PATHOPHYSIOLOGICAL CHANGES IN THE SPINAL CORD FOLLOWING SINGLE MODERATE AND REPETITIVE MILD TRAUMATIC BRAIN INJURY

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Prologue

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List of abbreviations and acronyms

Abbreviation	Definition
AD	Alzheimer’s disease
ALS	amyotrophic lateral sclerosis
ATLS	advanced traumatic life support
BBB	blood brain barrier
BSA	bovine serum albumin
CCI	controlled cortical impact
CNS	central nervous system
CT	computed tomography
CTE	chronic traumatic encephalopathy
DAB	diaminobenzidine
DAI	diffuse axonal injury
DAPI	4',6'-diamino-2phenylindole
FPI	fluid percussion injury
FTD	frontotemporal dementia
GBD	global burden of disease
GCS	Glasgow coma scale
HD	Huntington’s disease
HRP	horseradish peroxide
I/A	impact/acceleration (model)
ICP	intracranial pressure
LFPI	lateral fluid percussion injury
MRI	magnet resonance imaging
PD	Parkinson’s disease
RNA	ribonucleic acid
RRM	RNA recognition motif
TBS	tris(hydroxymethyl)aminomethane base
TBI	traumatic brain injury
TTM	targeted temperature management
VMF	ventral median fissure
WD	weight-drop (model)

1. Introduction

1.1. Traumatic brain injury

Traumatic brain injury (TBI) presents a structural or functional impairment of the central nervous system (CNS) that is caused by external forces to the skull. This can arise from penetrating, blunt or blasting impacts that lead to potentially irreversible neurodegeneration (1). A meaningful and thoughtful discussion about TBI requires a clear and definite description of the topic itself and its key points. This study is designed to contribute to the ongoing debate about TBI in perspective to its impact on distant sites like the spinal cord. The Demographics and Clinical Assessment Working Group of the International and Interagency Initiative toward Common Data Elements for Research on Traumatic Brain Injury and Psychological Health has defined TBI as follows: "TBI is defined as an alteration in brain function, or other evidence of brain pathology, caused by an external force" (2).

TBI generally arises from a head injury that can be opened or closed and is often associated with skull fractures and damages of the surrounding soft tissue. The primary impact triggers physiological processes that can result in secondary pathology which develops over time. In this section, an insight on TBI, its classification, pathogenesis, and especially its effects on spinal cord, will be mentioned.

TBI has a significant epidemiological importance. Studies that aim to define the incidence report that especially mild TBI is less often brought to medical attention. Epidemiological numbers of TBI therefore tend to underestimate the actual socioeconomical burden that TBI has on our society. In 2016, The Global Burden of Disease (GBD) study pointed out that annually an estimated 55 million experience TBI worldwide, with 8.1 million living on with disability (3). Studies have shown that TBI is the leading cause of death in individuals younger than 41 years of age in North America, which implies how important it is to establish the best possible management and treatment (3). In Germany, TBI has an incidence of 332:100 000 citizens, which means that there are annually approximately 226 000 patients suffering from TBI (4). It has been estimated that 2.7 billion euro is spent per year on the management and care of TBI patients in Germany (5,6). Because of the irreversible character of many head injuries, preventative measures are crucial. They aim to reduce the mortality rate of TBI. For example, in Germany, thanks to the prevention, TBI mortality was reduced from 27.2 per 100 000 citizens to 9 per 100 000 citizens in the period from 1972 to 2000 (7). This was favored by the changes of laws regarding safety regulations and the policy of wearing helmets in traffic. These numbers very well implicate how significant the management of head

injury and TBI is. Additionally, to the preventative measures, research about TBI must aim to create a better and thorough understanding of how to manage and treat the outcomes of TBI. New findings about the dynamics of TBI pathogenesis can possibly change present guidelines for the better and new innovative methods for a therapeutic approach can generate a better life quality.

There are several ways TBI can occur. The etiology of TBI differentiates between blunt head injuries, penetrating head injuries and blast injuries. Blunt head injuries are the most common injuries to induce TBI. Falls are the leading cause of blunt injuries, most commonly affecting children, adolescents, and elderly people (8). The second most common reasons for blunt injuries are motor vehicle accidents (9). Sport injuries, especially in collision sports, are important as they have been reported to be linked with the development of mild TBI (10).

Penetrating head injuries are a less common causative event for TBI, and they describe the penetrating assault of a foreign body to the head or face such as high-velocity missile injuries (gunshots, and non-missile low velocity injuries) (11,12).

Blast injuries are injuries caused by a very high-pressure wave that potentially induces TBI. This is common in active military or war zones (13).

In general, TBI results primarily due to high accelerative impacts to the brain parenchyma and to surrounding tissues which leads to the tissue damage and induces pathophysiological changes.

Classification of TBI is an important aspect for understanding and optimizing brain injury management. TBI can be classified in various regards. It depends on the structures that are affected, the way the injury has developed and the severity of the injury itself. This results in a heterogenic presentation of the patients that suffer from head injuries and their associated diseases. As a fact, already within minutes, secondary injuries can develop following the primary impact of a head injury, which explains why the outcomes of TBI can vary and present in such different ways and grades of severity.

Further distinguishment is made according to the integrity of the meninges. An opened head injury is present when the dura mater is breached, whereas a closed head injury is characterized with an intact meningeal layer (14). As already mentioned, the distinguishment of penetrating, blunt and blast injuries can be used to differentiate and classify types of TBI.

The most common way to distinguish various forms of TBI is according to the severity of the injury by using the Glasgow Coma Scale (GCS). The GCS score has been designed to evaluate a patient’s responsiveness by 3 aspects (verbal, motoric and eye opening). It allows medical practitioners a quick neurological assessment in cases of head injuries. According to a

patient's ability to respond to the given stimulations it is possible to achieve between 3 and 15 points. The worst score is 3 points, and it represents an imminent comatose state or brain death. A score of 15 means that the patient is fully conscious without any restrictions (15). A mild TBI resembles scores between 13 and 15, and it is associated with normal neuroimaging findings. Concussions meet the criteria for mild TBI and therefore fall into the range of a GCS score between 13 and 15 as well. Moderate TBI equals a GCS score from 9 to 12, while severe TBI is defined as any GCS below 8.

1.2. Clinical features and initial management of traumatic brain injury

Once an individual has experienced a head injury there are many different clinical pictures that can present as the injury progresses. The general symptoms vary according to the severity of the impact, the location, and the nature of the TBI. General neurologic symptoms include headaches, cases of amnesia, and alterations in the state of consciousness, orientation, and confusion. The intracranial pressure (ICP) is very important for the brain's ability to function. Any alterations in the ICP can cause measurable and detectable behavioral changes, and changes in the state of mood. Aggressive behaviors, dizziness and especially vomiting and nausea are characteristics of increased ICP as well as various syndromes that develop because of cerebral herniations. In focal brain trauma it is common that deficits are presented as isolated symptoms like sensory or motor loss (vision, smell, taste, speech), as only a specific region is affected by the injury, whereas elsewhere in the CNS normal cerebral function may be maintained. The inability of a patient to maintain body positioning, due to the occurrence of motoric hypertonia or dystonia, leads to an abnormal posturing which is often seen in patients with TBI with brainstem involvement. These clinical pictures indicate a very severe injury that must be dealt with immediately, as they indicate injuries of fatal nature (16).

As mentioned above, TBI develops because of a head injury, so the clinical picture is accompanied with symptoms of associated injuries like fractures of the skull. Liquorrhea, cerebrospinal fluid rhinorrhea, hematomas, cranial nerve palsies, facial swellings, and deformations may also be present.

TBI can present with relatively simple and mild symptoms, but due to the dynamic processes that can be induced, the outcome can worsen rapidly. A structured and organized approach is evident to ensure the best possible outcome for the patient. The Advance Trauma Life Support algorithm (ATLS) was designed to define a clear and structured sequence of diagnostic and life supporting measures to decrease the incidence and power of secondary brain

injury (17). It is important that therapeutic actions that ensure normotension, normocapnia, normoxia and euglycemia should never be delayed due to diagnostic methods which assess the injury and its severity. Especially, hypotension can significantly worsen the TBI outcome and should always be managed accordingly. Further, the transfer to centers with neurosurgical departments and equipment for neuroimaging should be kept as short as possible. Any delay during the transport would contribute to inadequate management of the injury and its outcome.

As part of the primary survey of the American College of Surgeons and the Committee on trauma the focus is concentrated on clinical problems that appear to be life-threatening. The ABCDE scheme aims to assure vital functioning and resembles a structured initial examination of a patient in an emergency (18). Excessive bleeding must be ruled out at first before the airway patency is evaluated. From there on the protocol proceeds with A (airway) on to B (breathing), C (circulation) and D (disability) to E (environment or exposure) the various assessment aspects are processed and the patient is repeatedly evaluated according to this scheme (17,19,20).

The secondary survey concentrates on a more detailed historical evaluation after the primary survey was concluded and the patient appears to be stabilized. It consists of a focused history, a physical examination, continuous monitoring of vital signs, emergency consultations and a potential transfer to institutions that are well equipped for neurological evaluations and trauma care. Lastly, the focus can be directed at the complementation of the third-party medical history and a generally more multifaceted care for the patient (17).

1.3. Pathophysiology of traumatic brain injury

1.3.1. Primary brain injury

From the pathophysiological perspective TBI can be divided into primary and secondary brain injury. The primary brain injury is defined as the brain injury that develops as a direct consequence of the trauma from the head injury. A focal primary brain injury collectively includes intracranial hemorrhages. The site of bleeding further distinguishes the condition of bleeding within the skull. Epidural, subdural, subarachnoid, or intracerebral bleedings are severe injuries that contribute to the development of an increase in ICP which could further trigger secondary damages. Cerebral contusions and coup-countrecoup injuries are as well focal brain injuries that can be a result of the initial head injury. Intracerebral or intracerebellar hematomas and lacerations of the brain parenchyma conclude the often seen set of focal brain injuries after a head trauma. Diffuse primary brain injuries include mild TBI, cerebral edema and diffuse axonal injury (DAI).

DAI is a neuronal injury that is secondary to acceleration and deceleration forces and is caused by multifocal shearing forces and can cause severe neurological injuries resulting in coma or a persistent vegetative state (21,22). The acting mechanical force ruptures important structures that maintain a stable architecture of the axons like the cytoskeleton. The entire disruption of axonal cells leads to completely inadequate neuronal communication and therefore resembles severe neurologic deficits. The impact and presentation of DAI at remote sites to the head injury will be highlighted in more detail later in the text (section 1.5.2. Impact of traumatic brain injury on spinal cord).

1.3.2. Secondary brain injury

In opposite to primary brain injuries, many of the arising secondary changes are preventative. Head trauma triggers various physiological changes of the soft tissues and parenchyma of the CNS and affects the surrounding structures which can elicit and induce further damage, some of which will be mentioned in the following section.

Secondary brain injuries usually develop because of the disruption in physiological balances in the CNS metabolism. This disruption of homeostatic measures may contribute to the development of pathological findings like brain edema. Brain edema can often appear because of a damaged blood brain barrier (BBB), an excessive release of excitatory neurotransmitters, neuronal depolarization or cerebral vasodilation which contributes to an increase in ICP. Additionally, inadequate mitochondrial functioning can cause neuronal misfunctioning and therefore induces neuronal cell death. Further, due to stress-induced glycemic disbalance, vasoconstrictive or vasodilative disorders of cerebral blood vessels represent the endothelial response of a head injury. This can lead to cerebral hypoxemia and cerebral hypoxia. Both conditions contribute to the development of irreversible damage to neuronal tissue and establish variations of disability depending on the site of ischemia. An injury to the hypothalamus and the pituitary can trigger neurogenic fever and, in some cases, create a central diabetes insipidus. The loss of cerebral autoregulation makes the CNS more susceptible to the alterations in the systemic blood pressure and the systemic oxygenation, which might promote secondary brain damage as it cannot sustain an adequate perfusion and oxygenation (19,22,23).

Another set of disorders arises from imbalanced electrolytes like sodium and potassium. Sodium-disorders are often self-limiting in cases of brain injury but in symptomatic hypernatremia, the treatment of the underlying cause is crucial to avoid further development of

pulmonary or cerebral edema. Symptomatic hyponatremia is also known to increase the risk of central pontine myelinolysis and once it is detected, a gradually correction of sodium levels is indicated (24).

The body's thermoregulation also appears to be affected by an TBI insult. Central hyperthermia is associated with focal damage to the thermoregulatory center in the hypothalamus. This condition is seen in 4-37% of patients with TBI in the US (25). Neurogenic hyperthermia should be treated aggressively as it is believed to be a major factor with negative impact on the TBI outcome (26). The goal of the targeted temperature management (TTM) is to avoid secondary brain damage by ensuring euthermia with a body temperature between 36 and 37.8°C (27). Thermal imbalances are believed to contribute to a breakdown of the BBB and thus increase neurodegenerative processes that have been partially mentioned in the previous text. Measures to achieve TTM include cooling of body surface, endovascular cooling or the application of antipyretics (28).

1.3.3. Neuroimmunity and neuroinflammation

The mechanical impact that triggers TBI elicits an immunological response from the CNS. In order to induce repair of damaged neuronal tissue and to clean the site from necrotic debris and cells, there is a quite different immunological answer that takes place in the CNS, as the BBB, the cerebrospinal fluid-blood barrier and the blood-spinal cord barrier avoid the migration of many immunological cells from the systemic compartment to the damaged brain or spinal cord (29).

Mainly the innate immune system contributes to the immediate development of inflammatory processes in damaged CNS tissue as the adaptive immune system is more prone to contribute to the development of secondary brain injury (30,31). The neuroinflammatory responses are known to play a major role in the nature of various neurodegenerative diseases like Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD) (32). This section will state in more detail how various cells of the CNS and immune system respond in innate and adaptive ways to TBI.

Neutrophils are the most common leucocytes that are produced by the bone marrow. Following TBI microbiological cascades activate adhesive proteins at the site of injury in order to recruit and accumulate neutrophils (32). They are observed at the highest numbers in the meningeal spaces approximately after 2 hours after the injury has occurred and are seen in higher numbers in the brain parenchyma after 24 to 48 hours. Even within minutes after TBI,

neutrophils can be detected at injured areas, which indicates their major role in the early pathogenesis of TBI (33). At the site of injury neutrophils work in a phagocytic manner to clear the tissue from necrotic debris. Adhesive processes of the endothelium favor the neutrophil recruitment and have been shown to be a major contributor to the development of edematous processes (34).

Microglia reside in neuronal tissue and play vital neuroinflammatory roles. They derive from the yolk-sac during embryonic stages and in adult stages they present in a vast heterogenicity (35). Together with blood-borne macrophages, microglia make up to 10% of the brain cell tissue (36) and they promote homeostasis and overall brain function via eradication of metabolic waste products and the breakdown of damaged cells (37). Once the CNS must react to infection, neurologic disorders, or injury as in TBI microglia function in various ways. They are equipped with highly motile projections that interact with neighboring cells like astrocytes, neurons, and endothelia. These interactions take role in the creation of the immune response in the CNS. Activation of microglia triggers morphological adjustments and functional changes. Increased proliferation, release of messenger molecules and a more amoeboid shaping of the cells elicits the immune response that is supported by microglia.

