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Chronic histopathological and behavioral changes after experimental traumatic brain injury

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1. Introduction

1.1 Traumatic Brain Injury

1.1.1 Definition

Traumatic brain injury (TBI) is defined as a non-degenerative lesion to the skull and intracranial structures (brain parenchyma, meninges, cerebral vessels) caused by direct or indirect external force [1].

1.1.2 Epidemiology and Etiology

TBI is a major public health problem in developed as well as developing country as it is a major cause for death and disability, especially in children and younger (<45 years) patients. Incidence data varies considerably between countries and studies as there is no unambiguous definition of TBI and as mild cases of TBI that are not treated in a hospital often go unreported [2, 3]. In 2011, the incidence of TBI in Germany was to be 332/100,000; in most cases 90.9%), TBI was considered mild, moderate (3.9%), or severe cases (5.2%) were much less frequent [4]. Recently published data [5] (Figure 1) record a mean incidence of 287.2 / 100,000 people per year in Europe, with enormous differences between countries (from 50 to 650/ 100,000) that are more likely to reflect differences in design of the study rather than true variation [3]. In a comprehensive review in 2016 that investigated fatal TBI cases and cases requiring hospital treatment in all European countries, rough incidence of TBI in Europe ranged from 47.3 per 100,000, to 694 per 100,000 population per year in country-wide and from 83.3 per 100,000, to 849 per 100,000 population per year in regional-level studies. Mortality resulting from traumatic brain injury ranged from 9 to 28.1 per 100,000 population per year (country-level), and from 3.3 to 24.4 per 100,000 population per year in regional-level studies. In another systematic review published in 2006, incidence rate of TBI in Europe

was approximately 235 per 100,000, mortality rate 15 per 100,000 population. This study included all TBI cases and found that in hospitalized patients the ration of mild, moderate, and severe TBI was approximately 22:1.5:1 [6]. In the United States incidence of TBI is estimated around 180-220/100,000, resulting in approximately 600,000 TBI cases per year, with 10% of these cases being fatal injuries [7].

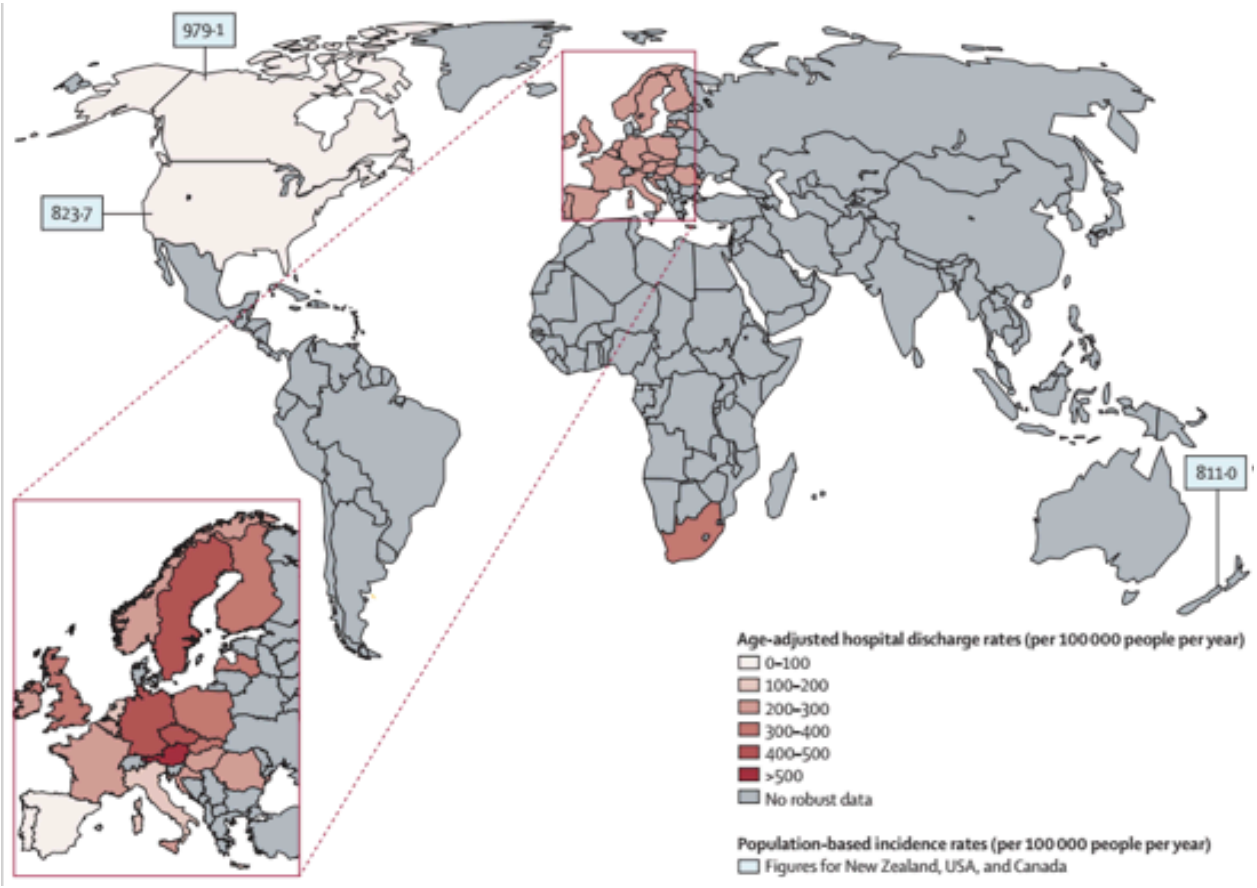


Figure 1: Worldwide incidence of traumatic brain injury [5]

In China (population 1.3 billion), there is no reliable nationwide data on the incidence of TBI. Report from the 1980s and one study investigating civilian inpatients of Chinese Military Hospitals from 2001-2007 state an incidence of 55.4 – 64.1 per 100,000 per year; These numbers are much lower than the estimates for other countries which probably reflects incomplete case ascertainment [8-11]. The World Health

Organization (WHO) estimates that the incidence of (mild) TBI may even be significantly higher than previously described in the literature [3, 12], because of different case definitions and methods of data collection. Furthermore, as motorization will probably increase, especially in developing countries, overall TBI incidence and mortality worldwide is predicted to significantly increase in the next decade [13].The most common mechanisms of injury of TBI are traffic accidents and falls [3]. The etiology is age-dependent: in childhood and in elder patient, TBI is most often caused by falls while traffic and motor vehicle accidents account for the majority of TBI in early to mid-adulthood [14]. Most of TBI cases were caused by traffic accidents [15, 16], while falls account for the major part of the TBIs in Europe, most notably in elderly patients [2, 3, 15, 17]. Young men (15-24 years) are the high-risk population for TBI [18, 19] due to the traffic accidents [15, 16]. Alcohol is associated with the leading causes of TBI [15]. Over time, the contribution of traffic accidents to total TBI events is declining in industrialized countries due to preventive measures (safety belts, helmets for cyclists [20] and motor-bikers) [15]; at the same time the percentage of aging patients in the same regions will probably increase the frequency of fall-related TBI. In developing countries, however, motorization will sharply increase in the near future, therefore the number of traffic accidents, and, thus, traffic-accident-related TBI, may also increase in these countries.

1.1.3 Classification of Traumatic Brain Injury

Traumatic brain injury can be categorized by severity, injury mechanism, and leading pathophysiololgy. For clinical practice, the classification of severity based on the Glasgow Coma Scale (GCS) is widely used as an assessment tool in emergency care. The GCS, first describe by Teasdale and Jeannett in 1974 [21] is a clinical score used to describe the general level of consciousness of TBI patients. GCS evaluates of three tasks/ functions:

eye opening (E, maximum of 4 points), verbal response (V, max. 5 points), and motor (M, maximum of 6 points) (Figure 2), maximum score is 15 points in healthy patients, minimum score of 3 points (deep coma) [21].

	Eye	Verbal	Motor
1	Does not open eyes	Makes no sounds	Makes no movements
2	Opens eyes in response to painful stimuli	Incomprehensible sounds	Extension to painful stimuli
3	Opens eyes in response to voice	Utters incoherent words	Abnormal flexion to painful stimuli
4	Opens eyes spontaneously	Confused, disoriented	Flexion / Withdrawal to painful stimuli
5	N/A	Oriented, converses normally	Localizes to painful stimuli
6	N/A	N/A	Obeys commands

Figure 2: Glasgow Coma Scale [21]

When initial GCS score after TBI is 13-15 head injury is classified as “mild”; moderate head injury is defined by a GCS score of 9-12. When consciousness is severely impaired after TBI, i. e. the GCS score is 8 or less, brain injury has to be considered severe [21]. GCS is not valid in intubated patients, intubation and sedation also may alter motor and eye response. Also, GCS is used when the doctor first sees the patient after the injury, before intubation. This classification allows for a rough estimate of prognosis and outcome after TBI: In mild traumatic brain injury there is no or only a short-lasting of disturbance of consciousness (<30 minutes), and usually there is no structural damage visible in imaging; even though the majority of patients are asymptomatic or suffer only

from transient headache and amnesia, they may experience persistent headaches as well as long-lasting neurocognitive disorders [22-24]. Moderate brain injury is defined as a brain injury resulting in a loss of consciousness from 20 minutes to 6 hours [25] and severe brain injury is defined as a brain injury resulting in a loss of consciousness of greater than 6 hours [25]. Imaging usually demonstrates structural brain damage such as contusions, hemorrhage (epidural, subdural, subarachnoidal, and intraparenchymatous), edema, or ischemia [26, 27]. These may be accompanied by epilepsy, hydrocephalus and pituitary dysfunction [28-30]. The mortality rate for TBI patients with an initial posttraumatic GCS of 3 was 78.4%; initial GCS of 4, 55.9%; and initial GCS of 5, 40.2% [31]. If GCS is higher than 8 points, the mortality is only 15%, and approximately 80% of patients have a good neurological outcome [32]. However, while GCS scoring is routinely and widely used after TBI, the definitions mild/moderate/severe are not consistently applied in everyday clinical practice, therefore this categorization is of limited use [27, 33]. Another way to classify TBI is based on the mechanism of head injury. TBI can be described as direct or indirect, dull or penetrating, acceleration or deceleration injury [27, 34]. Direct dull, and indirect acceleration / deceleration head injury are more likely to result in diffuse tissue injury, e. g. the so-called diffuse axonal injury (DAI) [35, 36], direct penetrating injury to vascular injuries [34]. The literature also describes the so called infringement mechanism after missile and blast injuries which is mainly important in the military field [35, 37]; brain damage is the result of several factors: the explosion resulting in overpressure which leads to the formation of brain edema, fragments causing penetrating injuries to skull and the brain tissue, and heat development resulting in burns [38]. There are also image-based or morphological classifications of TBI such as the Marshall score which was first described in 1992 and later modified, it remains one of most commonly used systems for grading acute TBI on the basis of computed tomography (CT) findings [39]. It classifies TBI according to lesions found in computed tomography scans [40, 41] and mainly focusses on two features: the degree of brain

edema / swelling, as determined by midline shift and / or compression of basal cisterns, and the presence and size of contusions / hemorrhages referred to "high or mixed density lesions" [40]. The Marshall Score distinguished six categories, diffuse injury I (no visible intracranial pathology), diffuse injury II (midline shift of 0 to 5 mm, basal cisterns remain visible, no high or mixed density lesions >25 cm^3), diffuse injury III (swelling, midline shift of 0 to 5 mm, basal cisterns compressed or completely effaced, no high or mixed density lesions >25 cm^3), diffuse injury IV (shift, midline shift > 5mm, no high or mixed density lesions >25 cm^3), evacuated mass lesion V (any lesion evacuated surgically), and non-evacuated mass lesion VI (high or mixed density lesions >25 cm^3, not surgically evacuated) [40]. Marshall CT classification was related with the mortality and the frequency of elevated intracranial pressure (ICP) [39]. Rotterdam scoring system was developed recently [41], which specifically described the status of the basilar cisterns and the degree of midline shift, along with presence of intraventricular hemorrhage and subarachnoid hemorrhage [42-45]. This scoring system was used to compare types of mass lesions, recognize the more favorable prognosis associated with epidural hematomas [43, 46, 47]. The study of Liesemer et al showed that the survival of children with TBI was better than adults in Rotterdam CT score categories representing less severe injuries, but higher score categories meant worse survival in children [48].

