR test from the results of the 15 cm injury. Animals with acute deficits observed following mTBI in behavioural tasks fully recover two months after the injury, with no gross changes in the integrity of the corpus callosum.

Mild TBI in spontaneously hypertensive rats causes significant working memory deficits. Hypertension-associated pathologic changes in the brain most likely exacerbates the TBI-induced excitotoxicity, causing long-term cognitive dysfunction.

In repetitive mild TBI, the interval between the successive injuries plays a critical role in determining the extent and persistence of cognitive impairment. A repetitive mild TBI model, with an inter-injury interval of 24 h, causes persistent cognitive deficits, as seen in the NOR task. However, no gross changes in the levels of GFAP, pTau or S100B were observed two months following injury, as well as no DAI immunopositivity was detected in the pontomedullary junction.

Furthermore, treatment with memantine at 1.0 mg/kg was successful in attenuating cognitive deficits in an rmTBI injury model, in the NOR test.

It has to be mentioned that there are certain limitations of the test batteries used in showing subtle but persistent consequences of TBI. For example, the MWM test was not found to be sensitive enough in determining the magnitude of spatial memory deficits following TBI in the present experimental design, since no baseline or pre-injury measurement was possible in the test due to possible task-habituation effects. Moreover, APP, which is a commonly used histopathological biomarker of DAI, was found to be inconclusive following TBI in our experimental models. It is likely that in order to assess DAI, APP could be more prominent marker only if immediately assessed following the trauma, as the neuroinflammatory response clears up the damage caused by secondary axotomy.

We conclude that the 15 and 25 cm injuries in the Marmorou paradigm are mild in nature and cause no long-lasting functional deficits. However, the 25cm injury, in conjuction with hypertension, still exhibited persistent cognitive impairments. Moreover, we conclude that the 15 cm repetitive mild TBI treatment with an inter-injury interval of 24 h is an efficient model to study the outcome of multiple concussions. Also, while GFAP levels corresponded with the severity of the injury (seen in the 150 cm injury group only), GFAP did not predict the outcome of rmTBI. Finally, based on our findings, we can conclude that rmTBI causes long-term behavioural deficits with no gross axonal injury, which could be reversed with the treatment with memantine. In future research, an increased understanding of rmTBI and its neuropathological effects will enable the identification of molecular targets specific to rmTBI and ultimately help in development of novel, effective therapeutic treatments.

8. SUMMARY

Mild traumatic brain injury (mTBI) is most often associated with short-term cognitive dysfunction that tends to resolve within three months of injury. Not only does pre-existing comorbid conditions, such as hypertension, increase risk of mortality in TBI, repetitive mTBIs may increase the risk of developing neurodegenerative disorders, such as dementia, in old age. Therapeutic intervention to treat TBI-related cognitive deficits is an unmet medical need. Using the Marmarou impact acceleration model, two mild TBI models were designed, induced from the height of 15 and 25 cm. Furthermore, we tested the 25 cm injury in spontaneously hypertensive rats to assess the behavioural outcome and utilised the 15 cm injury to design two repetitive mild TBI (rmTBI) models, with short and long inter-injury intervals. Finally, in order to treat rmTBI-induced cognitive impairment, different doses of NMDAR antagonist, memantine, were given at sub-acute and chronic stages of injury. Novel object recognition (NOR), and Morris water maze (MWM) tests were used to assess behavioural outcome. We found that mild TBI evoked from a height of 15 cm caused no significant long-term neurocognitive alterations. Animals with acute deficits observed following mTBI in the behavioural tasks fully recovered by two months after the injury, also showing no remaining gross changes in the integrity of the corpus callosum. In addition, we also found that the 25 cm injury did not appear to be significantly different in the NOR test from the results of the 15 cm injury. Working memory deficits two weeks following a 25 cm TBI treatment were observed in spontaneously hypertensive (SHR) and control normotensive rats at two weeks following a 25 cm TBI treatment. Hypertension-associated pathologic changes in the brain could explain the deficits in the NOR test at sub-acute phase. In repetitive mild TBI, the interval between the successive injuries plays a critical role in determining the extent and persistence of cognitive impairment. We found that, compared to sham-injured and single mTBI, an rmTBI evoked from a height of 15 cm, with 24 h inter-injury interval, caused persistent neurocognitive alterations 8 weeks following the last injury. Finally, glutamatergic NMDA receptor antagonist memantine at 1.0 mg/kg dose was efficient in reversing working memory deficits in the NOR test 6-9 weeks following repetitive injury. Glutamate-mediated excitotoxicity plays an early and critical role in the cascade of secondary injury events following TBI, and in the present experiment, memantine appeared to be effective in putatively attenuating NMDAR overactivity even several weeks after the injury.