Either they can support the healthy growth and maintenance of the body, or they contribute to damage of tissues leading to pathological conditions. Their homeostatic functions involve aiding learning and memory by producing brain-derived neurotrophic factor, taking part in normal synaptic pruning, and clearing cellular debris and protein aggregates through phagocytosis (35). However, when activated during pathological states, microglia can also damage normal healthy neurons and synapses, promoting the formation of β-amyloid or other misfolded proteins that cause neurodegenerative diseases. Microglia can produce cytokines such as interleukin-1α, tumor necrosis factor, and C1q, which have been linked to the creation of neurotoxic astrocytes. Moreover, microglia can also perform various functions in adaptive immunity, including roles in antigen presentation and immune regulation (38).

Recent evidence suggests that microglia and brain macrophages play a crucial role in neurodegenerative diseases, contrary to earlier beliefs that their function was mainly related to phagocytosis of cell debris. Animal studies revealed that these cells can have either a protective or pathogenic effect, depending on the experimental conditions (38).

Astrocytes are known to contribute to the maintenance of homeostasis of the CNS (39). Following TBI, and in the process of neuroimmunity, astrocytes may change from a resting state to become active and change their characteristics in various regards. Activated astrocytes are described to be hypertrophic, to increase their secretory activity and to present in larger

numbers. Together with an increased expression of intermediate filaments and the secretion of neuroinflammatory mediators and neurotrophic factors a process called reactive astrocytosis is described (40). Especially at the site of neuronal damage reactive astrocytosis can be observed. Research has revealed that reactive astrocytes can be transcriptionally and functionally diverse and can act either protectively or generate toxicity (41). Hypertrophic and proliferative changes are believed to play a major protective role to secure intact neuronal tissue (42). Further beneficial actions of reactive astrocytosis include the astrocytic expression of several glutamate transporters that help to regulate and decrease extracellular glutamate. This inhibits excitotoxicity which is a major contributor for neurodegeneration (32). However, in other inflammatory and degenerative conditions, reactive astrocytes seem to actively contribute to the process of injury formation. As mentioned previously, the cytokine mediated activation of astrocytes by microglia can convert astrocytes into a phenotype that promotes disease and neurodegeneration as it has been described in AD, PD, and ALS (38).

The adaptive or acquired immunity consists of mainly two branches, the humoral and cell mediated components and resembles the organism's ability to react to a specific pathogen due to antibody production and detection of antigenic substances (32). Once the innate immune system presents specific antigens, the adaptive immunity can identify them and induce a targeted lysis or degeneration of the pathological source. In neurodegenerative processes, the adaptive immune system can implement chronic immune responses that might be triggered by TBI and elicit neurodegeneration.

Understanding the role of T-cells in neuroinflammation is essential for interpreting the mechanisms underlying various neurological disorders and developing targeted therapeutic interventions. T-cells are a major component of the adaptive immune system, and they are essential for the cell-mediated immunity. They originate from lymphoid progenitor cells in the bone marrow and differentiate in the thymus (43). The mechanism for T-cell activation follows a cascade of complex microbiological events including the secretion of T-cell-derived-cytokines that are supporting the formation of an immune response by intercellular communication and activation of other cellular components of the immune response. Antigen presentation of several cells that are part the neuroinflammatory response mediates and prolongs the survival and proliferation of T-cells as a costimulatory signal. Cytotoxic T-cells (CTLs) can detect marked cells and structures and induce their eradication (44).

B-cells go through several phases as they elicit their function of the adaptive immune response. As T-cells they can bridge the BBB to accumulate and proliferate at the damaged site of the CNS. B-cells are as well secreting cytokines, contributing to antigen presentation and

especially their antibody production elicits the adaptive immune response. Secretion of autoantibodies and the development of autoimmune neuroinflammation has been linked to neuroinflammatory disorders like neuromyelitis optica (45). Furter by isotype switching and affinity maturation long-living memory B-cells are carrying out some of the actions of the adaptive immune response (46). Studies have shown that there is no significant change in the behavior of B-cell-mediated immunity responses following severe TBI in comparison to healthy individuals (47).

1.3.4. TAR DNA-Binding Protein - 43

TAR DNA-Binding Protein - 43 (TDP-43) is a ubiquitous protein in all our body cells that is found in the nucleus but can be present in the cytoplasm regulation of ribonucleic acid (RNA). TDP-43 is a multifunctional RNA-binding protein and is one of the heterogenous ribonucleoproteins that play an important role in regulation of gene expression and messenger RNA (mRNA) processing in the cells. TDP-43 is a part of a vast variety of intracellular processes for transcriptive, translational, and homeostatic cascades.

The N-terminus and C-terminus regions of TDP-43 create the protein's ability to interact with several neighboring proteins and molecules that participate in RNA processing during the many stages in the life cycle of RNA. There are two RNA Recognition Motif (RRM) domains that can bind almost all types RNA molecules in a sequence-targeted manner, from pre-mRNA immediately after it has been processed by RNA Polymerase II, to their following processing steps (48)(49). Therefore, not only mRNA is partially processed by actions of the TDP-43 protein, but also non-coding RNA molecules interact with TDP-43. This processing mechanism may include splicing besides several binding and processing actions of RNAs with undetermined functions (48). Figure 1 represents some of the yet described interactions of TDP-43 during RNA processing. The obvious multifunctionality of TDP-43 in RNA processing indicates its importance in gene expression and increases the attention that research has brought up within the past two decades to understand the involvement of TDP-43 in neurodegenerative processes more thoroughly. The fact that TDP-43 interacts with various RNA species highlights the belief that a proteinopathy of TDP-43 has a multifactorial extent.

The recognition of the linkage of TDP-43 in several neurodegenerative diseases, including ALS and frontotemporal dementia (FTD) started in 2006 (48,50). In these diseases, TDP-43 aggregation and mislocalization contribute to neurodegeneration. The exact pathogenesis of these neurodegenerative processes is yet not fully understood but it is believed

that TDP-43 aggregates can disrupt the processing of RNA and cause neuronal disfunction by impaired protein homeostasis. Not only the gain and overproduction of TDP-43 contributes to homeostatic impairment. The altered functionality leads to the loss of the physiological properties of TDP-43. This may even lead to the activation of specific caspases to induce apoptotic events (51).

Current research aims to describe the pathogenesis of TDP-43 proteinopathies in more detail. This way new starting points for the development of neurodegeneration could be used to intervene the pathological onsets.

Overall, better understanding of the intracellular cascades in which TDP-43 is involved in, can lead to a promising new therapeutic set, which could be used to avoid and prevent the establishment of neurodegenerative diseases like ALS and FTD. Current therapeutic approaches to alleviate the proteinopathy of TDP-43 aggregation include the oral administration of Withaferin-A. Withaferin -A is an antagonist of the nuclear factor-κB and elicits autophagy of TDP-43 aggregates. Therefore, mitigation of pathogenesis and amelioration of neuronal deficits can be achieved (52,53). Further, preclinical studies have shown that E6, a monoclonal full-length antibody, interferes especially with the cytoplasmic fraction of TDP-43 and can lead to a reduction of the TDP-43 proteinopathy responsible for the development of ALS and FTD mouse model. This study from the CERVO Brain Research Center implicates an influence on the functioning of lysosomal machineries and proteasomes (54).

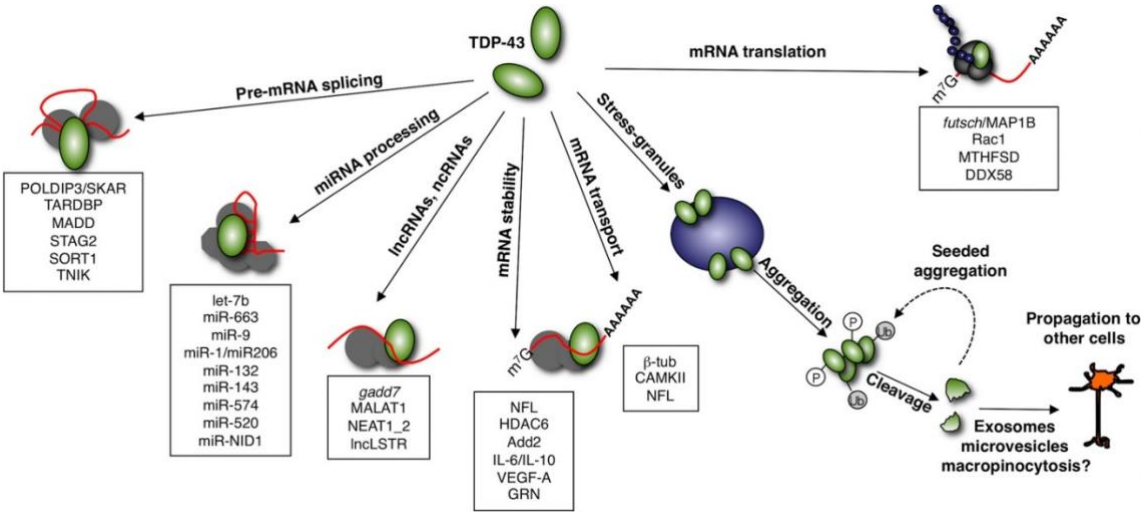

Figure 1. Diagram of known TAR DNA binding protein 43 kDa (TDP-43)-regulated cellular functions in the nuclear and cytoplasmic compartments. The boxes below each function report only the names of the RNA targets that have been extensively validated at experimental level. Possible causes of aggregation following stress granule formation and the consequent seeded spreading of the disease are also indicated. From publication by Ratti A, Buratti E. "Physiological functions and pathobiology of TDP-43 and FUS/TLS proteins" in: J Neurochem. 2016;138 Suppl 1:95-111. doi: 10.1111/jnc.13625 (48).

Repetitive mild TBI is reportedly in close connection to the early onset of neurogenerative disorders like ALS and AD, and studies have proven that there is a contribution to the development of TDP-43 pathologies (55,56). Alterations in the expression of TDP-43 and the occurrence of neuritic pathology is proven to be correlated. FTD and hippocampal sclerosis dementia are some types of dementia that can be linked to TDP-43 alterations as mentioned in previous sections (57).

1.4. Outcomes of traumatic brain injury

The outcomes that can result from TBI present a broad and heterogenous spectrum. TBI is the most common cause of death in young adults worldwide. As the CNS has been damaged, alterations and changes in emotional, behavioral, cognitive, and physical ways can impact one's individual quality of life (58). Brain trauma affects families, communities, interpersonal and occupational functioning in negative terms. The neuronal misfunctioning caused by TBI can lead to cognitive disturbances like insomnia, learning difficulties and many more imbalances in functioning, affecting attention, memory or coordinative manners (59). Damage of the centers of language and social interactions can cause other long-term difficulties which create huge imbalances in social functioning. Dysphagia and dysarthria are conditions that have been linked to the outcomes of TBI which emphasizes the severity of pathology that patients have to face in cases of TBI (60). Studies have shown that up to 22% of TBI survivors suffer minor depression, and 26% tend to develop major depression at 1-year post-TBI (61). Neurologic disturbances like increasing rates of seizures or the implementation of epileptic disorders are described in correlation with TBI as well (62).

1.5. Effect of traumatic brain injury in the spinal cord

1.5.1. Morphology of the spinal cord

The spinal cord is a cylindrical structure that runs through the vertebral column and extends from the brainstem to the first or second lumbar vertebra. The spinal cord is divided into four regions: cervical, thoracic, lumbar, and sacral. Each region contains a specific number of spinal nerves that innervate different parts of the body. The cervical region contains eight pairs of nerves, the thoracic region has 13 pairs, the lumbar region has six pairs, and the sacral region has four pairs. The spinal cord is composed of gray and white matter. The gray matter contains neuronal cell bodies, dendrites, and glial cells, while the white matter contains myelinated and unmyelinated axons that transmit signals to and from the brain. The spinal cord

also contains a central canal filled with cerebrospinal fluid that provides nourishment and cushioning to the nervous tissue (63). It is important to note that mice spinal cord has a similar morphology to human spinal cord, since it is a model species used in this research.

1.5.2. Impact of traumatic brain injury on the spinal cord

Traumatic brain injury and spinal cord injury are two distinct injuries that can occur independently or in combination. However, while TBI primarily affects the brain, it can have secondary effects on the spinal cord due to the proximity and interdependence of these two structures. Athletes participating in contact sports quite often experience repetitive mild TBI which is closely linked to the development of neurodegenerative conditions like chronic traumatic encephalopathy (CTE). Especially the repetitive occurrence of mild TBI is linked to the neurodegenerative conditions not only at sites of the injury. Extensive tau-immunoreactive pathology can affect distant structures like the brainstem and spinal cord (64). Repetitive TBI is known to cause changes in the physiological function of TDP-43 and therefore implicate neurodegeneration. Further, systemic responses to TBI can affect the spinal cord, as the autonomic nervous system, which controls involuntary functions such as heart rate and blood pressure, contributes to the dynamics of pathogenesis of secondary injuries following TBI.

TBI can induce a mislocalization of TDP-43 into the cytoplasm where the structural characteristics of TDP-43 show high properties for aggregate formation. This aggregation is a pathological key factor in the pathogenesis of several neurodegenerative diseases such as ALS. TDP-43 aggregates have been shown to spread across cells and therefore contribute to the progression of the disease at distant sites of the injury (65). The propagation of aggregated soluble TDP-43 can occur via axonal connections and synaptic communication among neuronal cells which leads to the physical dissemination of pathological TDP-43 aggregates and increases the pathology of ALS (55). The dissemination could possibly be decreased by converting the solubility of TDP-43 aggregates into insoluble species. This would hypothetically decrease the propagation of ALS pathology and minimize its severity in the clinical picture (55).

Already mentioned previously DAI is a type of TBI that can evoke following head injury (66) that mostly occurs following high-velocity accidents including motor vehicles (67). Every tenth patient with TBI is estimated to present with some degree of DAI (68). Every fourth patient of these suffering from DAI might lose their life due to the severity of the injury. DAI is a clinical diagnosis as the pathophysiological changes are usually hard to visualized with

imaging techniques like computed tomography (CT) or magnetic resonance imaging (MRI). Therefore, the actual incidence of DAI might be higher that currently described in the literature. Laboratory findings that may assess the presence or severity of DAI are currently not accessible, but research aims to detect biomarkers that indicate post-traumatic injury and could be used to evaluate DAI presentation (69). The pathophysiological changes mainly present due to the disconnection of neuronal interactions and communications that are carried out by the axonal projections of neurons. The shearing forces of the initial impact of a head injury can introduce physical damage to cytoskeletal architectures and thus cut signaling pathways. This may result in proteolysis of neurons as well as further phenomena like swelling and multifactorial neuronal damaging. Distant structures can no longer interact with the brain and present clinical pictures that may arise by misfunctioning of these remote centers. The severity of the injury can be classified by "The Adams Diffuse Axonal Injury Classification" that differentiates three different degrees (mild, moderate, and severe) considering the affection of different structures of the CNS (70). The degree of the injury often resembles the extent of the clinical presentation and ranges from headaches to vegetative states and coma. DAI often presents with bilateral neuronal deficits in patients due to the damaged white matter mostly of the temporal and frontal regions of the brain as well as the corpus callosum and the brain stem. The treatment concentrates on the management of secondary injury and rehabilitative measures for the best recovery possible. There has been less investigation about the presence of DAI at distant sites than the injury itself but previous experimental studies have been shown that there is an effect of DAI at remote sites as in the spinal cord (71).