1.1.4 Pathophysiology of Traumatic Brain Injury

1.1.4.1 Primary Brain Damage

1.1.4.1.1 Contusions

The primary brain damage occurs at the moment of direct, external violence on skull and brain tissue [49], its extents depends on the physical characteristics of the impact. It may

be focal (e.g. skull fractures, intracranial hematomas, lacerations, contusions, penetrating wounds) or diffuse (as in DAI [50]). The most common direct injuries are frontal/temporal contusions, epidural/subdural hematomas, traumatic subarachnoid hemorrhages, intracerebral / intraventricular hemorrhage[49, 51, 52]. The most common indirect injury observed is DAI [49, 51, 53].

Brain contusion is a bruising of brain tissue caused by TBI (Figure 3) [54] and formed in two ways: direct trauma and acceleration / deceleration injury. The incidence of contusion in severe TBI was around 20-30% [55]. This type of injury may lead to a decline in psychological function in the long term and may lead to cerebral herniation in an emergency situation, which is a life-threatening condition associated with brain edema [55]. Therefore, the purpose of the treatment is to prevent the ICP hypertention, and surgical resection was operated on that time. Except this typical contusion, multifocal hemorrhagic contusion is the other kind of contusion which is associated with broken capillaries occurring in grey matter under the cortex [56]. Most cases are caused by shearing injuries at the impact time and the outcome is poor if the patient is coma, or not [56].

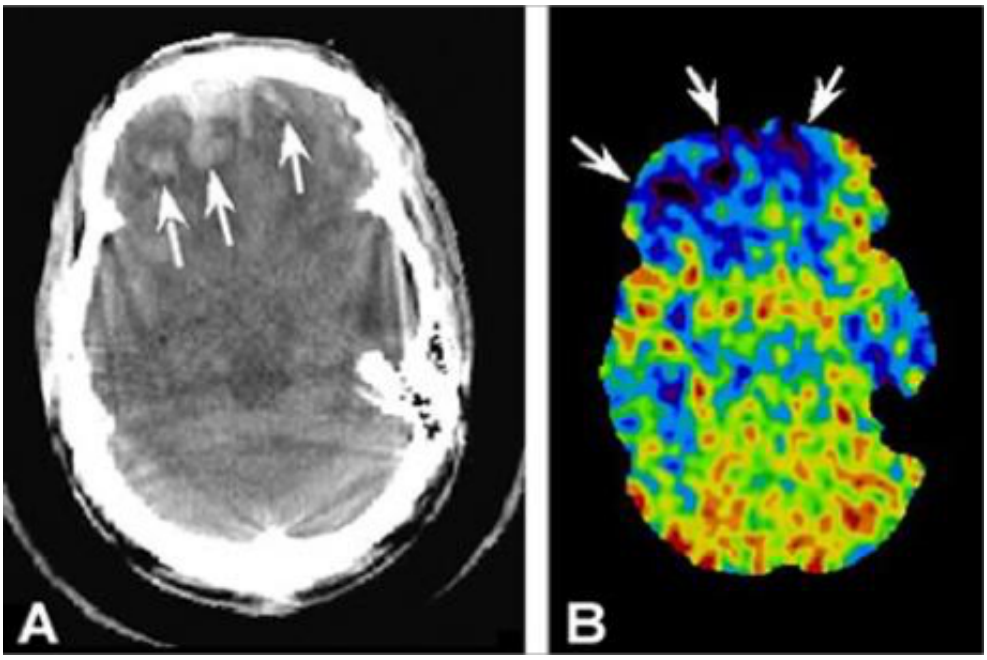


Figure 3: Comparison of a CT scan with a xenon blood-flow radionuclide scan. A. CT scan shows bifrontal contusions following severe head trauma (arrows). B. Companion CT scan showing xenon uptake demonstrates dark regions (arrows), indicating decreased perfusion in the contused brain [57].

1.1.4.1.2 Hemorrhage

Cerebral hemorrhage is a common TBI complication [58]. Its incidence is approximately 13 to 35 % in TBI patients [46, 59] and the increase of hemorrhagical volume happens in around 38 to 59 % [60-62]. Cerebral hemorrhage is divided into various types, such as delayed traumatic intracerebral hematoma [63], progressive hemorrhage [64, 65], traumatic intracerebral hemorrhage [66], and so on. Most of delayed traumatic intracerebral hematoma is related to traumatic intracranial aneurysm [67, 68]. Epidural hematoma, subdural hematoma, intraparenchymal contusion or hematoma, or subarachnoid hemorrhage result in the hemorrhagic progression [64]. One model of focal cortical contusion showd that progressive secondary hemorrhage was caused by

contusion injury [69]. Coronal brain sections in this study showed that the hemorrhagic lesions were between the cortex and the corpus callosum in the ipsilateral hemisphere on the early stage after TBI [69], however, hemorrhagic lesions on the surface views of the brain expanded to all directions, especially medially and laterally and even depper from 3h to 8 h after TBI (Figure 4) [69]. It was the same results as that in a controlled cortical impact model [70].

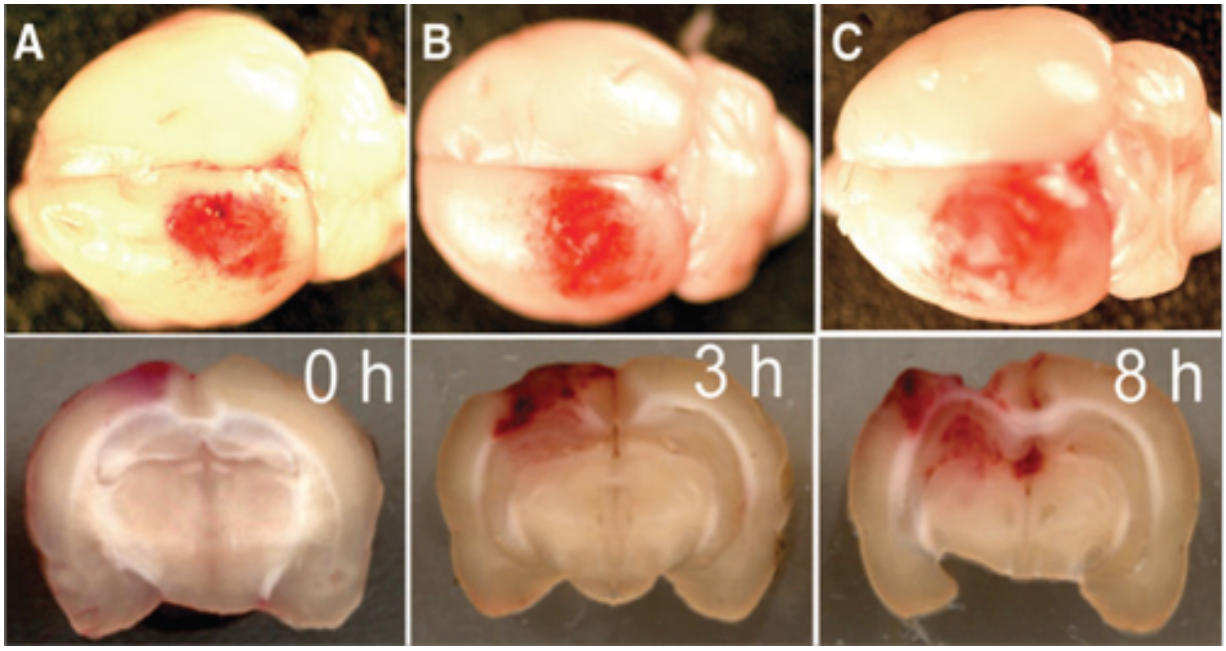


Figure 4: Contusion injury results in progressive hemorrhage of a brain contusion in a rat impact model. (A–C) Surface views (above), and coronal views of the same brains (below), from rats euthanized immediately after, 3 h after, or 8 h after contusion injury, as indicated [69].

1.1.4.1.3 Diffuse Axonal Injury

Diffuse axonal injury (DAI) was found in almost every severe TBI patient [71]. 56% were found with DAI in moderate TBI patients [50]. In DAI, stretching injury of brain parenchyma leads to an interruption of axonal transport, especially in the corpus callosum and in fiber tracts connecting the cerebellum and cerebellum [36, 53, 72, 73]. Ultimately, these processes can lead to the death of the axons. Histopathologic findings in patients with diffuse axonal injury can be graded as follows : Grade 1 - Axonal injury present mainly in parasagittal white matter of the cerebral hemispheres, Grade 2 - Grade 1 plus lesions in the corpus callosum, Grade 3 - Grade 2 plus focal lesions in the cerebral peduncle [53].

1.1.4.2 Acute Secondary Brain Injury

Secondary brain injury is tissue damage that results from a series of pathophysiologic changes that are triggered by the original trauma and the primary damage [74]. In contrast to the primary lesion whose extent is dependent on the force of the impact only and that can only be prevented, not treated, acute secondary brain damage develops in a delayed fashion over a period of several days. It is therefore amenable for treatment intervention several factors/ mechanisms that contribute to secondary brain damage will be explained in detail in the following paragraphs.

1.1.4.2.1 Biochemical Factors involved in Acute Secondary Brain Damage

Tissue injury sustained in the context of TBI leads to a variety of secondary cellular mechanisms that in turn trigger neuroinflammation and eventually lead to cell death. Among other things brain injury leads to an increase of excitatory amino acids (EAAs)

[75], such as gluatamate toxicity [75], endogenous opioid peptides (e. g. dynorphin-like immunoreactive material) [76, 77], extracellular potassium [78], cytokines [79-81], and intracellular magnesium [82-84]. Other factors that cause the development of cytotoxic brain edema are lactic acidosis due to anaerobic metabolic processes [85-87] and the calcium influx caused by oxygen radicals and oxidative stress which was produced by EAAs [88]. Otherwise, a rise in extracellular potassium leads to the formation of pro-inflammatory cytokines and further neuroinflammation [78]. In addition to the above factors, there are a variety of other pathomechanisms that contribute to the development of secondary brain damage after TBI. Thus, it could be shown that the kallikrein-kinin-system is activated after TBI and it increases the permeability of neurovascular and the formation of oxygen radicals which results in the post-traumatic brain edema [88-93]. Another factor that influences secondary necrosis growth is disturbed water homeostasis via aquaporins. Several studies show that increased expression of aquaporin-4 (AQP-4) occurs after TBI and AQP-4 plays a causative role in the development of brain edema via increasing the swelling of astrocytes [94-96]. Furthermore, impairment of mitochondrial function also plays a role in the development of secondary brain damage [88, 97]. Thus, it has been shown that apoptosis-inducing factor (AIF), a mitochondrial, pro-apoptotic molecule, enters the nucleus and leads to caspase-independent apoptosis induction [97]. Inhibition of AIF leads to a reduction of cell death [97]. Finally, there is an increased release of nitric oxide (NO) immediately after TBI [98-103]. However, this increase is only very brief, followed by a prolonged inhibition of endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) as well as an increase in inducible nitric oxide synthase (iNOS) [98-101, 104]. The overall result is an NO deficiency, which can be therapeutically influenced by inhalative application of NO, selectively dilates arterioles in the traumatic penumbra, and significantly reduces the secondary damage [105, 106]. Furthermore, the increase of iNOS seems to induce the formation of secondary damage, however, the inhibition of

iNOS reduces secondary necrosis growth [107]. Possible mechanisms of this NO-related damage include an induction of oxidative stress via the formation of peroxynitrite, a failure of energy production by inhibition of glycolytic or mitochondrial enzymes, and DNA damage or inhibition of DNA replication [108]. The other very important pathophysiological aspect of the secondary brain damage is triggered by inflammatory processes after TBI. It first comes minutes after TBI by exposing negatively charged surfaces, such as e.g. basal membranes and release of intracellular molecules, such as DNA, RNA and ATP, to activate the contact system [89]. This consists of the coagulation factors XI and XII, pre-kallikrein and the non-enzymatic cofactor kininogen, and leads the release of bradykinin enzymes from kininogen after activation [109]. In turn, bradykinin causes the increased release of NO, leads to vasodilation, the increase of vascular permeability and thus causes the formation of brain edema finally [90, 91, 93, 110]. Otherwise, vasopressin receptors play a role in the brain edema formation and the subsequent development of secondary brain damage after TBI [111] and the inhibition of vasopressin receptors could reduce brain edema formation after TBI. The immigration of leukocytes comes from hours to days after TBI, although it is still not clear whether these have direct influence on the development of secondary brain damage or not. For example, the adhesion molecule intracellular adhesion molecule-1 (ICAM-1) was knocked out in mice, and a marked reduction of brain edema in the experimental traumatic brain injury can be detected while the accumulation of leukocyte was not affected, which was a minor role of leukocyte immigration in the acute phase of secondary brain damage [112, 113]. While the role of cellular immune response in the acute phase of secondary brain damage was minor importance, and it seems that it could be even more important in the chronic phase of brain damage. So the activation of microglia and immigration of monocytes could take days to months and even up to 18-years after TBI [114, 115]. Overall, the acute secondary brain damage after TBI is mainly influenced by the activation of the contact system, while the cellular Immune

response mainly seems to be involved in the development of chronic pathologies of brain damage [89, 113, 116, 117].