To our knowledge, this is the first study to address targeting NMDAR at sub-acute and chronic stage, after a repetitive (5-hit) mild (15-cm) TBI. It is possible that the three-week period of time elapsed before the administration of memantine may be rather late after the initial insult and an additional pharmacological intervention administered closer to the time of injury may be likely to produce a more robust neuroprotective effect. Further research is necessary to find effective targets, as well as pharmacological agents, to treat and reverse most, if not all, of the cascade of metabolic events that occur following TBI.

9. ÖSSZEFOGLALÁS

Az enyhe koponyatraumát követő agysérülésekhez (mild traumatic brain injury, mTBI) gyakran társul rövid távú kognitív diszfunkció, mely azonban a sérülést követő három hónapon belül spontán javulhat. Azonban egyrészt a már korábban fennálló társuló alapbetegségek (komorbiditások), mint pl. a magas vérnyomás jelenléte, növelhetik a TBI elhalálozási kockázatát, másrészt az ismétlődő enyhe TBI események önmagukban is növelik a neurodegeneratív betegségek, mint például demencia idős kori kialakulását. A TBI-hoz kapcsolódó kognitív deficitek kezelésére egyelőre még nem találtak megfelelő terápiás beavatkozást. A jelen vizsgálatsorozatban az ún. Marmarou-féle szabadesésen alapuló gyorsulási-ütközési modellt használva legelőször kétféle enyhe TBI vizsgálatot terveztünk, melyekben a traumát előidéző súlyt 15 illetve 25 cm magasságból ejtettük a koponyára. Ezt követően, a 25 cm magasságból kiváltott TBI viselkedési hatását megvizsgáltuk spontán magas vérnyomásos (spontaneously hypertensive, SHR) patkányokon. Illetve, felhasználtuk a 15 cm magasságból kiváltott enyhe koponyatraumát további ismétléses enyhe TBI (repetitive mild TBI, rmTBI) modell kialakítására, úgy, hogy egyik esetben rövid (5 perc) , másik esetben hosszú (1 nap) időt hagytunk a traumás sérülések között. Végezetül, magatartásfarmakológiai vitsgálatban egy glutamáterg NMDA receptor antagonistát, a memantine-t alkalmaztunk különböző dózisokban a koponyasérülés szubakut és krónikus szakaszaiban azért, hogy a rmTBI kezelés által kiváltott kognitív deficitet ellensúlyozzuk. A viselkedést nyílt porond teszttel (OFT), új tárgy felismerési teszttel (NOR) és Morris-féle vízi útvesztő teszttel (MWM) határoztuk meg. A 15 cm magasságból kiváltott enyhe TBI nem okozott hosszú távú neurokognitív változásokat. Emellett, nem találtunk különbséget a 25 cm és 15 cm magasságból kiváltott TBI hatásai között sem. Ugyanakkor, két héttel a 25 cm magasságból okozott enyhe koponyatraumás sérülés után munkamemória deficitet mutattunk ki a hipertenzív patkányokban a normotenzív kontroll csoporttal szemben. Emellett, a 15 cm magasságból, 24 óránként, 5 ütéssel okozott ismétlődő enyhe TBI kezelés maradandóbb neurokognitív változásokat okozott, mint az egyszeri súlyos koponyatrauma, ahogyan azt a 8 héttel az utolsó sérülést követő NOR feladatban kimutattuk. Végezetül, az 1.0 mg/kg dózisú glutamaterg NMDA antagonista memantine kezelés hatásosnak bizonyult 6-8 héttel sérülés után a munkamemória deficit visszafordítására NOR feladatban. Így következtetésképpen megállapíthatjuk, hogy NMDAR antagonisták alkalmazása hatásos lehet poszt-traumatikus magatartási deficitek kezelésére. Ugyanakkor, további kutatás szükséges ahhoz, hogy hatékony célmolekulákat, illetve farmakológiai ágenseket találjunk olyan kezelésekhez, mellyel a TBI-t

követő metabolikus események kaszkádjának nagy részét, vagy talán egészét, visszafordíthatóvá tudjuk tenni.