1.6. Experimental models of traumatic brain injury

Experimental animal models are necessary in the understanding of pathophysiological processes and the development of new clinical and therapeutic interventions following TBI. Depending on the type of brain trauma, they can be divided into focal, diffuse, and combined models of traumatic brain injury (72).

Focal traumatic brain injury experimental models involve the use of a controlled mechanical force to induce a localized injury to the brain tissue. These models aim to replicate the features of TBI observed in humans, including the formation of contusions, hemorrhages, and axonal injury. Contusions are the most common brain lesions following TBI and therefore an adequate mimic of the injury for experimental evaluation is of specific interest. One common method for inducing focal TBI in rodents is the controlled cortical impact (CCI) model. In this

model, a craniotomy is performed to expose the dura mater. Then, a pneumatically driven rigid impactor is used to impact the exposed brain tissue. The impact force, duration, and depth can be adjusted to produce different degrees of injury severity. The injury induces a contusion at the impact site and often leads to secondary injury, including inflammation, edema, and neuronal cell death. Disadvantages of the CCI may include the complete ipsilateral cortex destruction which cannot be compared with a TBI that a human could survive. Secondary brain injury may develop from the craniotomies which also avoid a comparable mimicking of TBI. Additionally, the lack of brain stem involvement and rather short-lasting unconsciousness limits the clinical relevance of this model.

Another model of focal brain trauma is the weight-drop (WD) model, in which a weight is dropped on the exposed skull of an anesthetized rodent (closed skull WD) or onto the exposed dura mater (open skull WD). The force and height of the weight drop can be adjusted to induce varying degrees of injury severity. This model can also produce contusions and axonal injury. Overall, the WD models offer a quick and dependable method for inducing substantial brain damage, neuroinflammation, and cognitive impairments in rats and mice, thereby providing valuable insights into the focal TBI. (73)

Both the CCI and WD models have been widely used to study the mechanisms underlying TBI and to test potential treatments for TBI in rodents. Diffuse traumatic brain injury experimental models involve the use of a non-localized mechanical force to induce injury to the brain tissue, mimicking the diffuse damage observed in human TBI. For induction of a diffuse TBI, commonly the fluid percussion injury (FPI) model, the impact/acceleration (I/A) model, the blast injury model and or a modified CCI model are used to observe and evaluate the pathology (73).

In the FPI, a craniotomy is performed to expose the dura mater, and a specialized device is used to deliver a brief, high-pressure pulse of fluid to the surface of the brain. The pressure wave induces diffuse axonal injury and can also lead to secondary injury, including inflammation, edema, and neuronal cell death.

The I/A model induces diffuse axonal injury without the presence of skull fractures and lesions of the parenchyma. It resembles injuries that are commonly observed in humans that experience injuries because of rapid changes in acceleration and deceleration. Most commonly these mechanical forces are seen in motor vehicle accidents or falls. Desirable aspects of the I/A model are the extensive axonal injury that can be produced by the absence of focal contusions, the moderate cost aspect, and the adaptability of the impact (74).

Another model is the blast injury model, which uses a shock wave to induce diffuse injury. This model replicates TBI resulting from explosive blasts, such as those experienced by military personnel. The shock wave can cause primary injury to the brain tissue, as well as secondary injury from inflammation and oxidative stress (73).

Modified CCI models use a pneumatically or electromagnetically controlled piston to induce very specific and accurate trauma.

Diffuse TBI models can be more challenging to reproduce consistently than focal TBI models, due to the variability of the injury induced by the mechanical forces. Nevertheless, these models have been used to study the mechanisms underlying diffuse TBI and to test potential treatments for this type of injury in rodents.

Combined TBI experimental methods in mice involve using multiple injury paradigms to simulate the complex and heterogeneous nature of human traumatic brain injury (TBI). These models seek to mimic the clinical scenario of patients who sustain brain trauma that involves a combination of focal and diffuse TBI, or TBI in combination with other types of injury.

The most used model to induce TBI is the lateral FPI (LFPI) model that has been first described by McIntosh et al. (1989). In this model a craniotomy is performed and covered with a plastic cap that is fixed with dental cement. The release of a pendulum will elicit a fluid bolus that runs through a reservoir filled with saline and will finally strike the intact dural surface, extending into the epidural space and causing deformation of the brain. This injury model is clinically relevant due to the significant amount of axonal injury observed (75).

2. Aims and objectives

The aim of this study was to determine the pathophysiological changes in the spinal cord 14 days after the administration of a single moderate TBI or last trauma of a repetitive mild TBIs in mice. This study used the LFPI model for inducing a single moderate TBI and a WD model to simulate the events of repetitive mild TBIs. Investigating the severity of damage following head injury in the distant structure like spinal cord was of interest in this study. The presence and extent of the histological damage in the mice spinal cord following the two different types of TBI was evaluated. In addition, the comparison of the neuronal and glial cells response in spinal cord following single moderate and repetitive mild TBIs was of interest. Further, due to the close linkage of TDP-43 proteinopathy and its role in the pathogenesis of neurodegenerative diseases, the determination of subcellular expression of TDP-43 in the spinal cord of the mice subjected to single moderate or repetitive mild TBIs was assessed.

3. Materials and methods

3.1. Study design

The study used in total 19 male mice of the black C57BL/6 strain. Due to the potential effect of estrogen on regenerative processes in neuronal tissue female mice were excluded from the study (76,77). Additionally, men are more prone to experience TBI especially in the population of young adults. It has been estimated that from TBI patients, 55-80% include men that are involved in traffic crashes, falls and other accidents including trauma during sport activities (78).

The animals were bred in the Center for Engineering and Breeding of Laboratory Mice at the Faculty of Medicine of the University of Rijeka. The experiments were performed at the Department of Basic and Clinical Pharmacology and Toxicology at the Faculty of Medicine of the University of Rijeka. The animals were held under controlled conditions assuring light and dark cycles of 12 hours, constant temperatures of 20 ± 4°C with constant access to food and water. The ethics committee of the Faculty of Medicine of the University of Rijeka approved the conductance of the experiments according to the legal provisions of the Republic of Croatia (NN 135/06, 37/13, 125/13, 55/13, 39/17) and according to the guidance of the European Parliament (210/6/EU). The principle of 3R (replace, reduce, refine) was used to optimize the experiments carried out and to reduce suffering of the animals.

3.2. Materials

3.2.1. Chemicals

The following chemical substances have been used throughout the histological analysis and immunohistochemical evaluation: bovine serum albumin (BSA), ammonium hydroxide (NH_4OH), diaminobenzidine (DAB) chromogen, 4',6'-diamino-2phenylindole (DAPI), nitric acid (HNO_3), ethanol, Entellan, formaldehyde (CH_2O), hematoxylin (Biognost d.o.o., Zagreb, Croatia), potassium permanganate ($KmNO_4$), chloroform, cresyl violet, xylene, citric acid ($C_6H_8O_7$), sodium carbonate, luxol fast blue dye (Biognost d.o.o., Zagreb, Croatia), nitric acid, 2-methyl-butane methanol, Mowiol® 4 -88, NaCl, acetic acid (CH_3COOH), paraformaldehyde, silver nitrate ($AgNO_3$), Streptavidin DyLight™ 488 conjugate, Streptavidin DyLight™ 594 conjugate, Streptavidin-HRP (eng. horseradish peroxidase, HRP) conjugate, tris(hydroxymethyl)aminomethane (Tris) base (TBS), Triton X®, Tween® 20, hydrogen peroxide (H_2O_2).

3.2.2. Drugs

For conducting the experiments, the usage of the listed drugs was necessary: nitric oxide (N_2O), saline (0.9% NaCl), isoflurane (Forane®, Abbott Laboratories d.o.o., Sittingbourne, UK), iodine, ketamine (Ketamidor®, Richter Pharma AG, Wels, Austria), oxygen (O_2), xylazine (Xylazine®, Alfasan International B.V., Woerden, The Netherlands), levobupivacaine (Chirocaine®, Abbott Laboratories d.o.o. Sittingbourne, UK), mupirocin antibiotic ointment (Betrion®, Pliva Hrvatska d.o.o., Zagreb, Croatia), protective eye drops (Systane® Gel Drops, Alcon, Geneva, Switzerland).

The primary antibodies used for carrying out the immunohistological experiments were against glial fibrillary acidic protein (GFAP), ionized calcium-binding adapter molecule 1 (Iba1), neuronal nuclear protein (NeuN) and TDP-43 (Table 1). Secondary antibodies used in this study are listed in Table 2.

Table 1. Primary antibodies

Epitope	Host and clonality	Manufacturer	Dilution
GFAP	mouse monoclonal	Cell Signaling Technology, Danvers, MA, SAD	1:100
Iba1	goat polyclonal	Abcam, Cambridge, UK	1:100
NeuN	mouse monoclonal	Chemicon, Millipore, Billerica, MA, SAD	1:100
TDP-43	rabbit polyclonal	Proteintech Group, Rosemont, IL, SAD	1:200

Table 2. Secondary antibodies

Epitope	Host and clonality	Manufacturer	Dilution
mouse imunoglobulin	goat polyclonal, biotinylated	Thermo Fisher Scientific, Rockford, MA, SAD	1:200
rabbit imunoglobulin	goat polyclonal, biotinylated	Thermo Fisher Scientific, Rockford, MA, SAD	1:200
goat imunoglobulin	goat polyclonal, DyLight594	Abcam, Cambridge, UK	1:200
rabbit imunoglobulin	goat polyclonal, $DyLight_{488}$	Thermo Fisher Scientific, Rockford, MA, SAD	1:200
mouse imunoglobulin	$AlexaFluor_{488}$ Conjugate	Cell Signaling Technology, Danvers, MA, SAD	1:200
rat imunoglobulin	goat polyclonal, biotinylated	BD Pharmingen	1:200

3.3. Methods

In this study two models of experimental brain trauma were used. The LFPI method has been used to induce a single moderate brain trauma and therefore the mice exposed to this type of injury will be referred to as the LFPI group. The WD method has been used to induce the repetitive mild TBI to a separate cohort of mice, and thus in the further text the mice that received such injury will be called the RT group of mice.

3.3.1. Single moderate traumatic brain injury model

The LFP injury model was modified for the use on mice in this research to induce the moderate brain injury (79,80). Mice of a weight between 26 and 33 g, and an age from 5.5 to 9 months were used. Isoflurane was used to induce and maintain anesthesia during the experiments, at a concentration of 3.5% for induction, and 1.2-1.5% for maintenance, in a 1:2 combination of oxygen and nitrous gas. After that, the rodents were mounted on a stereotaxic device and given eyedrops to protect them from drying out. The hair of the scalp was shaved off, the skin was cleaned with iodine and ethyl alcohol, and then a 0.5% levobupivacaine solution was subcutaneously (s.c.) administered. After a sagittal incision of the scalp, the place for the craniotomy was defined by using stereotactic measuring device. The craniotomy with a diameter of 2.5 mm was performed without harming the meninges and assuring a position above the left parietal cortex. Dental acrylic adhesive was used to position a pre-made hollow plastic Luer-Lock connector with a 2.5 mm diameter at the site of the craniotomy. Air from the Luer-Lock got expelled with fluid drops to assure a nice and reliable transmission of the pressure wave onto the CNS tissue. Once the LFP device got connected to the Luer-Lock and the animal elicits pawn reflexes, a single moderate TBI was administered. The control group of animals consisted of sham-injured animals, which were treated identically to the previously described group of traumatized animals, except that they were not subjected to experimental TBI. A total of 9 mice were used in the described part of the experiment, 5 of which were in the LFPI group and 4 in the control (Sham LFPI) group.

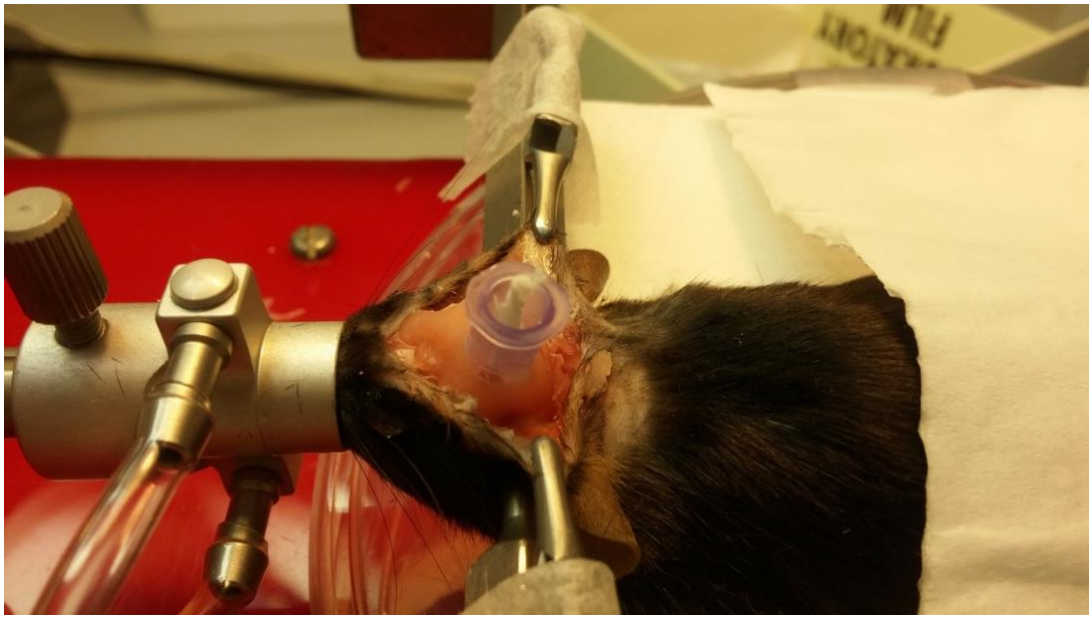

Figure 2. Anaesthetized mouse with craniotomy mounted on a stereotaxic device with an attached Luer-Lock connector covering the site of the craniotomy for the following administration of the lateral fluid percussion injury (LFPI). (The picture was taken by Tamara Janković)

3.3.2. Repetitive mild traumatic brain injury model

For the induction of the repetitive mild brain injury in mice we used a modification of the Marmarou weight drop model as described by Kane et al. (81). The mice used were 9 to 11 weeks old and weighed 22 to 25 g. They were briefly anesthetized with isoflurane and a mixture of oxygen and nitrous oxide in a ratio of 1:2, in a concentration of 3.5%. The mouse was in an anesthetized state and placed on an aluminum sheet covering a Plexiglas box when a weight from a height of 1m was dropped on the head of the mouse for repetitive mild TBI induction. As a result of being struck by a mechanical force, the animal tore through the aluminum foil, rotated 180°, and landed on the sponge located within the Plexiglas box that was described earlier. For the guidance of the 97-g steel weight, measuring 1.2 centimeters in diameter a vertical metal tube measuring one meter in length was used. Following the TBI, the animals were returned to their cages and given constant access to food and water. The experimental animals were subjected to mild repetitive brain traumas twice a day for five consecutive days, resulting in a total of ten mild repetitive traumas per animal. A control group of animals was used, which underwent a sham injury and were treated identically to the group of traumatized animals described earlier, except that they did not undergo experimental TBI. A total of 10 mice were used in the described part of the experiment, 5 of which were in the RT group and 5 in the control (Sham RT) group.