1.1.4.2.2 Cerebral Edema and Increased Intracranial Pressure

Cerebral edema is a determining factor in the development of secondary brain injury. Historically, two types of edema are distinguished: vasogenic and cytotoxic edema [88, 118, 119]. Both types of edema develop at different time points after trauma, they also may occur simultaneously [120]. Vasogenic edema is most prominent in the first few hours after TBI: early after trauma there is an acute vasodilation that leads to pooling of blood within the skull ("vascular engorgement") [121], there is progressive vessel wall in the cerebral microcirculation facilitating leakage of fluid into the interstitial space later and vasogenic edema primarily affects the white matter [122]. Cytotoxic cerebral edema is due to cellular swelling [123, 124]. It is thought to be the determining factor for traumatic brain damage [124-126], and it starts to develop within minutes and may persist for up to two weeks [127]. As described above, cell swelling may be due to inflammatory processes, release of toxic factors, and oxidative damage. The damage of the blood brain barrier (BBB) was also caused by activation of the Kinin-Kallikrein-System, vasopressin or Metallo-Matrix-Proteins (MMP) may facilitate cytotoxic edema because of BBB leakage [128, 129]. Cytotoxic edema affects predominantly the gray matter [122]. Subsequently to the development of posttraumatic brain edema, the intracranial volume gradually increases, and the severity of brain edema formation is dependent on the severity of trauma. As the brain is housed within a rigid container (skull), it can only tolerate small increases in volume within the intracranial compartment before intracranial pressure rises dramatically (Monro-Kellie doctrine [130, 131]). The intracranial volume (V i/c) consists of the following components:

V i/c = V (brain) + V (cerebrospinal fluid) + V (blood)

In the adult, the intracranial volume is about 1500 mL, the brain accounting for 85-90%, intravascular cerebral blood volume accounting for 10%, and cerebrospinal fluid (CSF) accounting for the remainder (< 3 %). When compensation strategies (expulsion of venous blood and CSF) exhausted ICP will increase in an almost linear fashion. This intracranial hypertension may lead to herniation [132] which may be life threatening if ICP increases massively in a short time [49, 133]. But also a gradual or moderate increase in ICP may have disastrous consequences as it may affect cerebral perfusion. Cerebral blood flow (CBF) is dependent on the cerebral perfusion pressure (CPP) which is defined as the difference between the mean arterial pressure (MAP) and intracranial pressure.

CPP = MAP – ICP

Relationship between cerebral perfusion pressure, mean arterial pressure and intracranial pressure [133].

If the ICP increased due to the cytotoxic edema, it critically restricted cerebral circulation especially the area around the primary damage continues. The penumbra concept describes tissue areas located around the primary lesion that do not like the primary lesion is irreversibly damaged but reversibly, and offered promise to rescue the brain tissue with the appropriate therapies [134], and it is more vulnerable to ischemic brain tissue than the intact brain tissue [135, 136]. Due to the blood circulation around 50% of the normal value which means the lack of oxygen and the tissue hypoxia, it causes mainly anaerobic metabolic processes, in turn, such as anaerobic glycolysis in the penumbra [137, 138]. The energy demand increases due to the immigration and activation of inflammatory cells [139]. An improvement in ischemia tissue can prevent irreversible injury and thus reduce the secondary brain damage [140]. However, it will be

irreversible necrosis in the primary injury and traumatic penumbra when the decrease of the cerebral blood circulation below the critical threshold 10 ml / 100 g*min^{-1} [141-143]. Depending on the cell death, it comes to leakage of the BBB and subsequently to the influx of molecules activated into the interstitial, e.g. intracellular proteins, and it is called vasogenic edema [88, 144, 145]. Ultimately, the temporal sequence of the post-traumatic brain edema underlying pathophysiological processes is not fully informed and remains the basis of controversial discussions [120, 146, 147].

1.1.4.2.3 Hydrocephalus

The syndrome of hydrocephalus following head injury was first recognized by Dandy and Blackfan in 1914 [148]. HC is common after TBI as high as 51% [149-152], it can present either as obstructive and non-obstructive hydrocephalus [153]. The non-obstructive type usually caused by the blood or blood degradation products impeding the flow of the CSF in the subarachnoid space and the absorption of CSF through the arachnoid villi is more frequent after TBI [154]. In obstructive hydrocephalus there is a mechanical blocking of CSF passage-ways, mainly by blood clots [153]. The characteristic symptoms of hydrocephalus consist of dementia, motor dysfunction, urinary incontinence, cognitive dysfunction, and so on [155, 156]. Hydrocephalus results in increased ICP, with transependymal exudation of CSF through the ependyma into the brain tissue [157]. Due to the ICP increased, cerebral herniation maybe occur in the acute phase, and this may manifest itself on CT or Magnetic Resonance Imaging (MRI) as ventricle increased [157]. Interstitial edema can be relieved by ventricular drainage [157].

1.1.4.2.4 Clinical Management

So far, therapy of traumatic brain injury is mainly symptomatic. In the early stages of TBI, the aim of clinical management is stabilization of the patients [158]; this includes general measures such as management of systemic blood pressure and oxygenation in order to ascertain adequate perfusion pressure to the brain. Life threatening conditions such as acute hydrocephalus and space occupying lesions such as epidural or subdural hematomas should be surgically treated in order to prevent herniation. In the further course the main goal of TBI therapy is to lower ICP, increase CBF, and to prevent/ alleviate cerebral edema formation. The guidelines of the Brain Trauma Foundation [159] recommends a graded approach that includes medical and surgical management strategies. A most effective tool to control intracranial hypertension is decompressive craniectomy (DC) (Figure 5) [160, 161], and it includes unilateral frontotemporoparietal craniotomy and bifrontal craniectomy in usual. While the ICP-lowering effect of this intervention is well documented the results regarding the long term neurological outcome vary: the DECRA study [162], the first large prospective, randomized, controlled, multicenter study evaluating the effect of DC, showed not an unequivocal positive effect on mortality and functional [162]; in a recent trial, the 'RESCUEicp' study, DC in patients with traumatic brain injury and refractory intracranial hypertension led to lower mortality, but also higher rates of adverse outcome [163]. In the later stages of TBI therapy, patients receive neurorehabilitation, treatment regimens include physical therapy, occupational therapy, speech therapy, psychological counseling, vocational counseling, and cognitive therapy [164]. Until now, there is no causal treatment strategy for posttraumatic brain damage resulting in neuroprotection. Despite many promising experimental studies, so far no therapeutical approach proved to confer clinical benefit in a clinical trial [52, 165, 166].

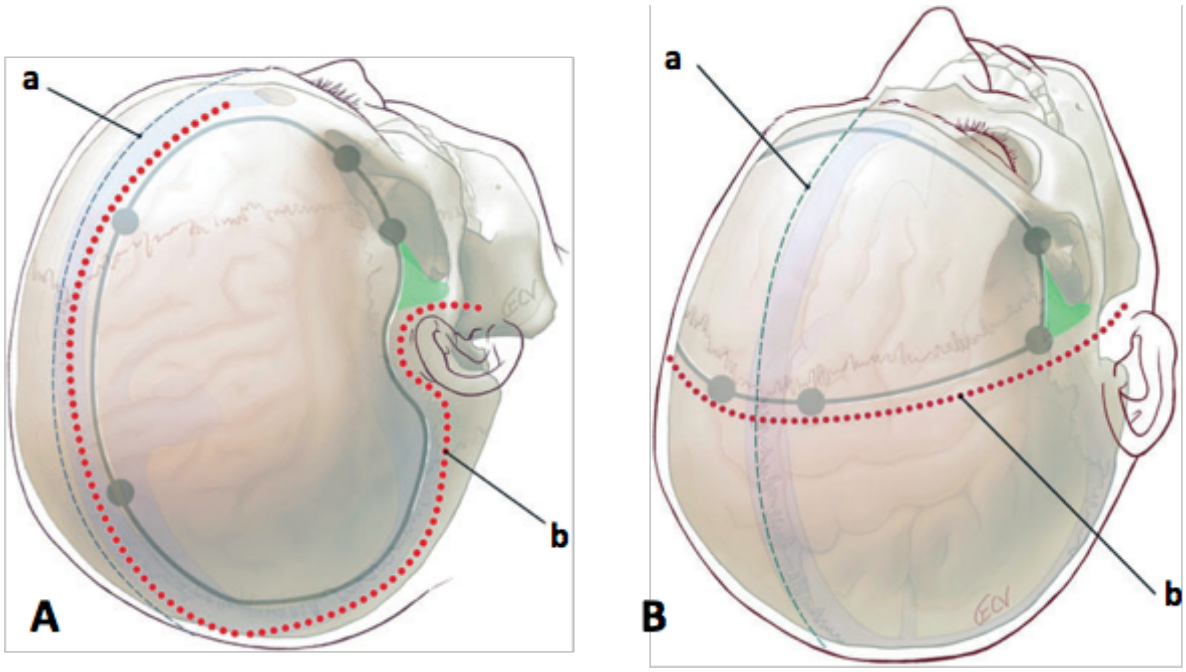


Figure 5: Decompressive craniectomy [160], A. Unilateral frontotemporoparietal craniotomy, a, midline; b, incision in skin.; B. Bifrontal craniectomy, a, midline; b, incision in skin.

1.1.4.3 Chronic Secondary Brain Damage

During the last decades, clinical and pre-clinical research efforts focused on the acute, life-threatening pathophysiological events after TBI, i. e. primary and acute secondary brain damage. Based on knowledge of the mechanisms of acute brain injury, treatment strategies in order to reduce acute posttraumatic brain damage were developed and tested with the intent of saving the patients' live and improving neurological outcomes. In recent times, however, it is increasingly recognized that the development of posttraumatic brain damage after a single TBI can continue for years and may lead to progressive deterioration over time [167, 168]. Also, TBI also is believed to be a risk factor for developing neurodegenerative disorders such as parkinsonism, cognitive decline and dementia [169]. Previous researches showed that occurrence of later-life

dementia is more likely when there is a history of a single TBI with loss of consciousness [170, 171]; furthermore it is reported that tau and amyloid pathology can be found in TBI survivors' brains decades after the initial injury [172, 173]. Another feature of chronic traumatic brain injury is called chronic traumatic encephalopathy (CTE), and its incidence was as high as 99% in National Football League (NFL) players in one study [174]. CTE is a progressive degenerative disease of the brain found in individuals with a history of repetitive brain trauma, symptomatic concussions as well as asymptomatic sub concussive hits to the head that do not cause symptoms. It was first described by Martland, H.S. in 1928 in pugilists as 'punch-drunk' syndrome [175], and in later studies observed mainly in athletes that regularly exposed to repetitive brain trauma, e.g. boxers, American football players, and ice hockey players. It remains unclear how high the risk of developing CTE is for professional sports players [176]; a recent autopsy study in professional American Football players reported evidence of CTE in up to 99% of individuals albeit in a highly selected group of subjects [174]. This sparked a controversial debate about sports associated health risks and medical standards after sport-related TBI, but the limitation of this study a highly selected group of individuals. TBI-associated dementia might be clinically different from Alzheimer's disease (AD) [177]. Although CTE is similar with AD, there are still significant differences. In general, the symptoms of CTE occur earlier than that of AD, which CTE generally present in one's 40s, otherwise symptoms in most AD cases generally present in one's 60s [178]. Except the time of symptoms coming, while the first and most central symptoms of CTE show problems with judgment, problem solving, and aggression in usual [177, 179, 180], while the first and most central symptoms in AD involve memory problems [178]. In addition, these diseases are found to be different in postmortem neuropathological findings.