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11. PUBLICATIONS

List of peer-reviewed journal articles related to thesis

Tadepalli SA, Bali ZK, Bruszt N, Nagy LV, Amrein K, Fazekas B, Büki A, Czeiter E, Hernádi I. (2020) *Long-term cognitive impairment without diffuse axonal injury following repetitive mild traumatic brain injury in rats.* Behavioural Brain Res. 378:112268. doi: 10.1016/j.bbr.2019.112268. Q1, Impact Factor: 2.77

Szarka, N, Toth, L, Czigler, A, Kellermayer, Z, Ungvari, Z, Amrein, K, Czeiter, E, Bali, ZK, Tadepalli, SA, Wahr, M, Hernadi, I, Koller, A, Buki, A, Toth, P. (2019) *Single mild Traumatic Brain Injury Induces Persistent Disruption of the Blood-Brain Barrier, Neuroinflammation and Cognitive Decline in Hypertensive Rats.* Int J Mol Sci 20(13):3223. doi: 10.3390/ijms20133223. Q1, Impact Factor: 4.183

List of other peer-reviewed journal articles

Bali ZK, Bruszt N, Tadepalli SA, Csurgyók R, Nagy LV, Tompa M, Hernádi I. (2019) *Cognitive enhancer effects of low memantine doses are facilitated by an alpha7 nicotinic acetylcholine receptor agonist in scopolamine-induced amnesia in rats.* Front Pharmacol 10:73. doi: 10.3389/fphar.2019.0007. Q1, Impact Factor: 3.8

List of conference abstracts related to thesis

Tadepalli S. A., Bruszt N., Nagy L. V., Bali Zs. K, Czeiter E., Amrein K, Büki A., Hernádi I. (2019) Repetitive mild traumatic brain injury causes long-term cognitive impairment in rats. Conference of European Behavioural Brain Society, 2019, Prague, Czech Republic

Tadepalli S. A., Bruszt N., Nagy L. V., Bali Zs. K, Czeiter E., Amrein K, Büki A., Hernádi I. (2019) *Repetitive mild traumatic brain injury causes long-term cognitive impairment in rats.* Conference of The Hungarian Neuroscience Society, 2019, Debrecen, Hungary

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Tadepalli S. A., Bali Zs. K., Bruszt N., Czeiter E., Amrein K., Vranesics A., Berente Z., Büki A., Hernádi I. (2017) *Evaluation of Cognitive Dysfunction and White Matter Integrity following Mild Traumatic Brain Injury in Rats.* CNS Symposium, 2017, Pécs, Hungary.

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13. APPENDIX

Diffusion tensor imaging

Scanning was performed on a Bruker® PharmaScan® (4.7 T) small-animal MRI instrument. ParaVision Acquisition 6.0.1 software (Bruker) was used to create coronal plane minimum intensity projection (minIP) images. The DWI images were acquired with a navigator echo based spin-echo EPI sequence. All imaging data were first converted into a DICOM (Digital Imaging Communications in Medicines) format and stored in an isolated hardware in a local system. Any further processing was performed via DICOM-handling software packages. (3D-Slicer v4.6, Onis v2.5). Regions of interests (ROI) were manually circumscribed in the splenium of corpus callosum and the fractional anisotropy (FA) value of ROI's were calculated. If the FA value tends to move towards 0, diffusion is isotropic – uniform in all directions. If FA value tends to move towards 1, diffusion is anisotropic – diffusion is unrestricted in 1 direction, and restricted in others. Decrease in FA following TBI may indicate reduction in fibres of CC.

Diffusion tensor imaging analysis revealed no significant change in the integrity of the corpus callosum

Diffusion tensor imaging was performed to analyse the visualize white matter tractography **(Fig. A1).**

Fractional Anisotropy (FA) values for the pre-injury DTI revealed no difference between injury groups. Similarly no significant difference was obsereved in the FA value for CC at post-injury 1 week, and at post-injury 8 weeks (**Fig. A2**). Quantitative results indicate that the white matter intergrity did not suffer any change or reduction post-TBI of any severity.