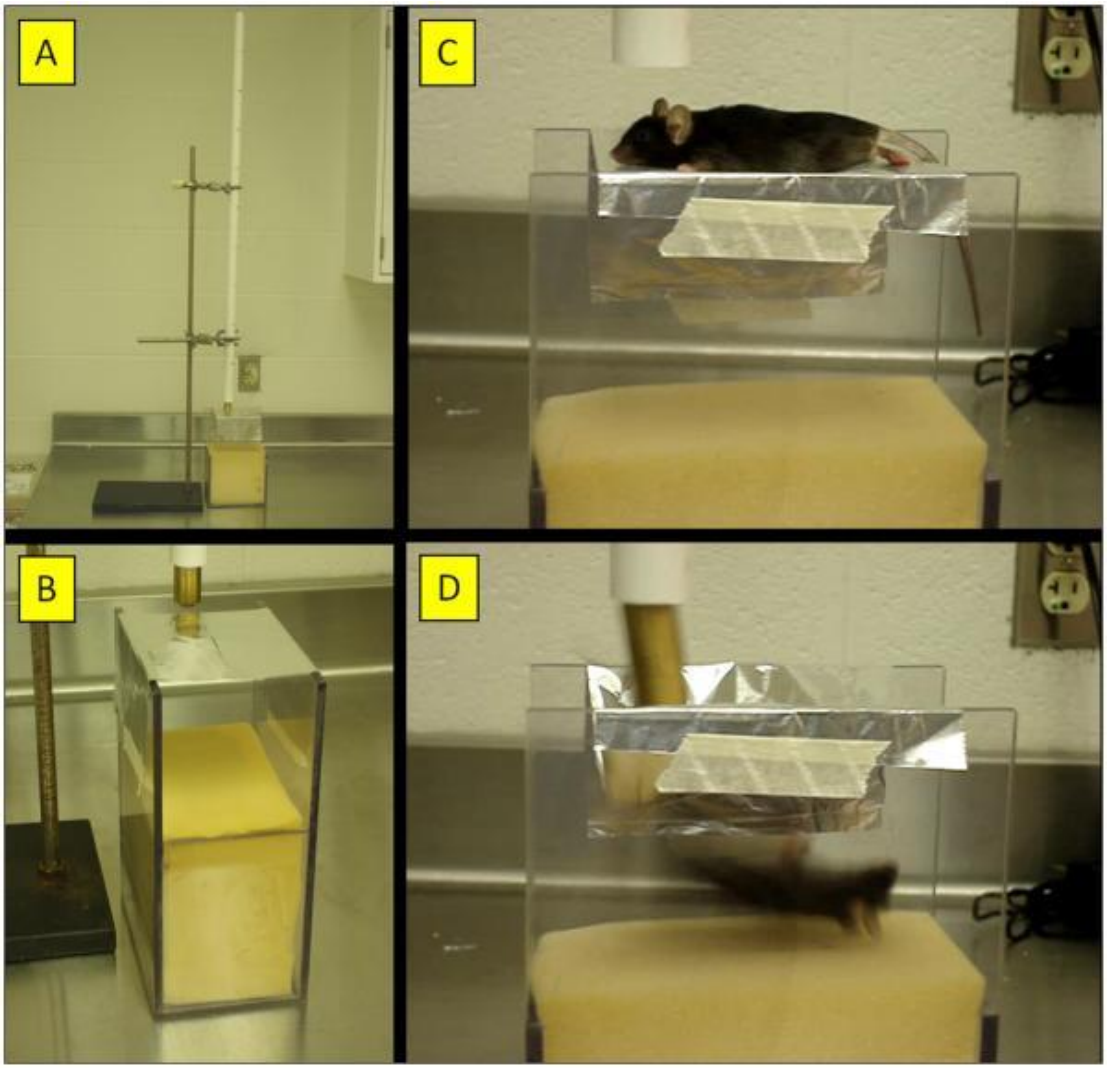

Figure 3. Illustration of experimental traumatic brain injury (TBI) in the mouse: a device for repetitive mild TBI with a weight-drop model. Device with associated Plexiglas box (A). Anesthetized mouse laid on a cut aluminum foil (B). The weight travels through the metal tube to the aluminum foil (C). The traumatized mouse turns 180° and falls through the aluminum foil onto the sponge inside the Plexiglas box (D).
From publication by Kane MJ, Angoa-Pérez M, Briggs DI, Viano DC, Kreipke CW, Kuhn DM. "A mouse model of human repetitive mild traumatic brain injury." in final edited form as: J Neurosci Methods. 2012 Jan 15; 203(1): 41–49 (81).

Experimental animals subjected to either a single moderate or repetitive mild experimental TBI were sacrificed on the fourteenth day after single brain injury or the last repetitive brain trauma, and they formed separate groups. The control group of animals, for each brain trauma type, consisted of sham-injured animals, and these mice were sacrificed one day after the sham procedure. The spinal cord tissue of sacrificed animals was further used for histological and immunohistochemical analysis.

3.3.3. Tissue preparation

Experimental animals were anesthetized with a mixture of ketamine and xylazine (100 mg/kg, 10 mg/kg, respectively) and then transcardially perfused, first with cold buffered saline, and prefixed with 4% paraformaldehyde. Fixation of the spinal cord tissue in the mentioned fixative was done overnight, and dehydration in 20% sucrose at 4 °C for 48 to 72 hours. The spinal cords were then quickly frozen in tissue freezing medium (2-methyl-butane) and stored at -80 °C until sectioning. With a cryostat, the cervical part of the spinal cords has been cut into 10 µm thick tissue sections, and afterwards dried for 3 hours in 50 °C to improve adhesion on the slides. After achieving room temperature, slides were stored at -80 °C until used for experiments.

3.3.4. Histological analysis

Detection of processes of demyelination was done with Luxol fast blue staining. Spinal cord sections reached room temperature and were then incubated at 50°C for 10 minutes. Removal of fatty excess was carried out by immersion in a mixture of alcohol and chloroform (ratio 1:1) at room temperature for 3 hours. Dyeing with Luxol fast dye was performed overnight at 50 °C. The next day, the sections were washed in 95% ethyl alcohol and distilled water, after which they were differentiated in lithium chloride for 15 seconds. They were immersed briefly in 70% ethanol and washed in distilled water. Immersion in increasing concentrations of ethanol was performed for dehydration before the sections were clarified in xylene and fixed with Entellan.

For the basic neuronal structural component identification and for the evaluation of the injury post-trauma cresyl violet staining was used. Frozen coronal sections of spinal cord tissue were brought to room temperature and then dried at 50 °C for 10 minutes. The sections were then directly immersed in a mixture of alcohol and chloroform 1:1 overnight and then rehydrated through 100% and 95% alcohol to distilled water. Sections were stained in a heated acidic 0.1% cresyl violet solution for 10 minutes, then washed in distilled water and then differentiated in 95% ethyl alcohol, dehydrated in 100% alcohol, and cleared in xylene. The sections were fixed with Entellan.

The visualization of nerve fibers has been carried out by the usage of Bielschowsky silver staining for a histological evaluation. Frozen sections of the spinal cord tissue were incubated at 50°C for 10 minutes after they have been brought to room temperature from the storage at -80°C. The sections were then immersed in a 20% silver nitrate solution and stayed

for 15 minutes at 37 °C in the incubator. After washing in distilled water, heated to 37 °C, the sections were immersed in an ammoniacal silver solution (20% silver nitrate and ammonium hydroxide). Sections were then immersed in a developing solution (20% formaldehyde, 0.5% citric acid and nitric acid with ammonium hydroxide) for 1 minute, then briefly rinsed in distilled water and immersed in a 5% sodium thiosulfate solution for 5 minutes. Furthermore, the sections were washed in distilled water and cleared in xylene. Overnight the sections dried and have been fixated with Entellan.

For each experiment, sections of at least three animals per group were used for immunohistological analyses, of which at least 3 sections of the spinal cord were analyzed for each animal.

3.3.5. Immunohistochemical protocol

For immunohistochemical analyses the chosen slides of spinal cord sections were thawed to room temperature before incubation at 50 °C for 10 minutes. Tissue permeabilization was performed with TBS-Triton X-100 (0.025%). Samples were further incubated in a buffer to block nonspecific binding sites using 5% BSA/TBS-Triton X-100 (0.025%) for 2 hours. The spinal cord slices were then incubated in the prepared primary antibodies (anti-TDP-43 1:200, anti-NeuN 1:100, anti-Iba1 1:100, anti-GFAP 1:100) diluted in the appropriate solution in 1% BSA/TBS-Triton X-100 (0.025%) at 4 °C overnight. The next day, sections were incubated in the appropriate biotinylated secondary antibody or fluorochrome-labeled secondary antibody, diluted 1:200 in the 1% BSA/TBS-Triton X-100 (0.025%) for 1 hour at room temperature. If the section was incubated with the secondary biotinylated antibody, streptavidin conjugated with the appropriate fluorochrome was used in the next step for 20 minutes at room temperature. The nuclear dye DAPI, at a concentration of 1μg/ml, was used to stain the nucleus for 10 minutes at room temperature. Spinal cord slices were embedded in Mowiol®, a fluorescent signal embedding medium.

During the research, double staining of the spinal cord was carried out by using a similar procedure as in single immunofluorescence labeling. For the double staining procedure two primary antibodies were used and applied separately on two consecutive days. If DAPI nuclear dye was used in addition to double immunofluorescence labeling, a triple immunofluorescence signal was obtained.

For each experiment, sections of at least three animals per group were used for immunofluorescent analyses, of which at least 3 sections of the spinal cord were analyzed for each animal.

4.Results

4.1. Histological evaluation of spinal cord changes following single moderate and repetitive mild traumatic brain injury

Standard histological staining with cresyl violet (Nissl) was used to visualize changes in neurons of the mice spinal cord tissue 14 days after single moderate LFPI (Figure 4) or last repetitive mild brain trauma (Figure 5), and their corresponding control groups. Significant histological changes were not detected in the cervical part of spinal cords neither after LFPI nor after RT. Representative microphotographs show neurons in the gray matter of the upper spinal cord without visible major morphological changes.

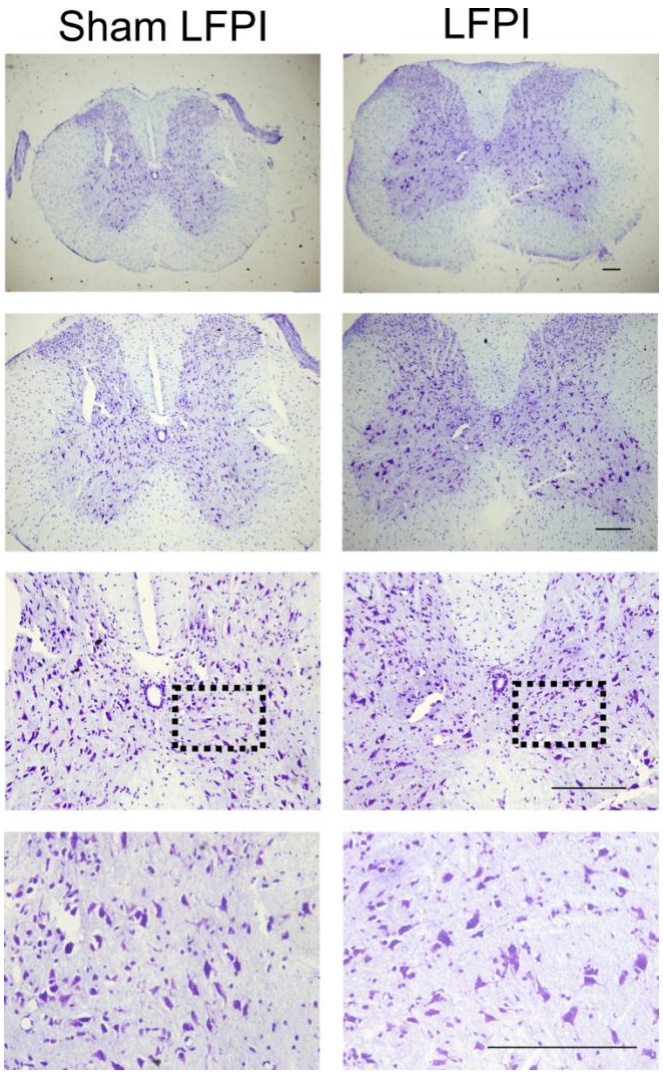

Figure 4. Pathohistological evaluation of neuronal degeneration in the cervical part of the mice spinal cord following single moderate traumatic brain injury. Representative microphotographs of the cresyl violet (Nissl) stained spinal cord sections of the animal of the control group (Sham LFPI) and mouse with lateral fluid percussion injury (LFPI), sacrificed 14 days after the brain trauma. Squares indicate regions of gray matter with abundant dark stained neurons. Scale bar = 200 μm at 4x, 10x, 20x and 40x magnification (from left to right).