On a histopathological level, CTE is characterized by accumulation of phosphorylated tau in sulci and perivascular areas of the cerebral cortex in 27 % CTE patients [181], and 95% of CTE cases showed widespread diffuse Aβ deposits [182, 183]. A recent study showed

that highest levels of abnormal tau protein appear in the CTE brain than that in AD brain (Figure 6) [184]. Except for the CTE patients with Tau-associated or Aβ-linked post-traumatic Alzheimer's disease (ptAD), little is known about the mechanisms of chronic posttraumatic brain damage [185-187]. The few available human post-mortem studies show that chronic traumatic brain damage is characterized by brain atrophy [188, 189] and may be associated with long-lasting neuroinflammation (activated microglia) evidenced for up to 18 years after TBI [114, 115]. These findings are corroborated by experimental studies in mice and rats showing long-term progressive brain atrophy after TBI [190, 191] as well as a long-lasting activation of microglia and monocyte invasion for up to 1.5 years after experimental TBI [192]. Whether this is the pathophysiological correlate of progressive neurocognitive decline and the cause of progressive neurodegeneration is still unclear. As there is uncertainty as to what exactly causes chronic posttraumatic brain damage there is no specific or targeted therapeutical strategy to alleviate or prevent it.

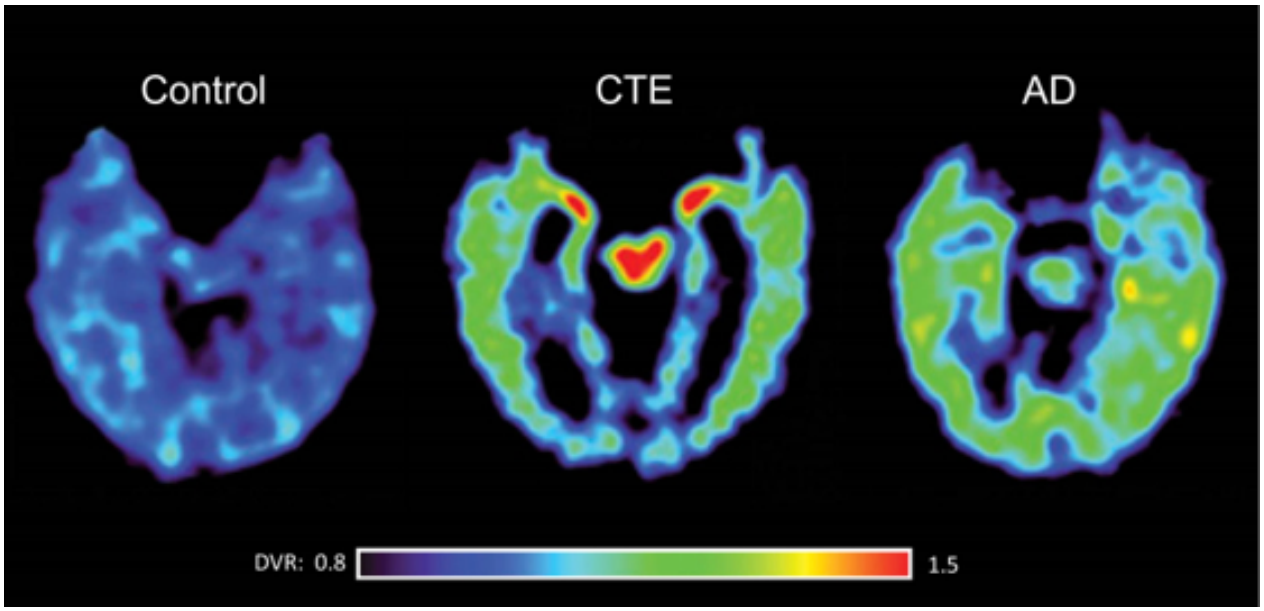


Figure 6: Left to right, brain PET scans of healthy control; former NFL player with suspected CTE; and person with AD. Areas with highest levels of abnormal tau protein appear red/yellow; medium, green; and lowest, blue [184].

1.2 Aim of the study

TBI causes long lasting deficits (like dementia, memory loss, depression) in patients that often aggravate over time. In order to further understand and – possibly even treat – chronic brain damage the time-course and the mechanisms of this phenomenon need to be clarified. So far, little is known about the mechanisms of chronic brain damage and how they are related and connected to the pathophysiology of acute (secondary) brain damage as most of our knowledge about post-traumatic brain damage is derived from short-term animal experiments or small human case series. The aim of the present study therefore was to characterize the development of chronic neuropathological and neurobehavioral changes in a widely used mouse model of experimental traumatic brain injury (the so-called controlled cortical impact model) over a period of 12 months.

2. Materials and Methods

2.1 Experimental Animals and Husbandry

Experiments were carried out on 6-8 -week-old male C57Bl/ 6 mice with a bodyweight of 20 – 22 g, which were obtained from Charles River Laboratories (Kisslegg, Germany). Mice had free access to water and food. The experimental animals were under a 12 hour-day/ night cycle. Prior to surgery, the mice were housed in groups of up to four mice per cage (207 x 140 x 265 mm, Macrolon II, Ehret Life Science Solutions, Emmendingen, Germany), after surgery animals were kept individually for one week and then put back together. Health screens and hygiene management checks were performed in accordance with FELASA guidelines and recommendations. All surgical procedures were carried out in accordance with the guidelines of the animal care institutions of the Munich University and approved by the appropriate authority, the Government of Upper Bavaria (protocol number 55.2-1-54-44-2017).

2.2 Randomization and Blinding

The experimental animals were randomly assigned to the experiment, allocation to different groups and time-points was achieved by drawing lots. The following groups/ time-points were examined: naïve animals (non-traumatized), sham surgery, post-traumatic groups evaluated at 15 minutes, 1, 7, 30, 90, 180, and 360 days after CCI. Figure 7 shows a graphical summary of the experimental groups. Surgical preparation and all postoperative behavioral tests were performed in a blinded fashion by a researcher unaware of group allocation and postoperative status of the animals.

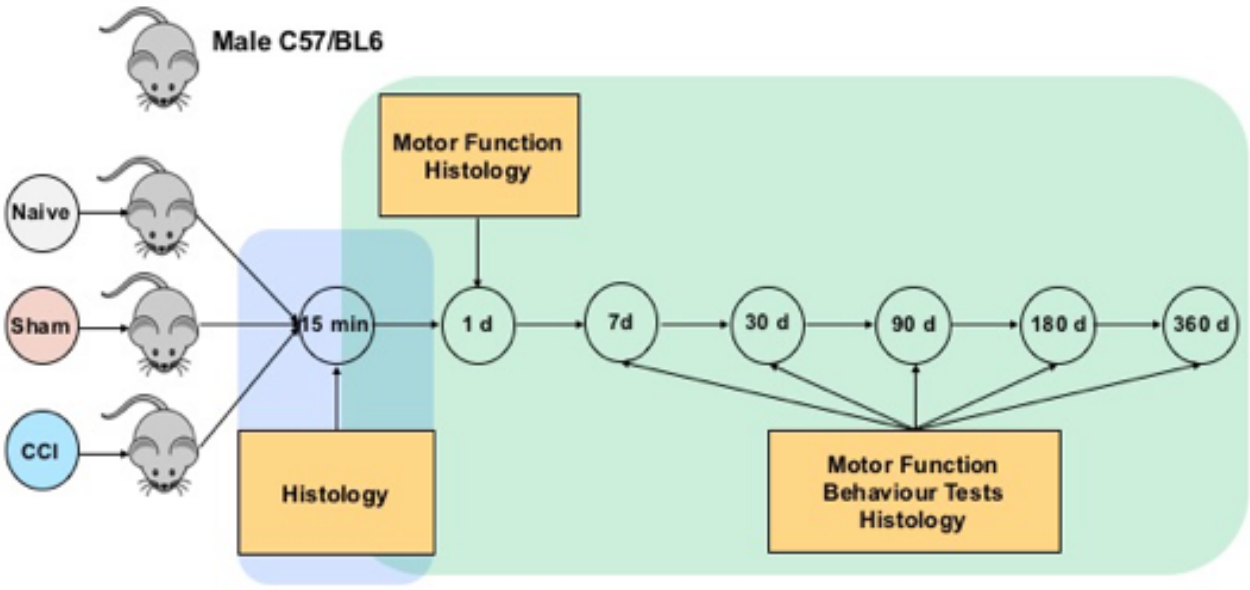


Figure 7: Schematic Overview of Experimental Groups

2.3 Anesthesia and Analgesia

30 minutes prior to surgery mice received an intraperitoneal injection of buprenorphine (Temgesic®, Reckitt Benckiser, Berkshire, UK, 0.1 mg/ kg bodyweight). Anesthesia was then induced in a specially prepared anesthesia chamber with 5% isoflurane (1ml / ml, 250 ml, CP-Pharma®, Burgdorf, Germany) over 45 seconds and then was continued at 1.5-2% isoflurane in an air/oxygen mixture (69% / 30%) applied by a respiratory mask. In order to avoid exsiccation of the cornea eyes were kept closed and protected with dexpanthenol cream (Bepanthen®, Bayer, Leverkusen, Germany). After completion of surgery animals received 100 % FiO_2 until mice fully regained consciousness. For the first three days, buprenorphine was injected every 8 hours as painkiller.

2.4 Experimental Traumatic Brain Injury – the Controlled Cortical Impact (CCI) Model

Traumatic brain injury was induced using the controlled cortical impact model. It was first described by James W. Lighthall in ferret in 1988 [193] and later adapted to other

species [194]. It has been established in our group approximately 15 years ago and has high reproducibility [105, 110, 194-197]. For surgery, animals were positioned in prone position with their head fixed in a stereotactic frame (Model 900 Small Animal Stereotaxic Instrument, David KOPF Instruments®, Tujunga, California, USA). After longitudinal skin incision along the sagittal suture (approximately 2 centimeters long) the galea aponeurotica is removed. Under the microscope (Leica M80, Leica, Germany) a right parietal craniotomy is performed using a diamond-tipped high speed drill (Rewatronik® Products, Wald-Michelbach GmbH, Germany; drill head GD890R, diameter 0.6 mm, Aesculap, Tuttlingen, Germany); the bone is not dissected at the sagittal suture. Location of the craniotomy, which is approximately 5 x 5 mm in size, is depicted in Figure 8A. The bone flap is then opened towards the sagittal suture exposing the intact dura mater (Figure 8B). The animal is placed under the impactor tip of the CCI device (Mouse-Katjuscha 2000, L. Kopacz, University of Mainz, Germany, Figure 8C), the tip is lowered to the dura under the microscope in order to allow exact perpendicular placement of the impactor bolt on the dura in the middle of craniotomy. Trauma induction is then carried out with a penetration depth of 1 mm, a dwelling time of the punch and an impact velocity of 8 m/s.