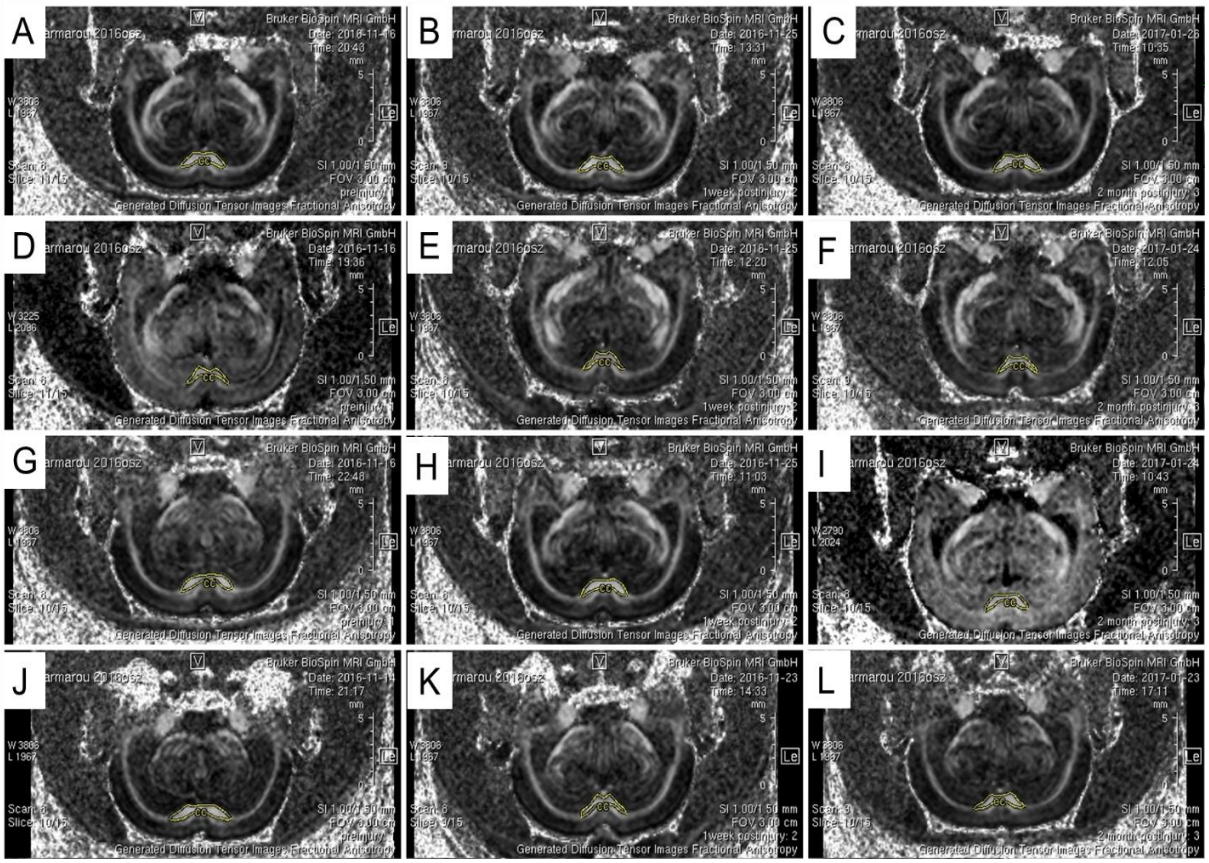

Figure A1*: Structural integrity of the corpus callosum (CC) before and following TBI, visualised using DTI. Top row: CC of a Sham animal before* ***(A)****, 1 week* ***(B)****, and 8 weeks* ***(C)*** *after TBI. 2nd row: CC of a Mild injury animal before* ***(D)****, 1 week* ***(E)****, and 8 weeks* ***(F)*** *after TBI. 3rd row: CC of a Mild2 injury animal before* ***(G)****, 1 week* ***(H)****, and 8 weeks* ***(I)*** *after TBI. Last row: CC of a Seve injury animal before* ***(J)****, 1 week* ***(K)****, and 8 weeks* ***(L)*** *after TBI.*