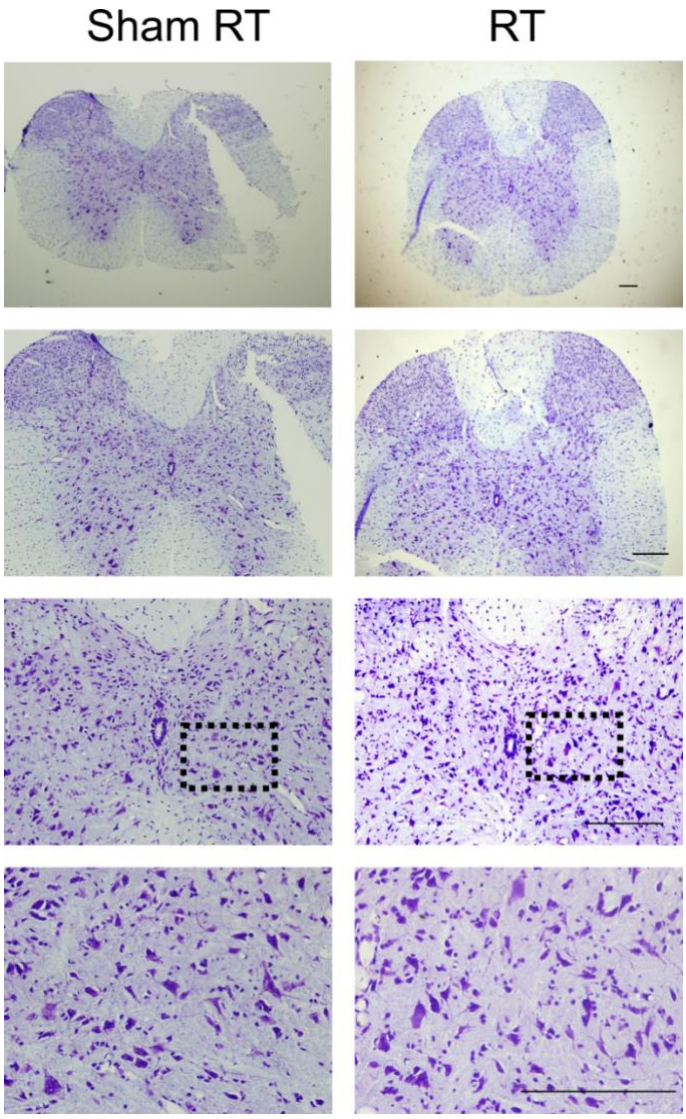

Figure 5. Pathohistological evaluation of neuronal degeneration in the cervical part of the mice spinal cord following repetitive mild traumatic brain injury (RT). Representative microphotographs of the cresyl violet (Nissl) stained spinal cord sections of the animal of the control group (Sham RT) and repetitively injured mouse (RT), sacrificed 14 days after the last brain trauma. Squares indicate regions of gray matter with abundant dark stained neurons. Scale bar = 200 μm at 4x, 10x, 20x and 40x magnification (from left to right).

To observe the integrity of myelinated neuronal fibers, Luxol fast blue staining with cresyl violet counterstaining was performed. Figure 6 shows the effect of a single moderate brain trauma on the myelination of the cervical part of the spinal cord and Figure 7 the myelination status after repetitive mild brain traumas. The Luxol staining did not reveal any changes in the white matter that could indicate a structural deformation in mice spinal cord at 14th day following single moderate LFPI or repetitive mild TBI. When compared to mice of their corresponding control group, there were no changes in structural arrangements in the spinal cords of injured animals. The microphotographs show a homogenous arrangement of the

white matter in both trauma and their corresponding control groups. Accordingly, the cresyl violet staining shows the morphological intact gray matter with no detectable damage.

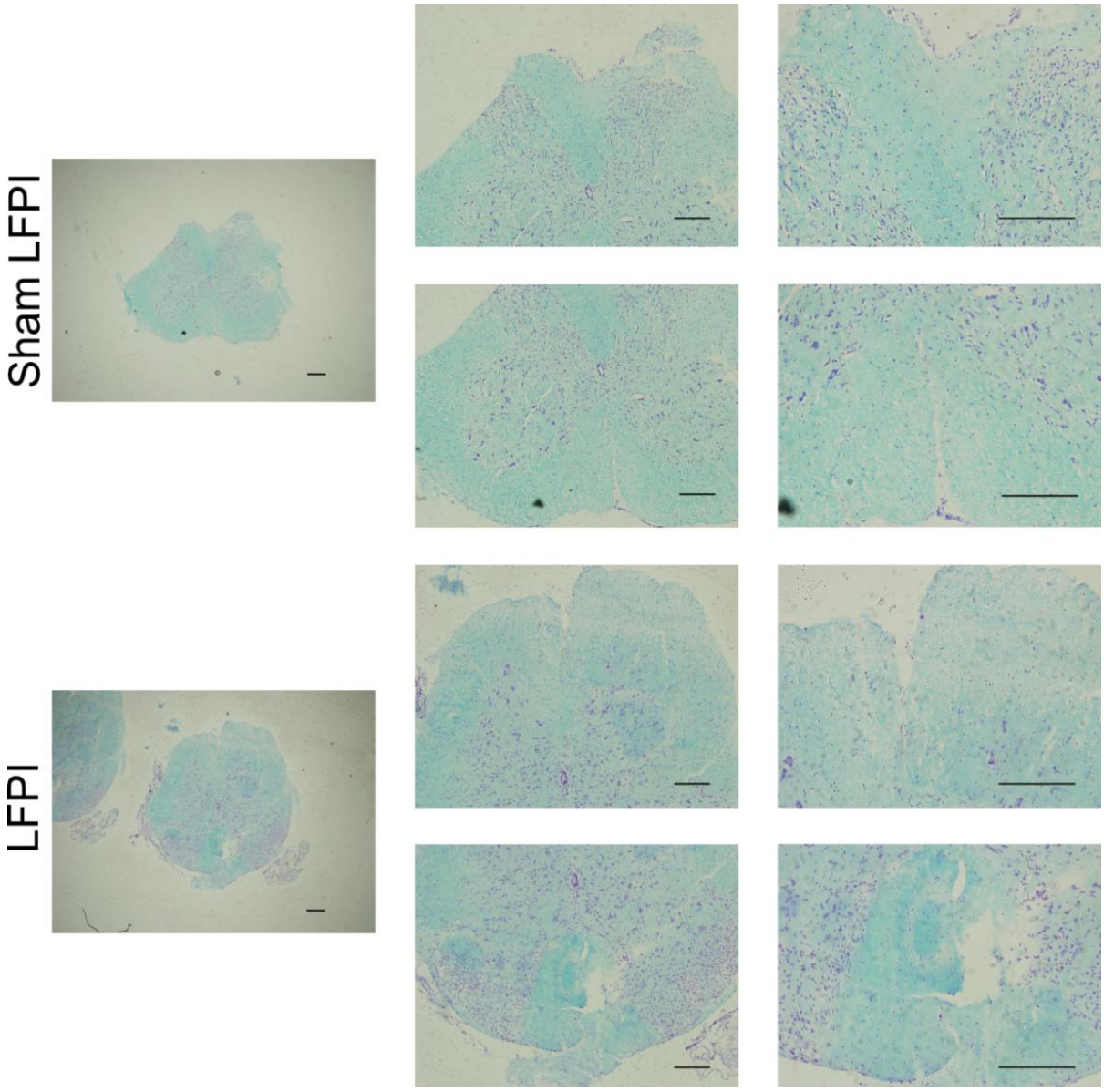

Figure 6. Integrity of myelinated neuronal fibers in the cervical part of the mice spinal cord following single moderate traumatic brain injury. Representative microphotographs of the Luxol fast blue stained histological preparations from the spinal cord of the animal of the control group (Sham LFPI) and mouse with lateral fluid percussion injury (LFPI), sacrificed 14 days after the brain trauma. Squares indicate regions of gray matter with abundant dark stained neurons. Scale line: 200 μm at 4x, 10x and 20x magnification (from left to right).

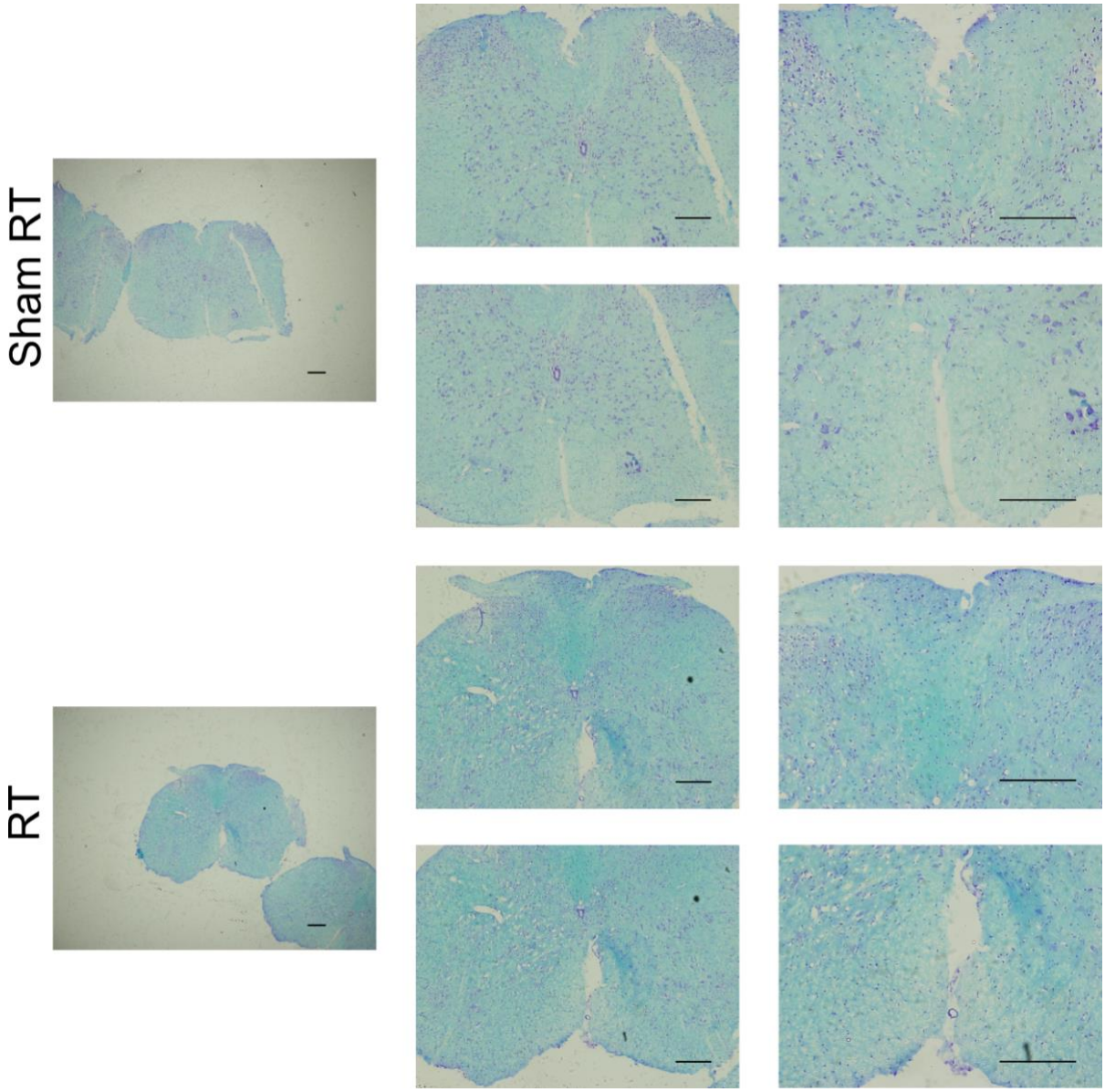

Figure 7. Integrity of myelinated neuronal fibers in the cervical part of the mice spinal cord following repetitive mild traumatic brain injury (RT). Representative microphotographs of the Luxol fast blue stained histological preparations from the spinal cord of the animal of the control group (Sham RT) and repetitively injured mouse (RT), sacrificed 14 days after the last brain trauma. Squares indicate regions of gray matter with abundant dark stained neurons. Scale line: 200 μm at 4x, 10x and 20x magnification (from left to right).

Bielschowski silver staining method was used for visualization and localization of spinal cord neuron degeneration. Figure 8 shows the result of the silver staining on the frozen sections of the spinal cord of mice sacrificed 14 days after receiving single moderate LFPI and their corresponding control groups. Microphotographs show a mild increase in the silver uptake in the mice subjected to moderate LFPI in comparison to their corresponding control group. Argentophilic structures of very different and irregular shapes are visible in the sections of both control and traumatized mice.

In the spinal cord sections of the mice subjected to repetitive mild TBI, accumulation of sliver was also mildly increased and argentophilic structures did not differ from the sections of its corresponding control group, as it is shown in Figure 9.

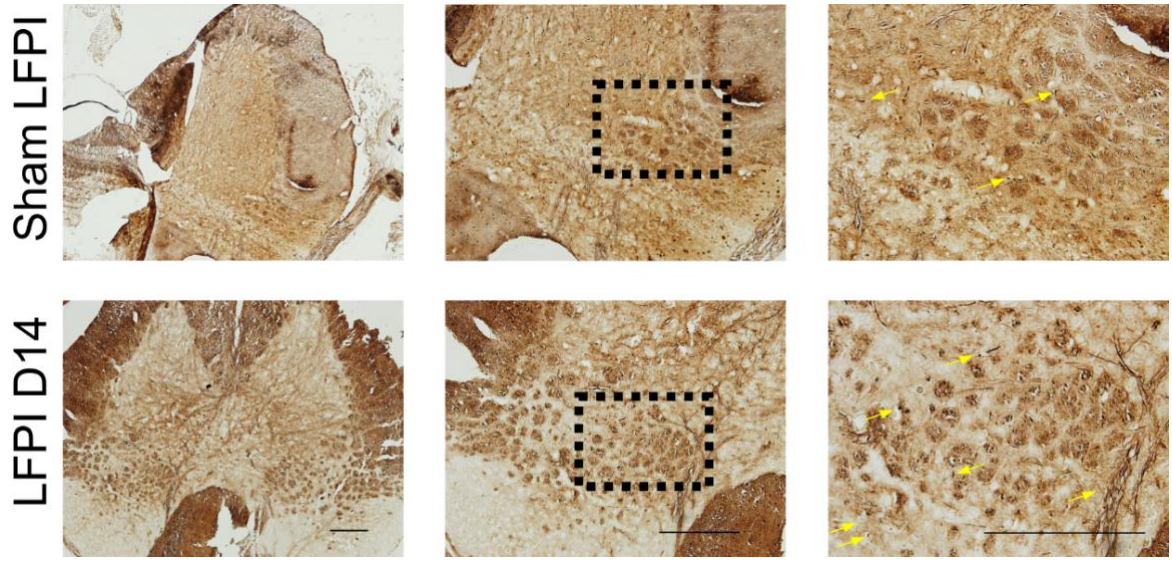

Figure 8. Neuronal degeneration in the cervical part of the mice spinal cord following single moderate traumatic brain injury. Representative microphotographs of the Bielschowski silver staining in the spinal cord sections of the animal of the control group (Sham LFPI) and mouse with lateral fluid percussion injury (LFPI), sacrificed 14 days after the brain trauma. Arrows show argyrophilic structures. Scale lines: 200 μm. Magnification 10x, 20x; magnified square (from left to right).