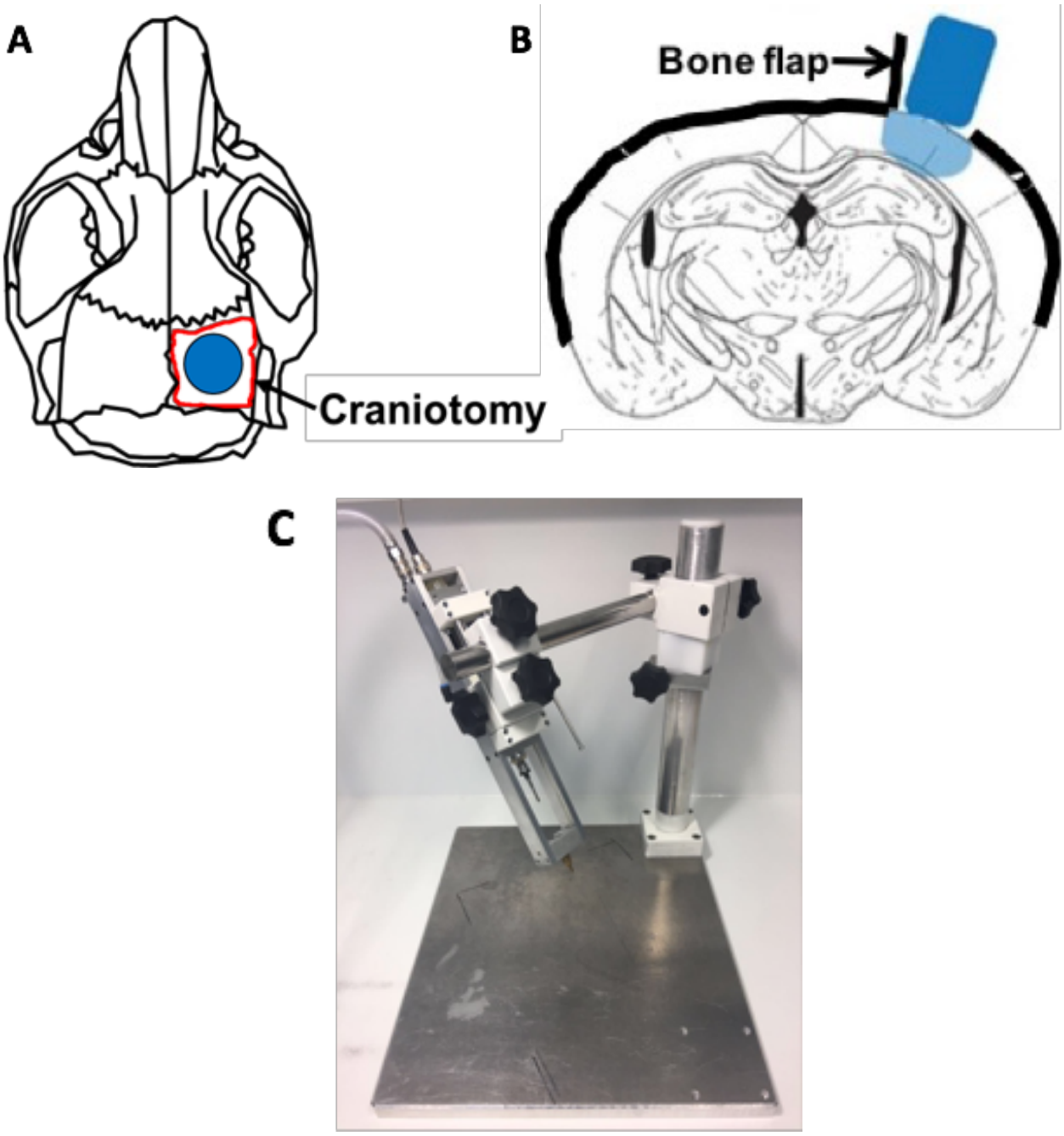


Figure 8: A: Position of cranial window and trauma. Red rectangle: craniotomy; Blue round shape: position of the trauma induction; B: Position of Bone Flap; C: CCI device

The cranial window was immediately closed by replacing the bone flap and fixing it using tissue glue (No.1469SB, 3M Vetbond™, USA). The skin wound was sutured by

monofilament sutures (6/0, SERAFIT®, SERAG-WIESSNER GmbH, Naila, Germany). Mice were then allowed to wake up, until recovery of spontaneous motor activity they received 100% O_2 via face mask. In order to avoid postoperative hypothermia, they were then placed in an incubator (MediHEAT ™, Peco Services, United Kingdom) heated to 32 ° C for 60 minutes. Sham operated animals underwent craniotomy and bold placement; however, the impact was not triggered.

2.5 Body Weight

Body weight as a parameter for general wellbeing of experimental animals was measured directly before the induction of anesthesia. After CCI it was determined daily for the first 7 days; afterwards, animals were weighed once a month until the end of the observation period using a precision balance (OHAUS®, Waagendienst Winkler GmbH, Munich, Germany).

2.6 Behavioral Testing

2.6.1 Evaluation of Motor Function - Beam Walk Assessment

As the posttraumatic brain damage in our model is located in the region of the murine motor cortex (hindpaw region) animals show distinct motor deficits after CCI. We therefore chose a paradigm that reliably detects motor and gait deficits, the beam walk test, which has been previously established in the CCI mouse model [194]. For the test, mice were placed on a wooden beam (1 cm width, 100 cm length, 100 cm above ground, see Figure 9 for pictures of the set-up). When crossing the beam, the time needed to completely traverse the beam is assessed; furthermore the number of missteps with the hindpaw contralateral to CCI is counted. Starting 3 days before trauma the beam walk test was performed at 1 day, day 3, and day 7 after trauma; afterwards, it was conducted every month until the end of the observation period.

Figure 9: Experimental Set-up for Beam Walk testing

2.6.2 Depression-like Behavior - Tail Suspension Test

Tail suspension is a test to evaluate depressive behavior in rodents [198]. For the test, the animal is suspended at the tail for three minutes. During this time, the animal is continuously video-monitored with a high resolution camera (EthoVision®XT, Noldus Information Technology, Netherlands) in order to assess mobility and immobility time, i. e. the time the mouse moves in order to recover balance/ the time the mouse does not move. For the test, a custom-made device (Figure 10) allowed for testing three animals at one time. In order to avoid climbing, a plexiglass cylinder (4 cm length, 1.6 cm outside diameter, 1.2 cm inside diameter, 1.6 grams) was placed on the tail (Figure 10). The tail suspension test was performed 90, 180, 270, and 360 days after CCI.

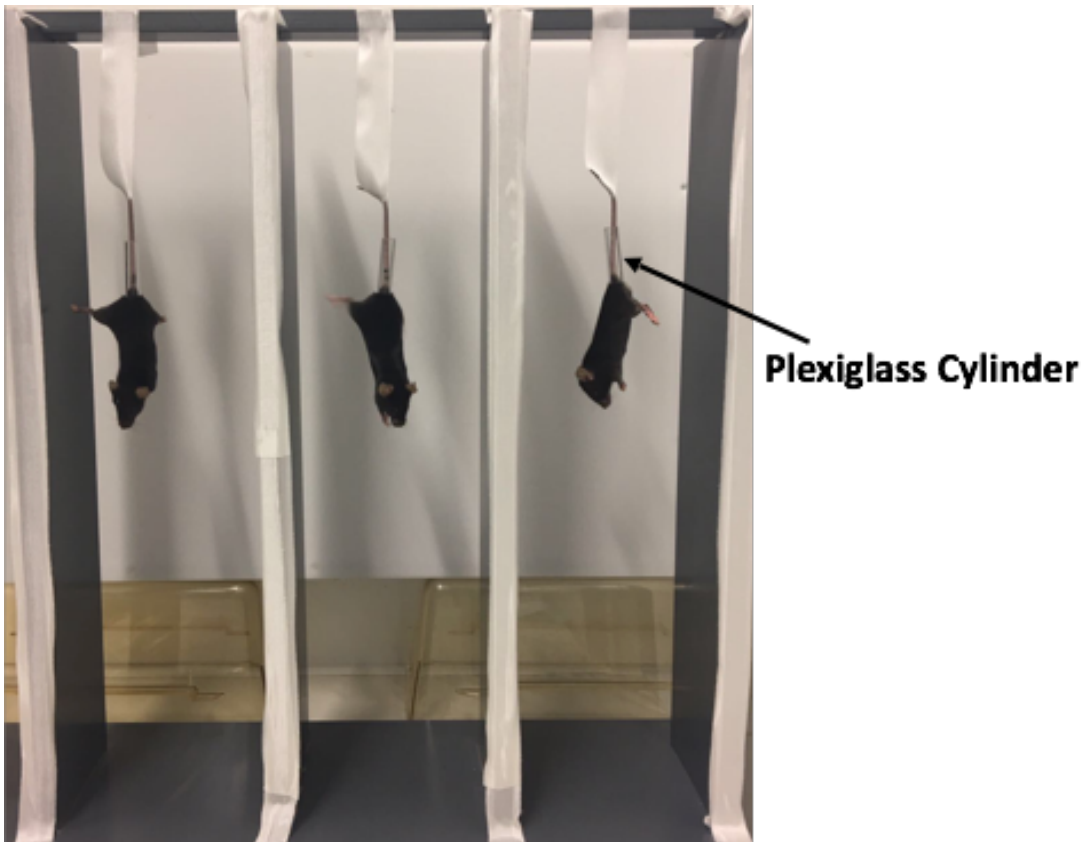


Figure 10: The Device for Tail Suspension

2.6.3 Spatial Orientation and Learning Behavior – the Barnes Maze Test

The Barnes maze is a test paradigm to study spatial learning and memory in rodents [199] based on the rodent's instinctive avoidance of open fields and their natural preference for dark and sheltered spaces. The test is performed on a round platform (diameter 100 cm) that is brightly lit and contains 20 identical holes (diameter 10 cm) spaced evenly around the perimeter (Figure 11A). Below one of these apertures there is a so called "home-box" (20 X 5 X 3 cm) that provides a sheltered space to escape the brightly lit open space of the platform. The aim of the test was for the mouse to correctly identify the home-box and arrive there as quickly as possible within the test duration of 180

seconds. The whole set-up was monitored with a high resolution camera, the animal´s performance digitally recorded; a software then allows to assess the time the mouse needs to arrive at the home-box (latency) as well as track the speed and the distance travelled (EthoVision®XT, Noldus Information Technology, Netherlands, see Figure 11B for photograph of the setup). Three marks are attached in different positions in order to guide the mouse towards the location of the home-box. Animals are trained in this test paradigm twice daily (with an interval of 4-5 hours) for 4 days, starting 5 days before the actual experiment begins. During training phase, in the mornings the mouse is placed under a glass cylinder (12 cm diameter, 22 cm height) in the center of the table for 120 seconds. After that, the mouse is slowly moved with the cylinder towards the home-box until the mouse can enter it; the animal is then left in the home-box for 60 seconds. In the afternoon, it started when the glass cylinder was moved out of the mouse, so the mouse could begin to search the home-box during 180 seconds. For the probe day, we did the procedure as the afternoon part and just recorded the latency, distance and velocity during 180 seconds. After surgery, test is performed at day 90, 180, 270, and 360 after the start of the experiment. Repetitive training trails of Barnes Maze were performed on Day 85-88, Day 175-178, Day 265-268, and Day 355-358.

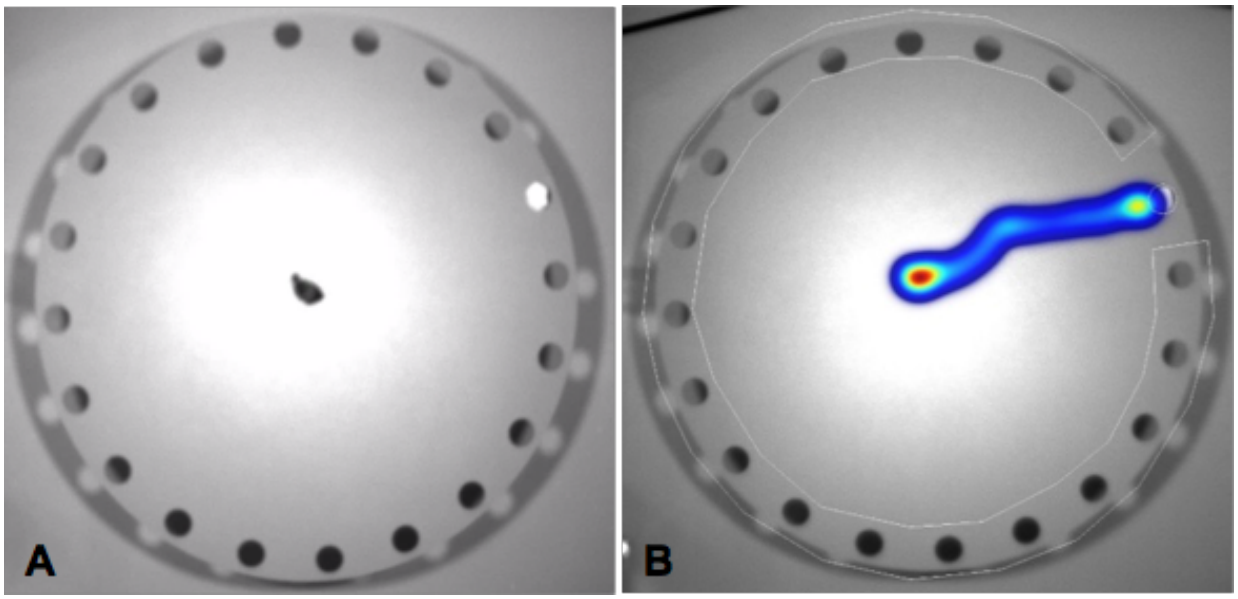


Figure 11: The Device for Barnes Maze

2.7 Histopathological Assessment of Posttraumatic Brain Damage Parameters

2.7.1 Tissue Harvesting

For histological evaluation, coronary floating sections of the brains were prepared as previously described [200]. At the end of the respective observation period, animals were sacrificed by transcardial perfusion after induction of deep anesthesia achieved by intraperitoneal injection of medetomdine (0.5 mg/ kg body weight; Domitor®, Pfizer, Karlsruhe, Germany), midazolam (5 mg/ kg, ratiopharm, Ulm, Germany), and fentanyl (0.05 mg/kg b.w., Jannsen Cilag, Neuss, Germany) 15 minutes before tissue removal. After placement and fixation of a cannula (21G, Safety-Multifly®-Needle, Sarstedt, Nümbrecht, Germany) in the left ventricle the animal was perfused with 30 ml of physiological saline (NaCL 0.9%, B.Braun AG, Melsungen, Germany) over 1 minute and then 50 ml of 4% paraformaldehyde (PFA) (Morphisto GmbH, Frankfurt am Main, Germany) over 2 minutes using a pressure-controlled perfusion system (Perfusion One, Lecia Biosystems, Richmond, VA, USA). After carefully removing the brain it was placed

in a tube with 4% PFA at 4°C for 12-16 hours for fixation and then stored in phosphate buffered saline (PBS) until further preparation.