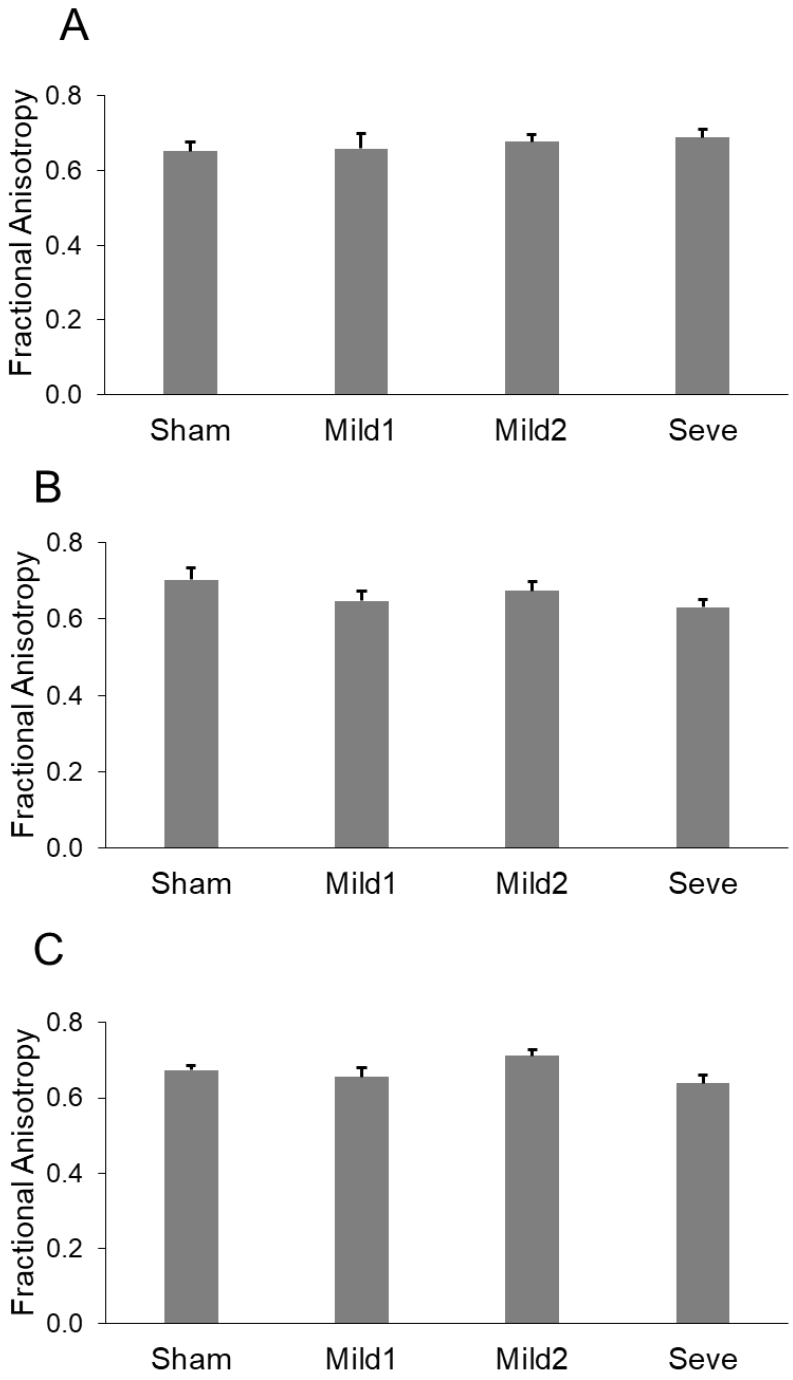

Figure A2: *Fractional anisotropy (FA) values for CC.* ***(A)*** *In pre-injury imaging, all the groups (n=3-4/group) indicated similar FA value for the CC.* ***(B)*** *No significant effect of injury was observed in FA values at post-injury 1 week (F(3,12) = 1.335: p=0.309), and* ***(C)*** *at post-injury 8 weeks (F(3,11) = 1.793; p=0.207).*

Statement on the originality of the PhD thesis and papers

Undersigned name: Tadepalli Sai Ambika

Birth name: Tadepalli Sai Ambika

Mother's maiden name: Bhagwatula Saroja

Place and date of birth: Nagpur, India. 1991.10.03

Today I submitted my PhD thesis entitled: Investigation of Behavioural and Neuropathological Alterations Caused by Repetitive Mild Traumatic Brain Injury in a Rodent Model

to Doctoral School of Biology and Sportbiology

Name of supervisor(s) Hernádi István

In addition I declare my doctoral dissertation submitted in the present case

- had no submitted other doctoral school (not domestic nor abroad university) earlier,
- my application to doctoral process is not rejected within two years,
- I had no unsuccessful doctoral procedure the last two years
- my doctorate degree has not cancelled within five years,
- my thesis is an independent work, I have not presented another person's intellectual property as it would be mine, the references are clear and complete, the false or falsified data are not used in my dissertation.

Date: 2020. 02. 26 .....

signature of candidate