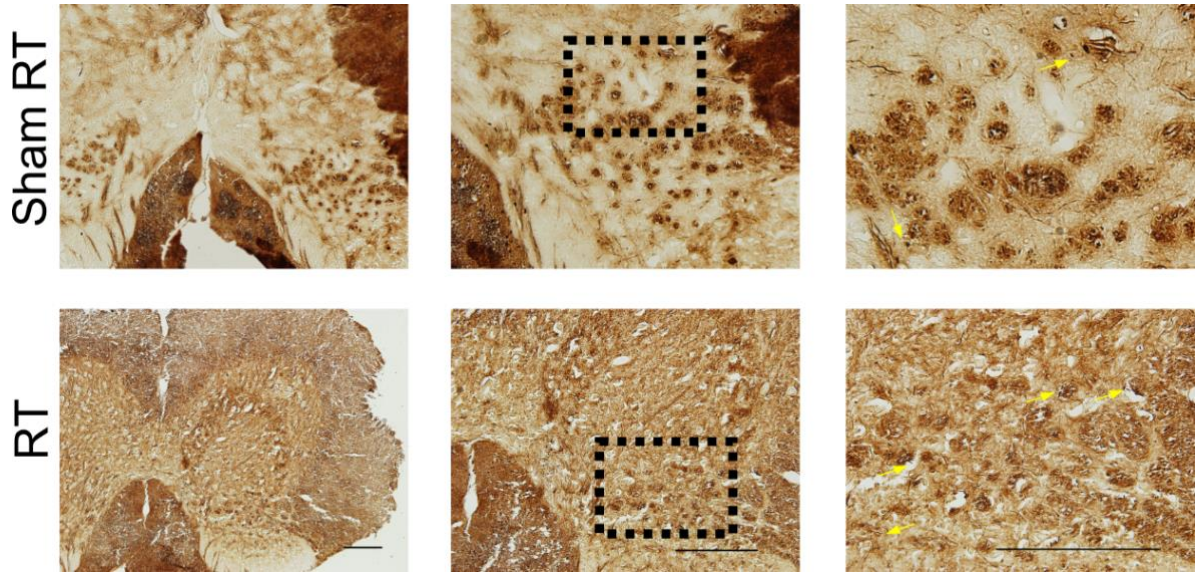

Figure 9. Neuronal degeneration in the cervical part of the mice spinal cord following repetitive mild traumatic brain injury (RT). Representative microphotographs of the Bielschowski silver staining in the spinal cord sections of the animals of the control group (Sham RT) and repetitively injured mice (RT), sacrificed 14 days after the last brain trauma. Arrows show argyrophilic structures. Scale lines: 200 μm. Magnification 10x, 20x; magnified square (from left to right).

4.2. Response of astrocytes and microglia following single moderate and repetitive mild traumatic brain injury

Further, we wanted to investigate changes in the morphology and expression of the glial cells in the spinal cord sections of the mice subjected to both types of injuries. For detection of astrocytes, antibody against GFAP was used to stain the astrocytic cytoskeleton. To detect the changes in morphology and the activation state of microglia, samples were stained with an antibody against Iba1. DAPI staining was used to visualize the nuclei of cells to locate their cell body.

GFAP expression at 14 days after single moderate LFPI is shown in Figure 10. Astrocytes were visible in both experimental groups, particularly around the spinal canal and ventral median fissure (VMF), but there were no differences in the GFAP immunostaining between the traumatized and animals of the corresponding control group.

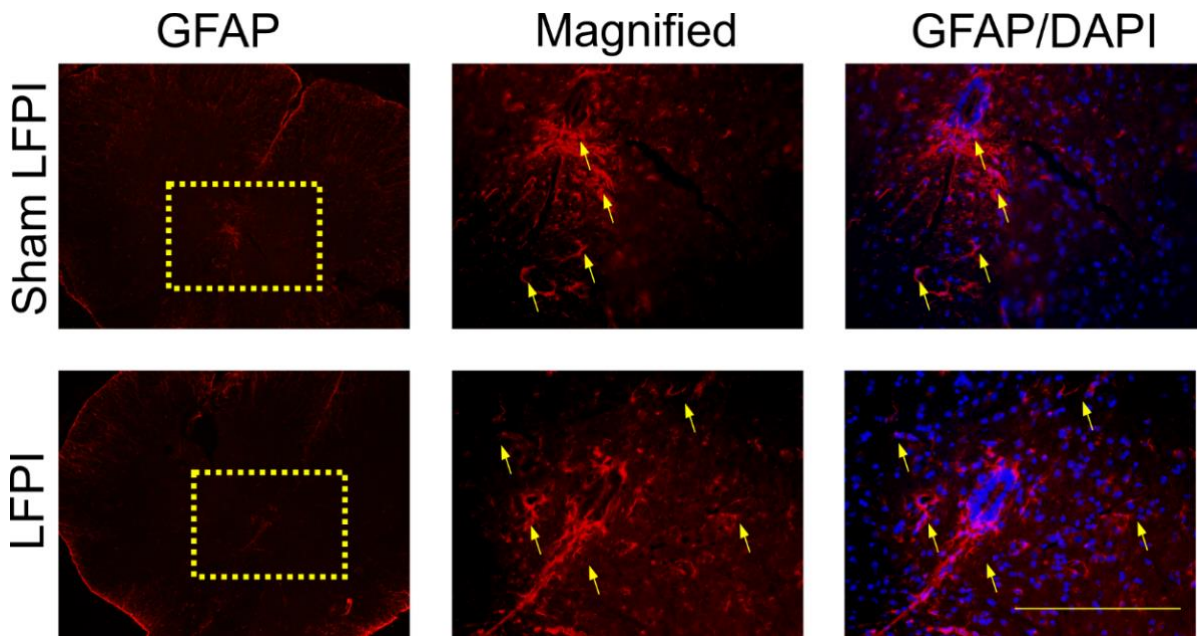

Figure 10. Activation of astrocytes in the cervical part of the mice spinal cord following single moderate traumatic brain injury. Representative microphotographs of the spinal cord sections immunostained with antibody against glial acidic fibrillary protein (GFAP) (red) and counterstained with nuclear dye (DAPI) (blue) in the animal of the control group (Sham LFPI) and mouse sacrificed 14 days after the lateral fluid percussion injury (LFPI). Arrows indicate GFAP positive cells (activated astrocytes). Scale line: 100 μm.

Activation of astrocytes after repetitive mild TBI is shown in Figure 11. Changes in GFAP immunoreactivity are visible in the injured mice in comparison to their corresponding control group. Hypertrophic astrocytes were detected around and above the spinal canal of the injured animals. Further, additional differences between the traumatized and control mice were

detected in the outer segments of the spinal cord as an increased GFAP immunostaining, indicating the presence of astrocytic activation.

Overall, reaction of astrocytes was more extensive after repetitive mild traumas than after single moderate TBI at 14th day postinjury.

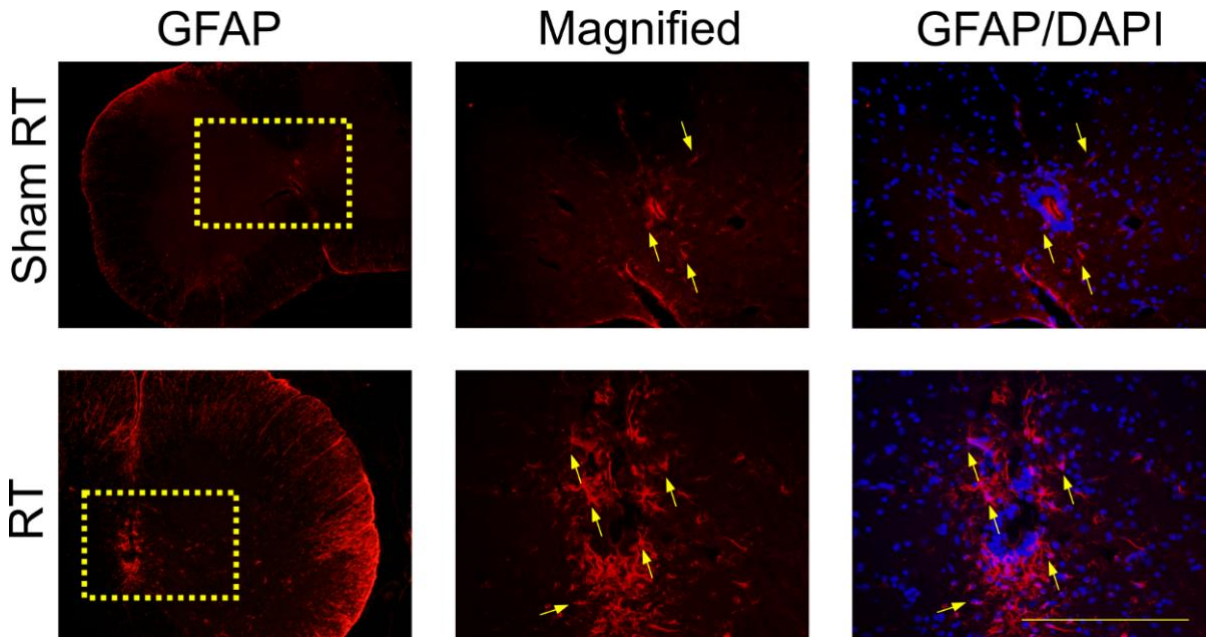

Figure 11. Activation of astrocytes in the cervical part of the mice spinal cord following repetitive mild traumatic brain injury (RT). Representative microphotographs of the spinal cord sections immunostained with antibody against glial acidic fibrillary protein (GFAP) (red) and counterstained with nuclear dye (DAPI) (blue) in the animal of the control group (Sham RT) and mouse sacrificed 14 days after the last RT. Arrows indicate GFAP positive cells (activated astrocytes). Scale line: 100 μm.

14 days after a single moderate LFPI, individual Iba1 positive cells were detected only below the central canal of the spinal cord and around the VMF. Their presentation did not differ from the cells detected in the corresponding control group (Figure 12).

Mice with induced repetitive mild TBI showed a significant activation of microglia compared to their corresponding control group. Figure 13 demonstrates ramified microglial cells that were detected below the central canal of the spinal cord and around the VMF. Also, activated microglia with visible ramifications extending from the cell body were present above the spinal canal, and around gracile nucleus, as shown in Figure 14.

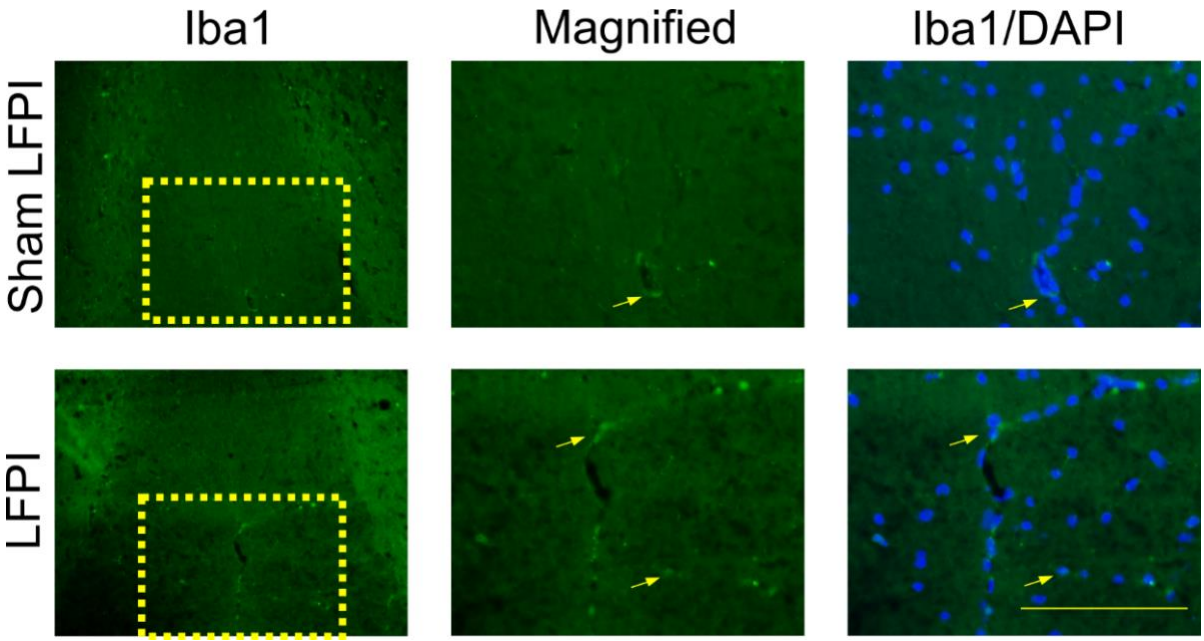

Figure 12. Activation of microglia in the cervical part of the mice spinal cord following single moderate traumatic brain injury. Representative microphotographs of the spinal cord sections immunostained with antibody against the ionized calcium-binding adapter molecule 1 (Iba1) (green) and counterstained with nuclear dye (DAPI) (blue) in the animal of the control group (Sham LFPI) and mouse sacrificed 14 days after the lateral fluid percussion injury (LFPI). Arrows indicate Iba1 positive cells observed in the white matter, below the spinal canal and around the ventral median fissure. Scale lines: 100 μm.

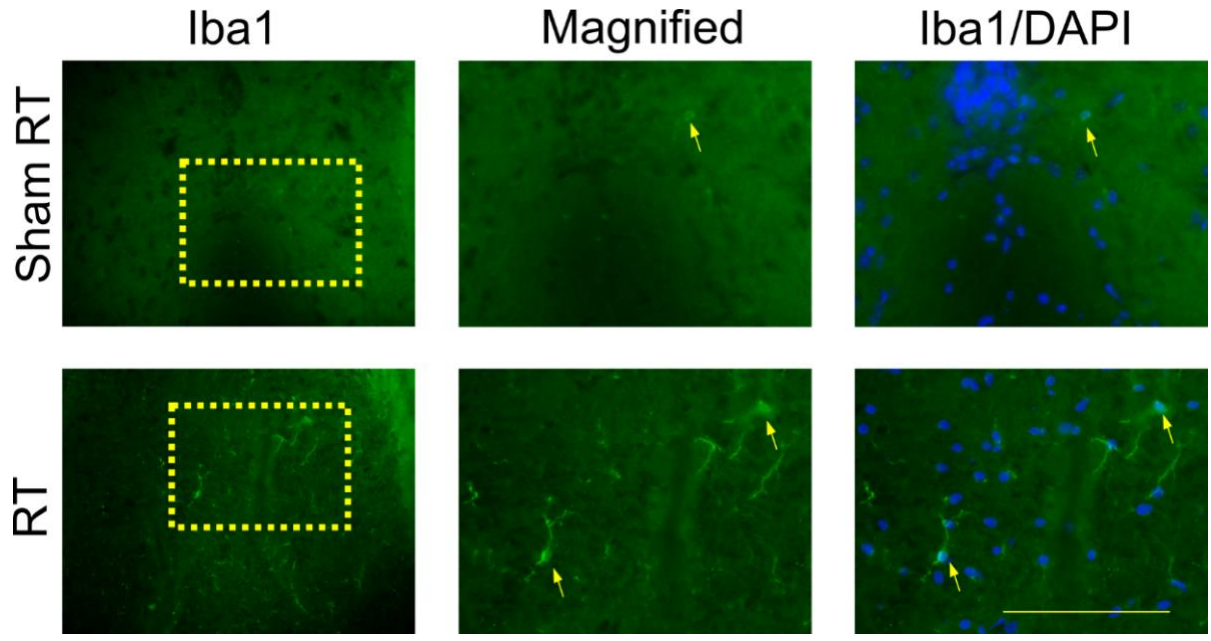

Figure 13. Activation of microglia in the cervical part of the mice spinal cord following repetitive mild traumatic brain injury (RT). Representative microphotographs of the spinal cord sections immunostained with antibody against the ionized calcium-binding adapter molecule 1 (Iba1) (green) and counterstained with nuclear dye (DAPI) (blue) in the animal of the control group (Sham RT) and mouse sacrificed 14 days after the last RT. Arrows indicate Iba1 positive cells observed in white matter, below the spinal canal around the gracile nucleus and especially around the ventral median fissure. Scale lines: 100 μm.