2.7.2 Preparation and Staining of Brain Sections

50 µm thick coronal sections were prepared using a vibratome (Leica VT1200S, Nussloch, Germany). After embedding the brain in 4% agarose (PEQLAB, VWR International GmbH, Erlangen, Germany) it was mounted on the stage of the vibratome, which was then set to the following parameters: 0.8 vibrating speed (amplitude) and 0.8 m/s advancing speed. Starting 1000 micrometers behind the olfactory bulb a total of 12 sequential coronal 50 µm thick sections were collected every 600 µm and applied to glass slides slides (ELKA, Glaswarenfabrik Karl Hecht GmbH & Co KG, Sondheim, Germany). The sections were then stained according to Nissl using cresyl violet (see Figure 12 for detailed protocol) and fixed and lidded (Eukitt®, O. Kindler GmbH & Co, Freiburg, Germany).

2 min	Fix sections with 70% Ethanol
10-15 min	Cresyl violet solution
	Rinse shortly with water (2 x)
2 min	Ethanol 70%
2 min	Ethanol 96%
2 min	Ethanol 100%
2 min	Isopropanol
5 min	Rotihistol I
5 min	Rotihistol II

Figure 12: Protocol for Nissl staining

2.7.3 Histological Quantification of Posttraumatic Brain Damage Parameters

2.7.3.1 Evaluation of Defect Volume

Gross pathology revealed that there is progressive tissue loss or defect formation in the zone of the former contusion as early as 7 days after trauma. Therefore, determination of contusion volume is not feasible at later observation time-points as there is no more contused/ necrotic tissue. In order to assess structural lesion size a different parameter for the groups that were observed from 7 days until 12 months after CCI was introduced: defect volume (Figure 13). Sections were placed under a microscope (Zeiss Axio Imager M2, Carl Zeiss Microscopy GmbH, Munich, Germany) and digitally photographed at 12.5 X magnification with a digital camera connected to the computer (Zeiss Axio Imager M2, Carl Zeiss Microscopy GmbH, Munich, Germany). With the help of an imaging software (AxioVision LE 4.8, Carl Zeiss Microscopy GmbH, Munich, Germany), the areas of the contralateral hemisphere (A), ipsilateral hemisphere (B), and respective ventricle area (C) were measured. Defect area, i. e. the area of "missing tissue" was then calculated as based on the ipsilateral hemisphere, contralateral hemisphere and respective ventricle area:

Defect Area = A – C –B (Figure 13).

The defect volume was then determined using the following formula:

$V_{Dir} = d*(E_1/2 + E_2 + E_3 \ldots + E_n/2)$ with d = 0.6 (0.6 for 600 µm, distance between two sections).

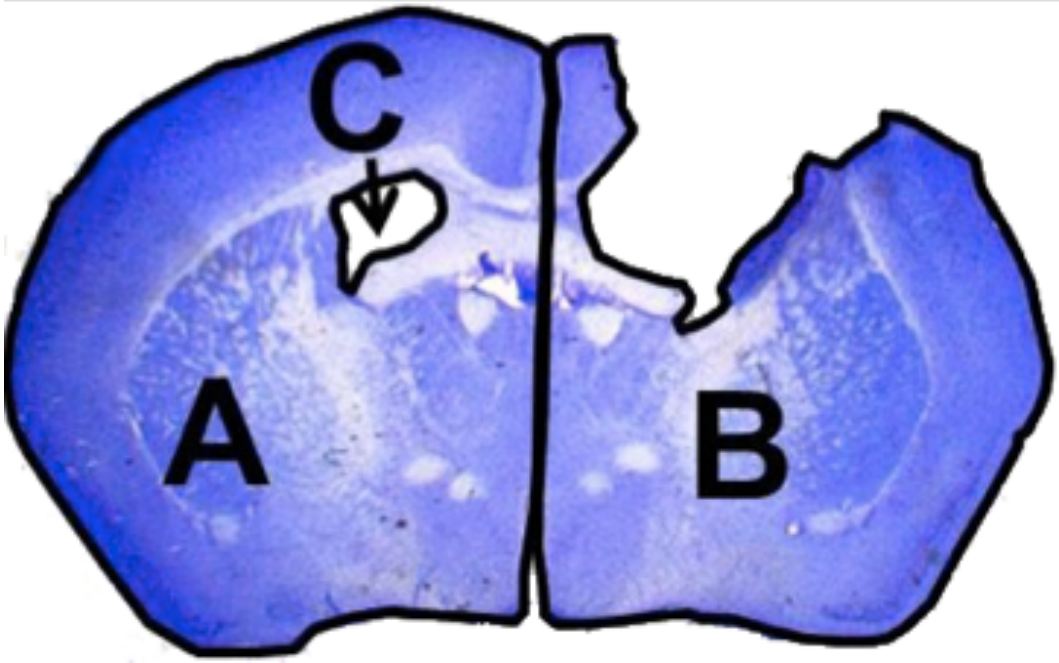


Figure 13: Histomorphometric Determination of Defect Volume

2.7.3.2 Determination of Tissue Atropy

Quantification of tissue atrophy was also performed histomorphometrically (see description above for set-up). For determination of atrophy one section at 1.0 mm posterior the Bregma level (see Figure 14A [201]) was imaged and the area of both hemispheres determined (see Figure 14B for exemplary photograph). Atropy of the hemisphere ipsilateral to the trauma is expressed as percentage of contralateral hemisphere volume.

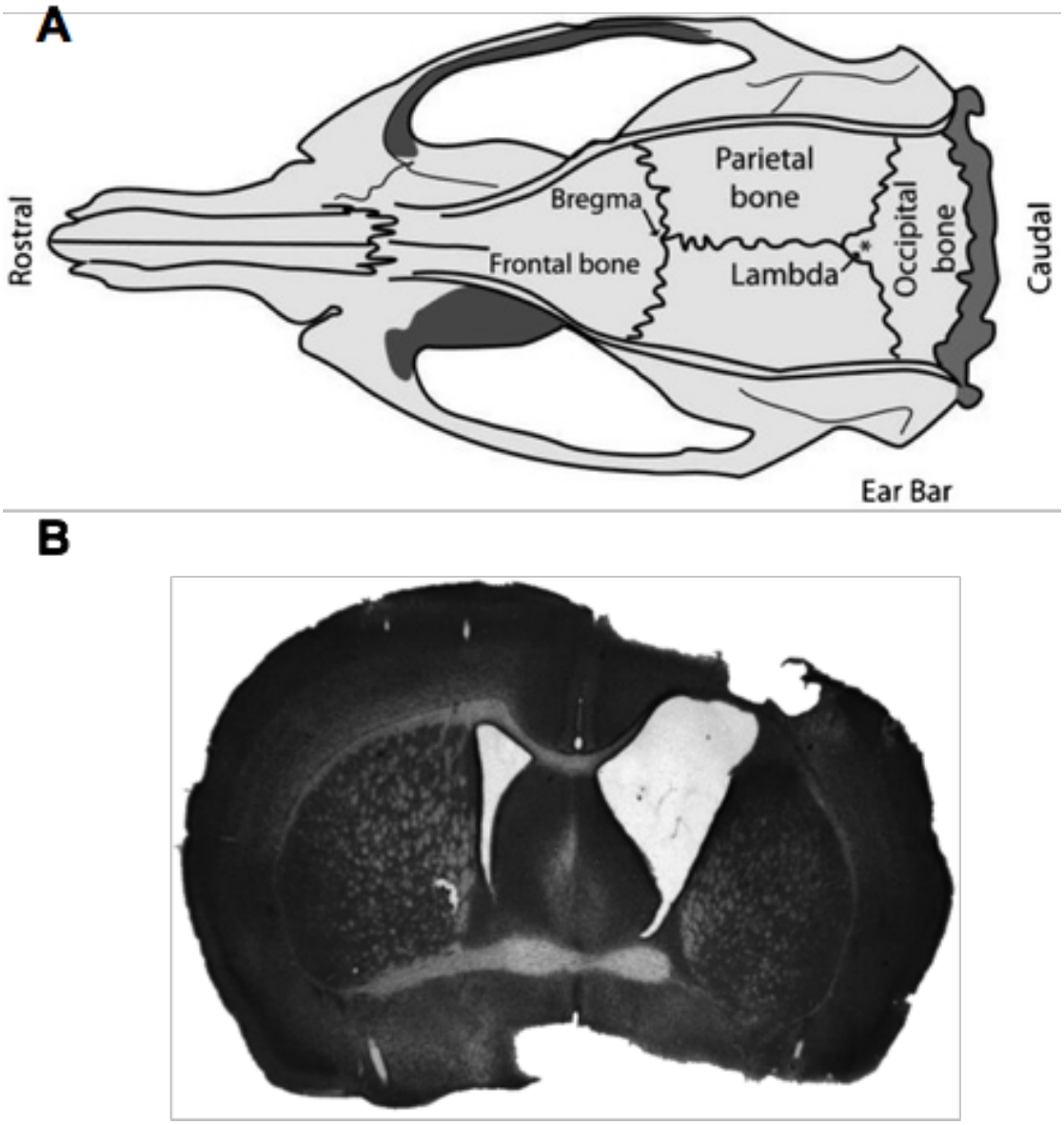


Figure 14: A. Schematic Drawing of a Mouse Skull [201]; B. Histomorphometric Determination of Atropy

2.7.3.3 Assessment of Hydrocephalus

Ventricular enlargement/ hydrocephalus was determined by histomorphometry at 1.0 mm posterior the Bregma level (see Figure 15 for exemplary picture) [202]. The area of

the non-traumatized hemisphere and the hemisphere ipsilateral to trauma were measured. Results for both ventricles are given in mm^2.

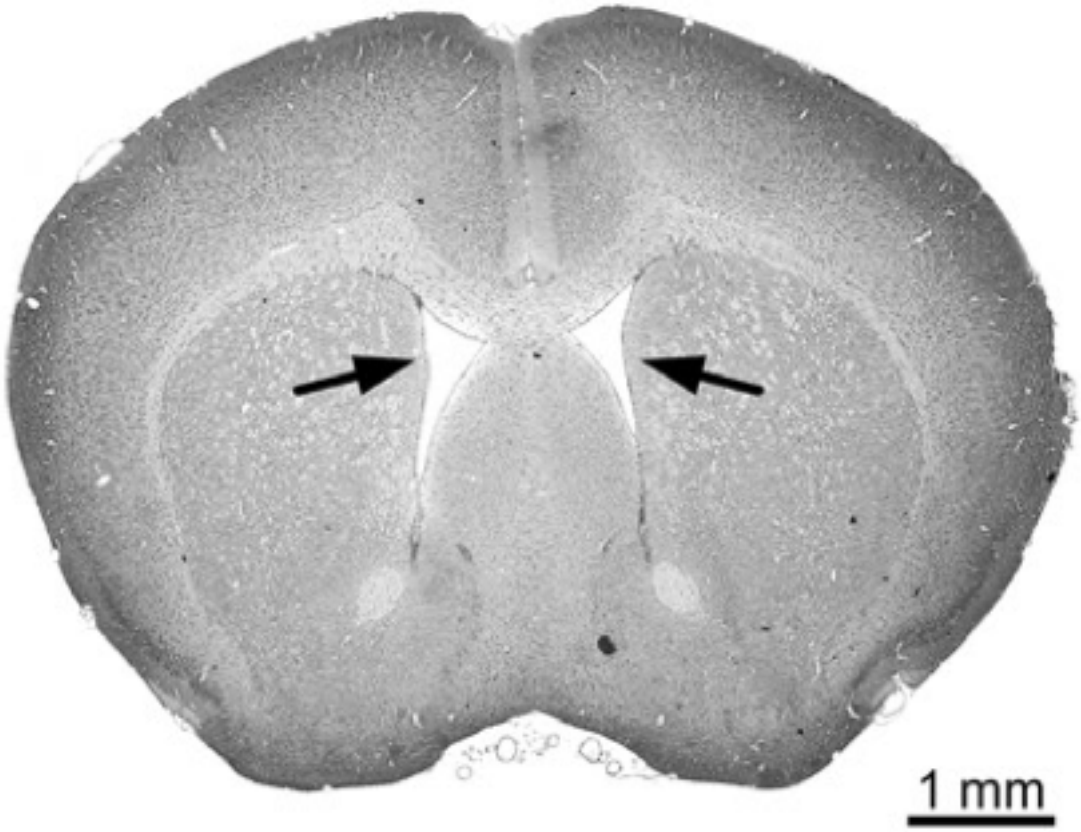


Figure 15: Histomorphometric Determination of Hydrocephalus [202]

2.7.3.4 Evaluation of White Matter Damage

White matter damage was assessed by measuring corpus callosum thickness using hisomorphometry as previously described [202]. Corpus callosum width was determined

as shown in Figure 16 in both hemispheres in a section obtained at 1.0 mm posterior the Bregma level. Results are given in mm.