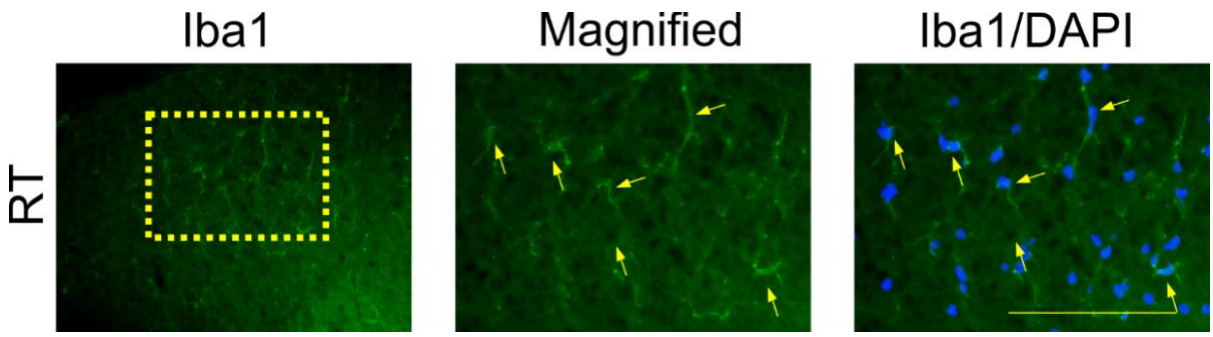

Figure 14. Microglial activation in the area of gracile nucleus of the spinal cord following repetitive mild traumatic brain injury (RT). Representative microphotographs of the spinal cord sections labeled with antibody against ionized calcium-binding adapter molecule 1 (Iba1) (green) and counterstained with nuclear dye (DAPI) (blue) in the mouse sacrificed 14 days after the last RT. Arrows indicate Iba1 positive cells. Scale line: 100 μm.

4.3. Expression and subcellular localization of TDP-43 in the mice spinal cord following single moderate and repetitive mild traumatic brain injury

The expression and intracellular localization of TDP-43 were examined using immunofluorescent staining in the cervical spinal cord sections of animals sacrificed 14 days after the single moderate TBI or last repetitive mild TBI, and their corresponding control groups.

Mislocalization of TDP-43 from the cell nuclei was detected in the mice subjected to single moderate LFPI but did not differ significantly from the mice of the corresponding control group (Figure 15).

Pronounced mislocalization of TDP-43 was evident in the mice that received repetitive mild TBI. The cytosolic TDP-43 expanding into dendritic extensions and axons was observed, with a more intense expression in the traumatized than in the control animals (Figure 16).

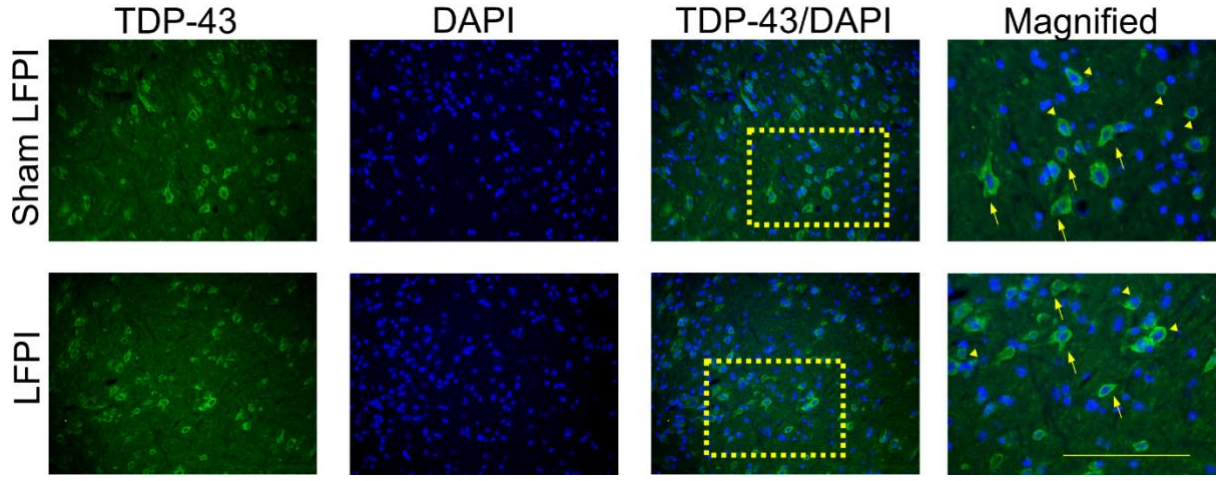

Figure 15. Expression and subcellular localization of TAR DNA-binding protein 43 (TDP-43) in the cervical part of the mice spinal cord following single moderate traumatic brain injury. Representative microphotographs of the spinal cord sections immunostained with antibody against the TDP-43 (green) and counterstained with nuclear dye (DAPI) (blue) in the animal of the control group (Sham LFPI) and mouse sacrificed 14 days after the single moderate brain trauma (LFPI). Scale lines: 100 μm.

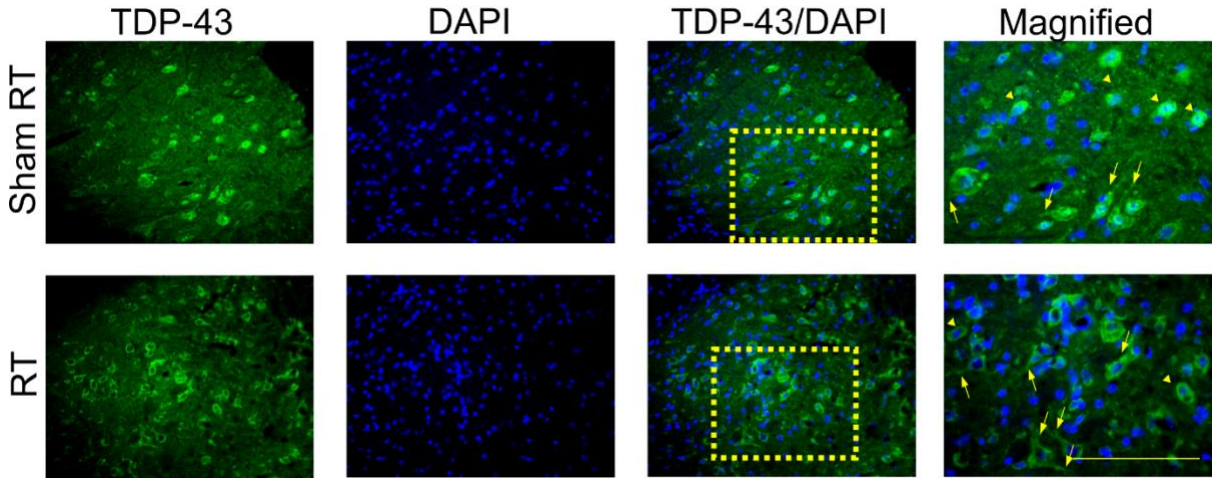

Figure 16. Expression and subcellular localization of TAR DNA-binding protein 43 (TDP-43) in the cervical part of the mice spinal cord following repetitive mild traumatic brain injury (RT). Representative microphotographs of the spinal cord sections immunostained with antibody against the TDP-43 (green) and counterstained with nuclear dye (DAPI) (blue) in the animal of the control group (Sham RT) and mouse sacrificed 14 days after the last RT. Scale lines: 100 μm.

Furthermore, we wanted to examine the expression and mislocalization of TDP-43 in certain types of spinal cord cells i.e., astrocytes and neurons. Double immunofluorescence staining was performed, with a nuclear dye DAPI for nucleus staining, which enabled the detection of the triple fluorescent signal. Astrocytes were labeled with anti-GFAP antibody (GFAP + TDP-43 + DAPI) and neurons with NeuN antibody (NeuN + TDP-43 + DAPI).

TDP-43 signal was detected in the nucleus of the astrocytes present around the central canal of the spinal cord after both types of injuries and in the control animals. However, mislocalization of TDP-43 from the astrocytic nuclei was not detected neither after the single moderate (Figure 17) nor after the repetitive mild (Figure 18) TBI, or their corresponding control groups.

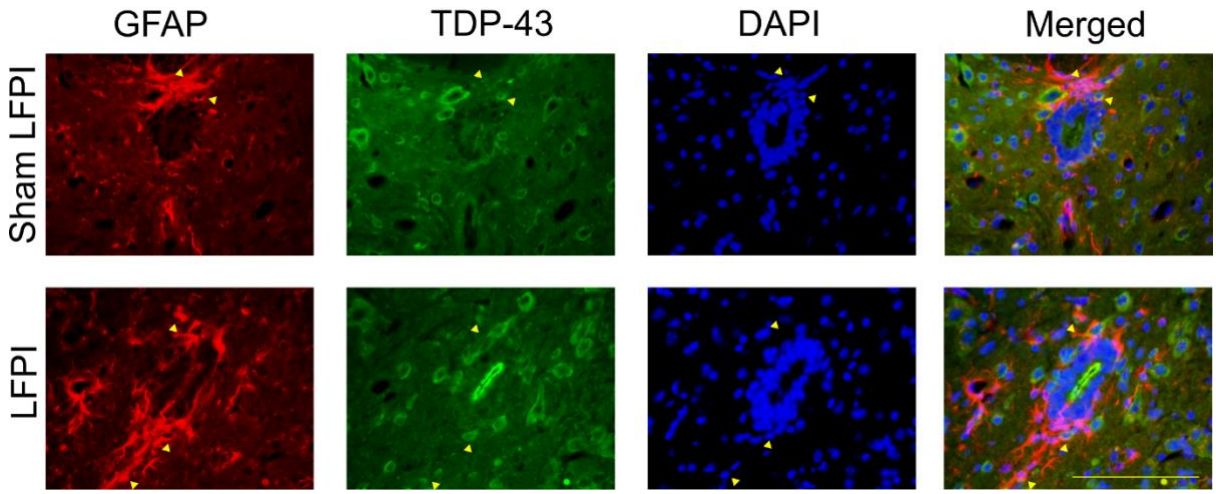

Figure 17. Cellular localization of TAR DNA-binding protein 43 (TDP-43) in astrocytes of the mice spinal cord following single moderate traumatic brain injury. Representative microphotographs of spinal cord sections immunostained with antibodies against the TDP-43 (green) and glial acidic fibrillary protein (GFAP) (red) and stained with nuclear dye DAPI (blue), in animal of the control group (Sham LFPI) and mouse sacrificed 14 days after the single moderate brain trauma (LFPI). Arrowheads indicate astrocytes with nuclear TDP-43 distribution. Scale line: 100 μm.

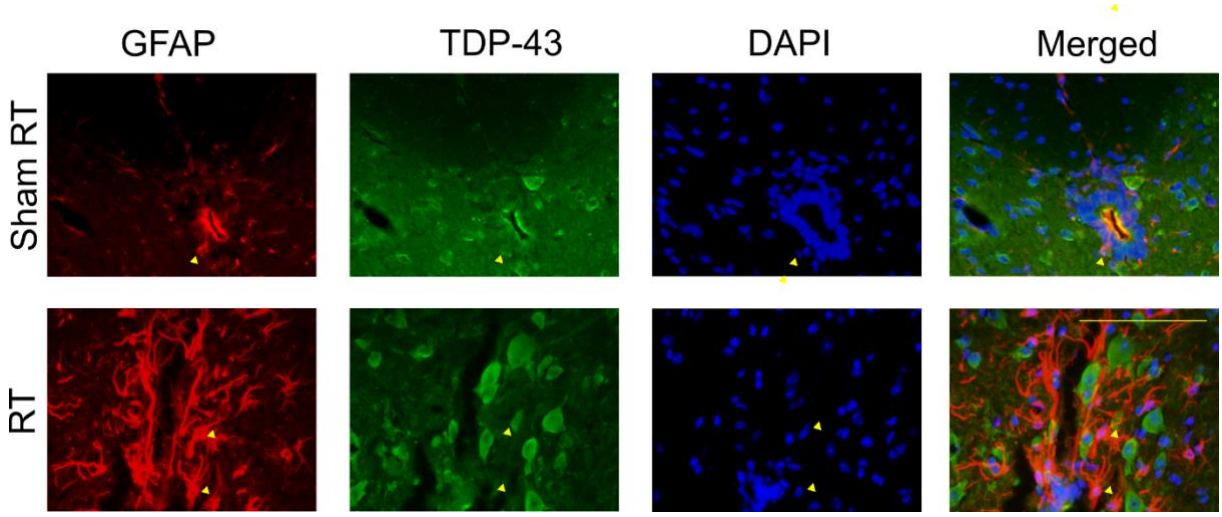

Figure 18. Cellular localization of TAR DNA-binding protein 43 (TDP-43) in astrocytes of the mice spinal cord following repetitive mild traumatic brain injury (RT). Representative microphotographs of spinal cord sections immunostained with antibodies against the TDP-43 (green) and glial acidic fibrillary protein (GFAP) (red) and stained with nuclear dye DAPI (blue), in animal of the control group (Sham LFPI) and mouse sacrificed 14 days after the last RT. Arrowheads indicate astrocytes with nuclear TDP-43 distribution. Scale line: 100 μm.

However, TDP-43 mislocalization from the nucleus to cytoplasm was detected in the neurons of the spinal cord sections from mice with a single moderate LFPI and the mice of the corresponding control group (Figure 19).

Mislocalization of TDP-43 from nucleus to cytoplasm of dendrites and axons of the spinal cord neurons was also visible in the sections of mice that received repetitive mild TBI and mice of corresponding control group (Figure 20).

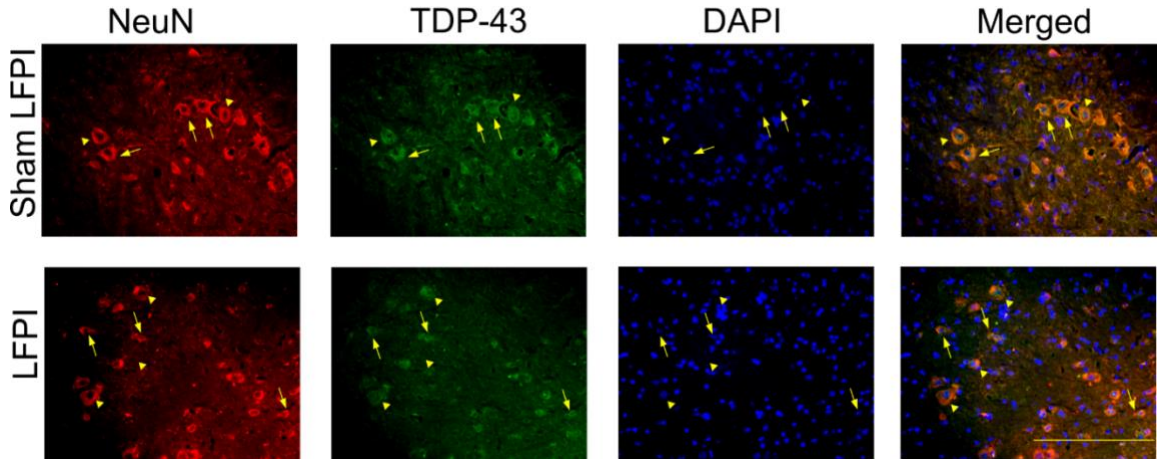

Figure 19. Cellular localization of TAR DNA-binding protein 43 (TDP-43) in the neurons of the mice spinal cord following single moderate traumatic brain injury. Representative microphotographs of spinal cord sections immunostained with antibodies against the TDP-43 (green) and ionized calcium-binding adapter molecule 1 (Iba1) (red) and stained with nuclear dye DAPI (blue), in animal of the control group (Sham LFPI) and mouse sacrificed 14 days after the single moderate brain trauma (LFPI). Arrowheads indicate neurons with predominantly nuclear, and arrows neurons with predominantly cytoplasmic TDP-43 staining. Scale lines: 100 μm.

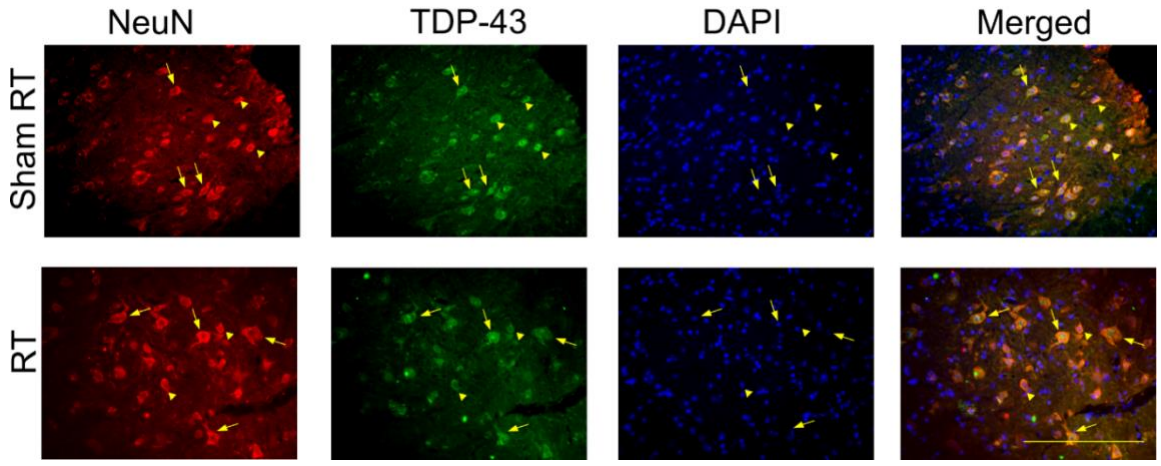

Figure 20. Cellular localization of TAR DNA-binding protein 43 (TDP-43) in the neurons of the mice spinal cord following repetitive mild traumatic brain injury (RT). Representative microphotographs of spinal cord sections immunostained with antibodies against the TDP-43 (green) and ionized calcium-binding adapter molecule 1 (Iba1) (red) and stained with nuclear dye DAPI (blue), in animal of the control group (Sham LFPI) and mouse sacrificed 14 days after the last RT. Arrowheads indicate neurons with predominantly nuclear, and arrows neurons with predominantly cytoplasmic TDP-43 staining. Scale lines: 100 μm.

5. Discussion

In this graduation thesis, the effects of a single moderate and repetitive mild brain trauma on the development of pathological changes in the mice cervical part of the spinal cord were studied. LFPI model was used for induction of the single moderate TBI, as it is considered as a highly reproducible model (79) that combines both focal and diffuse type of injury and reproduces injuries seen after falls and road accidents (82). Repetitive mild brain traumas are often seen in athletes involved in contact sports, military personnel, and victims of domestic violence (10,13,83), so this type of injury was induced with a WD model described with Kane et al (81). Kane's model can be considered as clinically relevant because after the induction of trauma, it allows a free movement of the mouse head and body which mimics an injury that includes a concussion.

Previous studies suggest that the deficit in motor skills after TBI propagates from the trauma injury site (84). For this reason, a subacute, 14-day time point was chosen to allow time for injury development in the distal regions of the CNS. Also, previous studies have shown subacute subcellular changes in some regions distant from the trauma site i.e., hippocampus in glial response and TDP-43 expression (85), and thalamus and cerebellum in neurodegenerative changes, cell death, glial activation, and synaptic changes (86,87).

The outcomes of this study present results that indicate detectable effects on distant structures to the initial head injury following both repetitive mild and single moderate TBI. TDP-43 mislocalization in neurons and activation of glial cells in the cervical part of the spinal cord 14 days after the last trauma was detected and will be discussed.

5.1. The effect of a single moderate and repetitive mild traumatic brain injury on the spinal cord damage

Histological evaluation did not reveal any detectable changes in the morphological presentation of the mice spinal cord after single moderate and repetitive TBI. Alterations in the physical appearance and organization of cells and tissue were not visible. Loss of neurons and connective tissue was not detected. According to our knowledge, there are no results in the scientific literature about changes in the spinal cord after a single moderate LFPI. However, from the original protocol of Carbonell et al (79). we can see that the changes in the distal regions from the injury site such as the dorsal hippocampus are not observed on the 14^{th} day after trauma. Also, mild trauma induced with the protocol of Kane et al (81). does not cause changes in cellular integrity even in the place of the applied trauma, so such results were

expected in the repetitive mild TBI group of mice. The physical impact of the initial head injury, 14 days after both single moderate and repetitive mild brain trauma was not significant enough to stimulate changes in morphology like hyperplastic arrangements of inflammatory cells, presentations of hypertrophic cells, or areas of atrophy.

However, significant accumulation of silver into spinal cord neurons, indicating the presence of neurodegenerative processes, was observed in the traumatized mice after both single moderate and repetitive mild type of brain trauma, with more evident increase after a single LFPI, which has not yet been investigated in the mentioned models of experimental TBI.

5.2. Response of astrocytes and microglia in the spinal cord after single moderate and repetitive mild traumatic brain injury

Reactive astrocytosis after single moderate type of brain trauma was evident around the spinal canal, but it did not differ from the findings in the spinal cords of mice from the control group. Also, only several Iba1 positive cells were observed in the white matter of the spinal cord, around the VMF, in both traumatized and corresponding sham operated mice.

Astrocytic hypertrophy was detected in the group receiving repetitive brain traumas. Also, increase in the presence of microglia was detected around the VMF and the area of gracile nucleus. Astrocytic and microglial changes in the corresponding control group were not detected. It has been previously observed that inflammatory changes may have a significant role in the dissemination of the repetitive injuries through the CNS (25).

5. 3. Single moderate and particularly repetitive mild traumatic brain injury induces TDP-43 cellular mislocalization

It's important to note that the absence of these distinct morphological changes in histology does not rule out the presence of TDP-43 proteinopathy. The results of this study show that the traumatic incident of a single TBI of moderate intensity is already capable of triggering similar neurodegenerative dynamics. Significant TDP-43 mislocalization from the nucleus to the cytoplasm, including dendrites and axons has been detected in mice subjected to LFPI. However, similar TDP-43 localization changes are visible in their corresponding control group of mice. More prominent TDP-43 mislocalization was detected in the mice of the repetitive mild injury group, compared to the mice of their matching control group. Slightly less severe presentation of cytoplasmic TDP-43 in neuronal cells after single moderate TBI, when compared with the results of repetitive mild TBI, is based only on the qualitative examination

of the microphotographs from immunohistochemical labeled slices and does not include quantitative results. The abnormal accumulation of TDP-43 protein may still occur in a more diffuse or soluble form, which can be challenging to detect using traditional histological methods. In such cases, additional techniques, such as biochemical or molecular analysis, may be required to identify TDP-43 pathology. The fact that TDP-43 protrusions into axonal and dendritic extensions of neurons after a single trauma were found, indicate that disseminating pathology is present and it strengthens the idea of using TDP-43 as a biomarker for neurodegenerative disorders.

TDP-43 proteinopathy may be characterized by the presence of cytoplasmic inclusions or aggregates in affected neurons and glial cells. In our study, mislocalization of TDP-43 was not detected in astrocytes of any of types of examined brain traumas. Despite that, TDP-43 was detected in the cytoplasm of neurons after both single moderate and repetitive mild TBI. To our knowledge such findings regarding TBI have not been published.

Repetitive mild TBI is especially associated with a much worse outcome and symptomatic presentation in cases of ALS. Repetitive mild TBI contributes to the loss of motor function and exacerbates the increase in pathological presentation in the peripheral nervous system (88). The effect of a single moderate TBI on the periphery and distant sites of the CNS is less described in the literature.

Previous studies describe that there is a change in the formation and behavior of TDP-43 after traumatic incidents. A dissemination of the pathology to remote areas is known to occur and is linked to the development of hypopituitarism or ongoing neurodegeneration, as described in CTE (25,55). The absence of morphological changes does not imply that the tissue in remote areas is not undergoing pathological changes in posttraumatic phases. There is evidence that cytoplasmic mislocalization of phosphorylated TDP-43 is present in motor neurons of living ALS patients, which is detectable already in early diagnostic stages. This establishes an interest in detecting TDP-43 abnormalities as it could participate in diagnostic manners as a fluid biomarker for ALS pathology (89). It is crucial to emphasize here that the diagnosis of TDP-43 proteinopathy is not solely based on histology but also includes clinical symptoms, neuroimaging findings, and other supportive evidence. Furthermore, ongoing research aims to develop more sensitive and specific diagnostic markers for TDP-43 proteinopathy to improve accuracy and early detection of these conditions.

The undebatable fact that TDP-43 aggregation plays a crucial role in neurodegenerative processes increases the interest for future research to target possible therapeutic approaches. The current treatment for TDP-43 proteinopathies concentrates on a multidisciplinary approach

and focuses on supportive care and alleviation of associated symptoms of ALS, FTLD, or other TDP-43 proteinopathy-linked conditions. This may include medications, physical and occupational therapies (90). There are also therapies targeting speech and swallowing for patients suffering from the outcome of TDP-43 proteinopathies, several assistive devices such as braces, walkers, wheelchairs, communication aids, and adaptive equipment can be utilized to improve mobility, communication, and daily functioning (91). In case the respiratory muscles are affected in ALS patients the implementation of respiratory support into the management of the disease is crucial. Non-invasive ventilation or similar support may assist breathing and therefore improves the quality of life (92). The mechanism of the disseminating events of proteinopathy after a head trauma must be investigated more thoroughly to uncover new targeting for possible treatments. Theoretically, the avoidance of dissemination of TDP-43 aggregates to distant sites should decrease the pathology and loss of neurons that is linked to known neurodegenerative changes.

6. Conclusion

To our knowledge this study is one of the first that compares the influence of a single moderate and repetitive mild TBI at remote sites from the impact, such as the spinal cord. We were able to rule out morphological changes following both types of the applied trauma. Nevertheless, this study shows that there are some discrete neurodegenerative changes in the tissue of the spinal cord following both types of brain trauma. Also, activation of astrocytes and microglia was detected in the spinal cord fallowing repetitive mild brain traumas. The presence of abundant TDP-43 mislocalization in neurons of the spinal cord after both moderate and mild TBIs, is very significant finding of this study, as it proves the dissemination of TDP-43 into axonal and dendritic protrusions of neuronal cells. This implicates that the further research should concentrate on a more thorough understanding of the aggregate formation and their spreading mechanism, as this could implement new therapeutic approaches to a set of neurodegenerative diseases and injuries with neurodegeneration as a secondary pathology, that currently depend mainly on supportive care manners.

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8. Summary

A traumatic brain injury (TBI) occurs when the skull is subjected to mechanical force, resulting in long-lasting alterations to the central nervous system (CNS). TBI often leads to neurodegenerative consequences, such as proteinopathies, including TAR DNA binding protein 43 (TDP-43), and activation of the glial cells. Recent research indicated that TBI induces changes in CNS regions further from the initial site of injury, such as hippocampus, thalamus and cerebellum. This study aimed to examine the pathophysiological alterations, including those related to TDP-43 proteinopathy, in the spinal cord of mice, 14 days following a single moderate TBI or last repetitive mild TBIs. The lateral fluid percussion injury method has been used to induce a single moderate brain trauma and weight-drop method has been used to induce the repetitive mild TBIs. Sham injured animals were used as a control group. Histological evaluation of the mice spinal cord sections did not reveal morphological changes following neither single moderate nor repetitive brain injuries. Glial response and cytoplasmatic mislocalization of TDP-43 were present after both single moderate and repetitive TBIs. Subtle pathological changes following different types of TBIs, detected at the remote sites from the impact, increase the interest for more thorough understanding of secondary injury triggering factors, like TDP-43 pathology. It seems promising that new therapeutic approaches for primary neurodegenerative conditions, like amyotrophic lateral sclerosis or Alzheimer's disease, but also injuries with neurodegeneration as a secondary event, like TBI, can be established by targeting cellular processes that contribute to the pathological processes of TDP-43.

Keywords: glia, mouse, neurodegeneration, spinal cord, TDP-43 proteinopathy, traumatic brain injury

Curriculum Vitae

Marc Jaeger is a medical student and was born in Hamburg, Germany in 1994. The year following his high-school graduation at the "Gymnasium Hochrad" in 2012, he worked at a dockyard for wooden boatbuilding and enrolled into studies of wood economy at the University of Hamburg. In 2015 he moved to Cologne to continue with a B.Sc. in Biology at the University of Cologne before he decided to enroll into the first generation of medical studies in English at the medical faculty of Rijeka in Croatia in 2017.

Throughout his studies he has done several internships at various institutions including the Dermatology and Molecular Biology Laboratory of the DERMATOLOGIKUM HAMBURG. Especially during his medical studies, he has done multidisciplinary internships at departments for surgery, internal medicine, and anesthesiology in Switzerland (Spital Thusis, Luzerner Kantonsspital, Spital Grabs, SGO Oberengadin).

Early on he showed great interest in sports and is a licensed Tennis coach. As a member of the 1st men's division in tennis sports of the Hamburger Polo Club and as the leader of the 2nd men's division he played tennis competitively on a semiprofessional level. Since his medical studies Marc Jaeger has represented the University of Rijeka and the Faculty of Medicine at national championships in tennis sports and he participated regularly in track, swimming and shows great interest in sailing sports.

After his graduation of medical studies Marc Jaeger will start his residency at the surgical department of the Spital Oberengadin in Samedan, Switzerland.