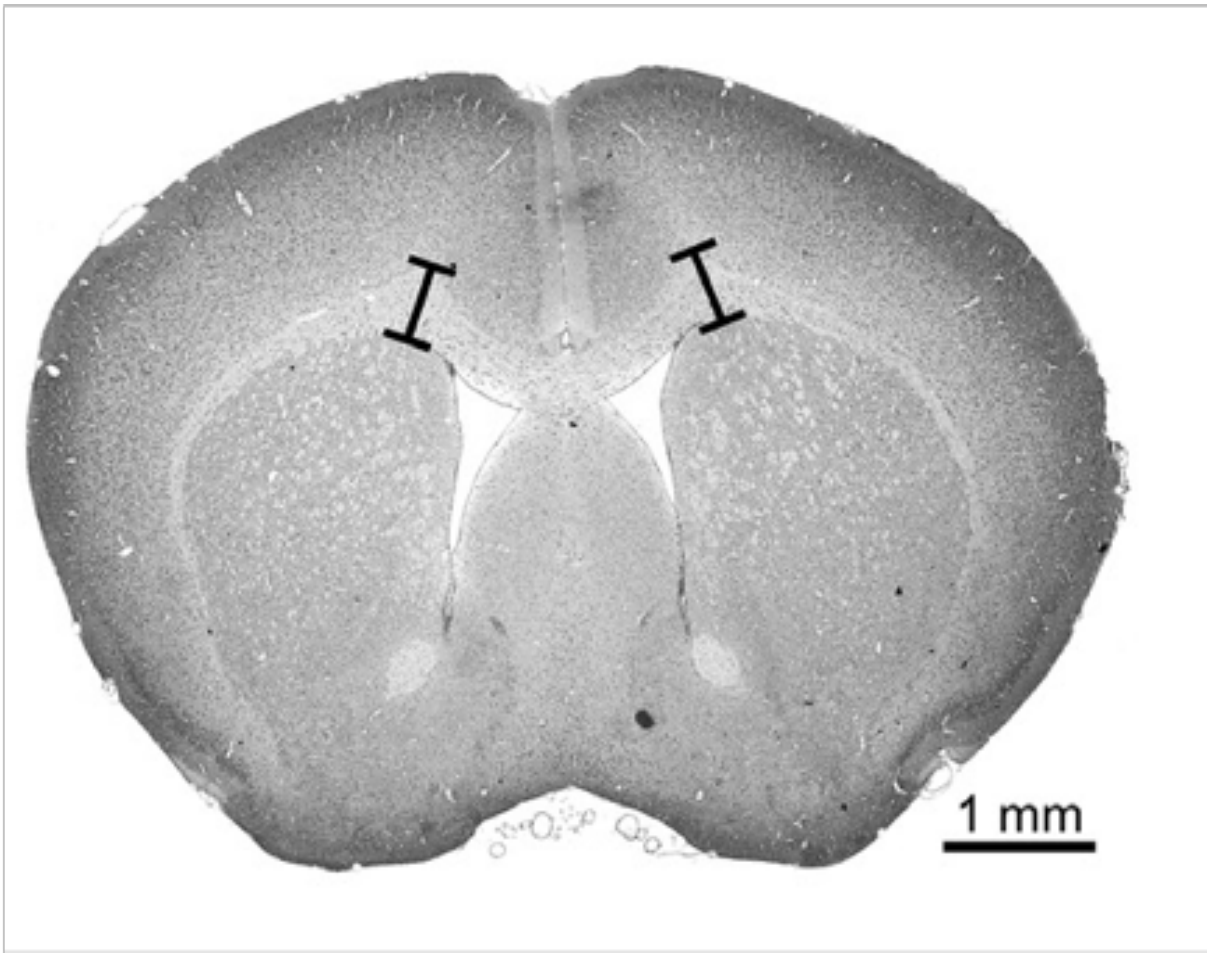


Figure 16: Histomorphometric Determination of White Matter Injury [202]

2.7.3.5 Evaluation of Hippocampal Damage

For assessment of hippocampal damage one section 2.0 mm posterior the bregma level was imaged by histomorphometry. Total hippocampal area was then measured for both

hemispheres (Figure 17). Results are given hippocampal area for the ipsi- and the contralateral hemisphere.

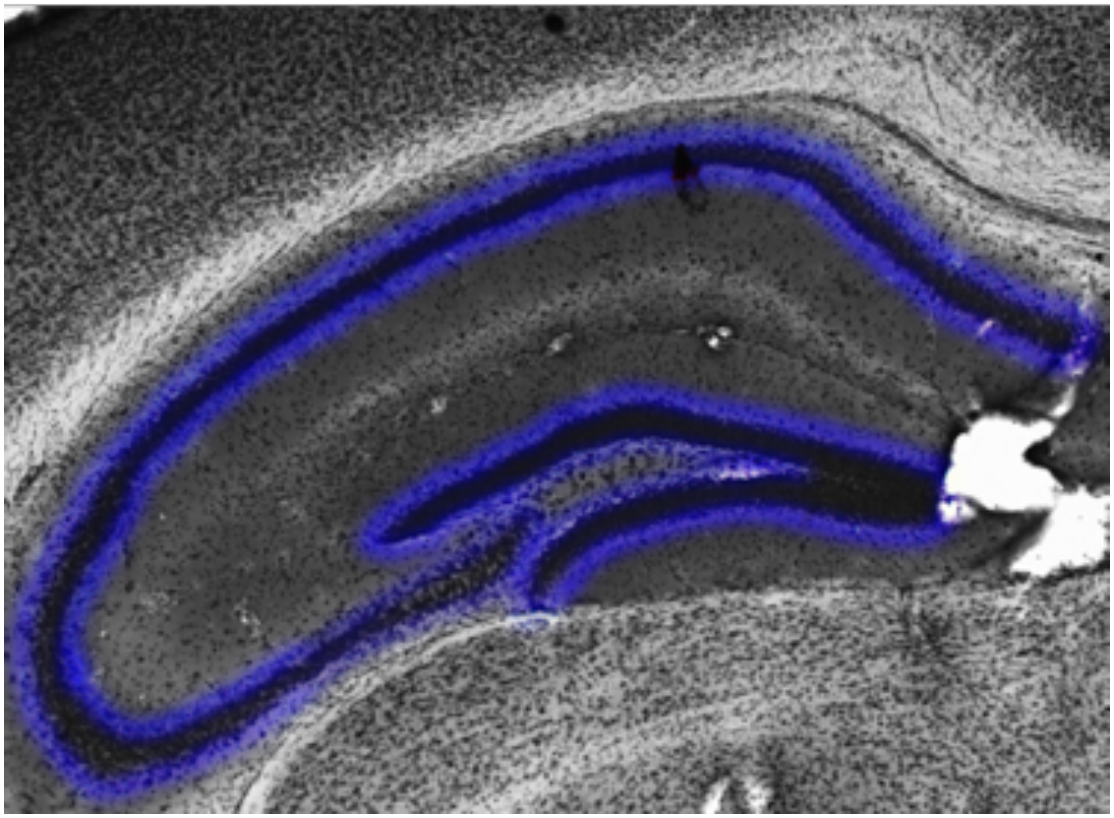


Figure 17: Histomorphometric Determination of Hippocampal Injury

2.8 Statistical Analysis

Statistical analysis was performed using a statistical software package (GraphPad Prism 6.0, La Jolla, CA, USA). Sample size calculations were performed with the following parameters: alpha error = 0.05, beta error = 0.2, calculated standard deviation ranged from 15 - 20% (depending on the parameter investigated), and biologically relevant difference of 30%. As sample size is below 25 per group in our study we used non-parametrical tests even when data passed the normality test. For comparisons

between two groups the Mann–Whitney test was used, Kruskal–Wallis analysis of variance on ranks followed by Dunn's method as a post hoc test was used to compare more groups. Differences between groups were considered significant at $P<0.05$. Except where indicated otherwise, all data are expressed as mean ± SD.

3. Results

3.1 Behavioral Changes

3.1.1 Body Weight

Before surgery, there was not statistically significant different on body weight between the different groups (24.3 ± 1.1 g for the naïve group, 24.2 ± 1.2 g for the sham group, 24.3 ± 1.1 g for the CCI group, n=10, each group). One day after operation, there was a significant loss of body weight in the CCI group as compared to naïve group (CCI group vs. naïve group $p=0.0023$), and there were no difference either between sham group and naïve group, or between sham group and CCI group. Animals in the sham and CCI group regained their pre-trauma body weight within 14 days after operation in (25.2 ± 0.9 g for the sham group, 24.8 ± 1.3 g for the CCI group). The weight of sham animals was reduced compared to naïve up to 270 d after operation. Over the observation period, the weight in naïve animals continuously increased up to 41.3 ± 1.9 g at 360 d. Sham animals showed a slight delay in weight gain but caught up with the naïve group at 360 d. After CCI, weight increased at a much slower rate for 360 d after trauma; animals were significantly lighter than the sham-operated or non-traumatized control group up to the end of the observation period (41.3 ± 1.9 g for the naïve group, 40.4 ± 2.3 g for the sham group, 34.4 ± 1.5 g for the CCI group; CCI vs. naïve, $p<0.0001$; CCI vs. sham, $p<0.0001$). Maximum body weight was significantly lower at all times (Figure 18).

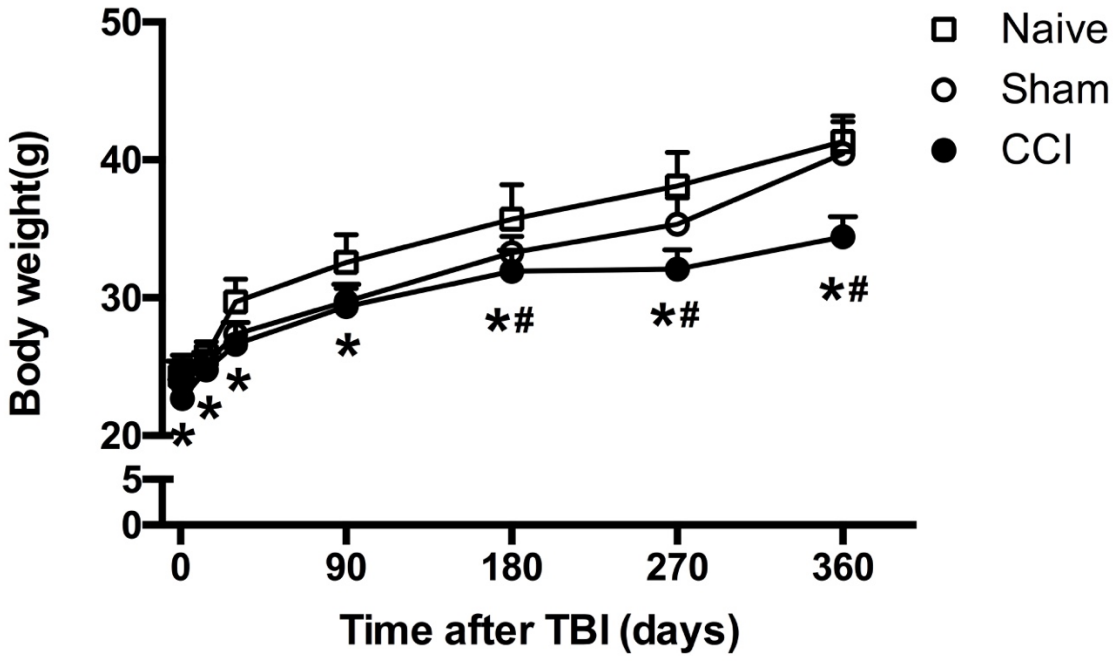
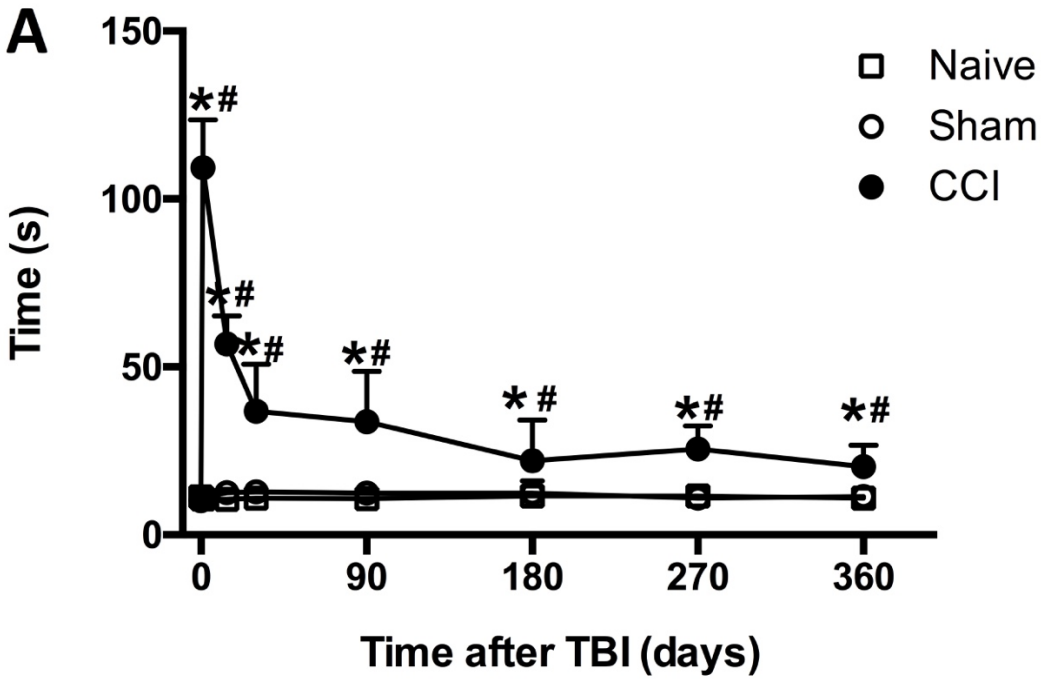


Figure 18: Weight loss over time: After CCI and sham surgery, mice lose weight. In the further course of the experiment, CCI animals gained weight significantly slower than sham-operated mice which catched up with the non-traumatized group after 360 d; they had a reduced maximum body weight (n=10; *, CCI vs. naïve, $p<0.01$; #, CCI vs. sham, $p<0.01$).

3.1.2 Motor Function – Beam Walk Test

Before start of experiments there was no difference between groups as far as the time needed to cross the beam (11.3 ± 2.3 s for the naïve group, 10.2 ± 1.0 s for the sham group, 10.5 ± 1.6 s for the CCI group, n=10, each group, Figure 19A) or the number of missteps (0.5 ± 0.7 for the naïve group, 0.6 ± 0.8 for the sham group, 1.0 ± 0.9 for the CCI group, n=10, each group, Figure 19B) was concerned. 24 hours after surgery, animals in the CCI group needed significantly longer to perform the task than before TBI (109.3 ±

14.3 s vs. 10.5 ± 1.6 s, $p < 0.0001$) while no change was observed in the naïve and sham mice´s performance. Although CCI mice gradually recovered within a month they did not regain their pre-trauma level, crossing time was still significantly elevated compared to baseline (10.5 ± 1.6 s) at 360 days (20.3 ± 6.4 s) after CCI ($p=0.0028$); also, crossing time was significantly longer than in naïve (10.9 ± 2.5 s) and sham mice (11.4 ± 2.6 s) at 360 days after CCI (CCI vs. naïve, $p=0.0045$; CCI vs. sham, $p=0.0044$). The results for the number of missteps showed a similar trend: there was significant worsening in the CCI group 24 hours after trauma (36.1 ± 4.0), compared to their own baseline (1.0 ± 0.9) performance ($p < 0.0001$), however, it remained stable throughout the observation period in the naïve group and sham group. Again, CCI mice gradually recovered within a month (9.8 ± 2.0), but their performance remained constantly worse than baseline (1.0 ± 0.9), naïve (1.0 ± 0.9) and sham group (1.1 ± 0.9) at 360 d after trauma. (CCI vs. baseline, $p < 0.0001$; CCI vs. naïve, $p < 0.0001$; CCI vs. sham, $p < 0.0001$).



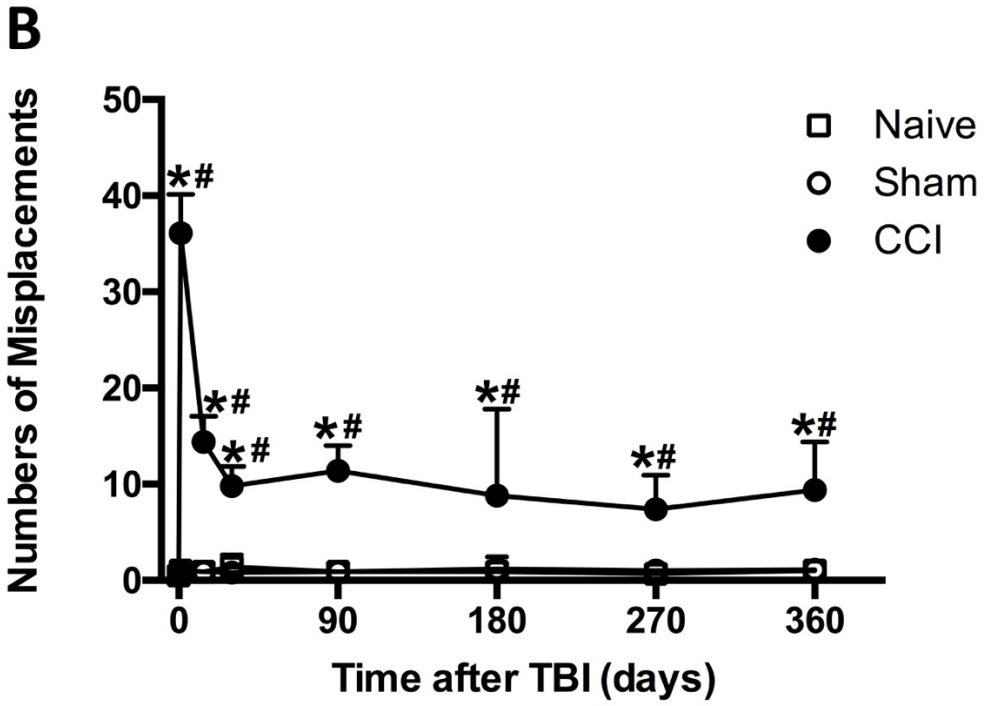


Figure 19: Motor Function assessed in the beam walk test. A. Time needed to cross the beam; B. Number of missteps. Directly after trauma there was a massive increase in time and missteps; the motor deficit recovers quite quickly but even after 6 months animals have not returned to their baseline value (n=10; *, CCI vs. naïve, $p<0.01$; #, CCI vs. sham, $p<0.01$).

3.1.3 Depressive-like Behavior – Tail Suspension Test

As described in the Materials & Methods section, tail suspension is a sensitive test paradigm that can detect depression like behavior; shorter mobility time is indicative for depression like behavior (Figure 20). Before the start of experiments there was no difference in mobility time among groups (33.0 ± 22.2 s for the naïve group, 31.0 ± 9.7 s for the sham group, 32.6 ± 7.4 s for the CCI group). Over the course of the experiment, mobility time declined in all groups, most probably due to habituation. However, 90 days

after trauma, animals in the sham group (12.0 ± 9.3 s) as well as the CCI group (13.3 ± 15.4 s) showed shorter mobility time compared to naïve mice (27.0 ± 16.1 s) (sham vs. naïve, p=0.0522; CCI vs. naïve, p=0.0455). Mobility time steadily declined in CCI animals up to the end of the observation time at 360 d after trauma (0.156 ± 0.493 s), indicating progressive depressive behavior, and was significantly reduced compared to sham (9.84 ± 8.43 s) and naïve (12.3 ± 13.5 s) animals at the same time (CCI vs. sham, p<0.0001; CCI vs. naïve, p=0.0002). Sham animals recovered by 180 days after surgery and reached the levels observed in the non-traumatized group by the end of the observation period.

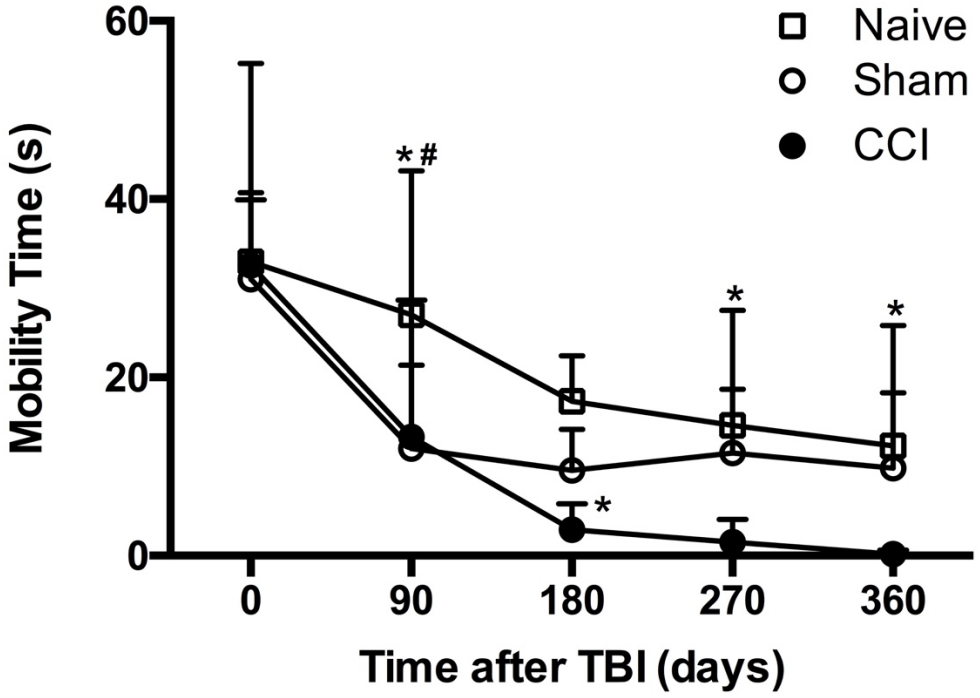


Figure 20: Tail suspension test. Mobility time progressively reduced in mice after TBI indicating depression like behavior (n=10; *, CCI vs. naïve, p<0.01; #, CCI vs. sham, p<0.01).

3.1.4 Spatial Orientation and Learning Behavior - Barnes Maze Test

Figure 21A shows representative summary heat maps obtained at 360 d after trauma. Starting 90 days after trauma, latency (Figure 21B), distance (Figure 21C), and mean velocity (Figure 21D) was investigated every 90 days. Latency was significantly elevated in the CCI group (65.03 ± 38.97 s) 90 d after TBI compared to sham operated (12.76 ± 10.20 s) or naïve animals (13.60 ± 9.946 s) at the same time (CCI vs. sham, $p<0.0001$; CCI vs. naïve, $p<0.0001$). Over the course of time, performance in the CCI group operated deteriorated up to the end of the observation period at 360 d after trauma (129.7 ± 66.01 s) and was significantly worse than that at 90 d after trauma ($p=0.0277$). While the naïve operated (16.39 ± 9.349 s) and sham operated (18.70 ± 13.23 s) group presented stable latency times at 360 d and significant better than the CCI group (CCI vs. naïve, $p=0.0002$; CCI vs. sham, $p=0.0002$). This indicates that there is progressive memory loss in TBI. Distance was constantly and significantly higher in the CCI group (260.7 ± 116.1 cm) compared to the naïve (137.4 ± 73.67 cm) and sham group (142.5 ± 104.1 cm) at 360 d (CCI vs. naïve, $p=0.0147$; CCI vs. sham, $p=0.0288$), and the mean velocity was lastingly and significantly lower in CCI (2.952 ± 2.751 cm/s) compared the naïve (8.928 ± 1.837 cm/s) and sham group (8.131 ± 2.206 cm/s) at 360 d (CCI vs. naïve, $p=0.0007$; CCI vs. sham, $p=0.0007$).

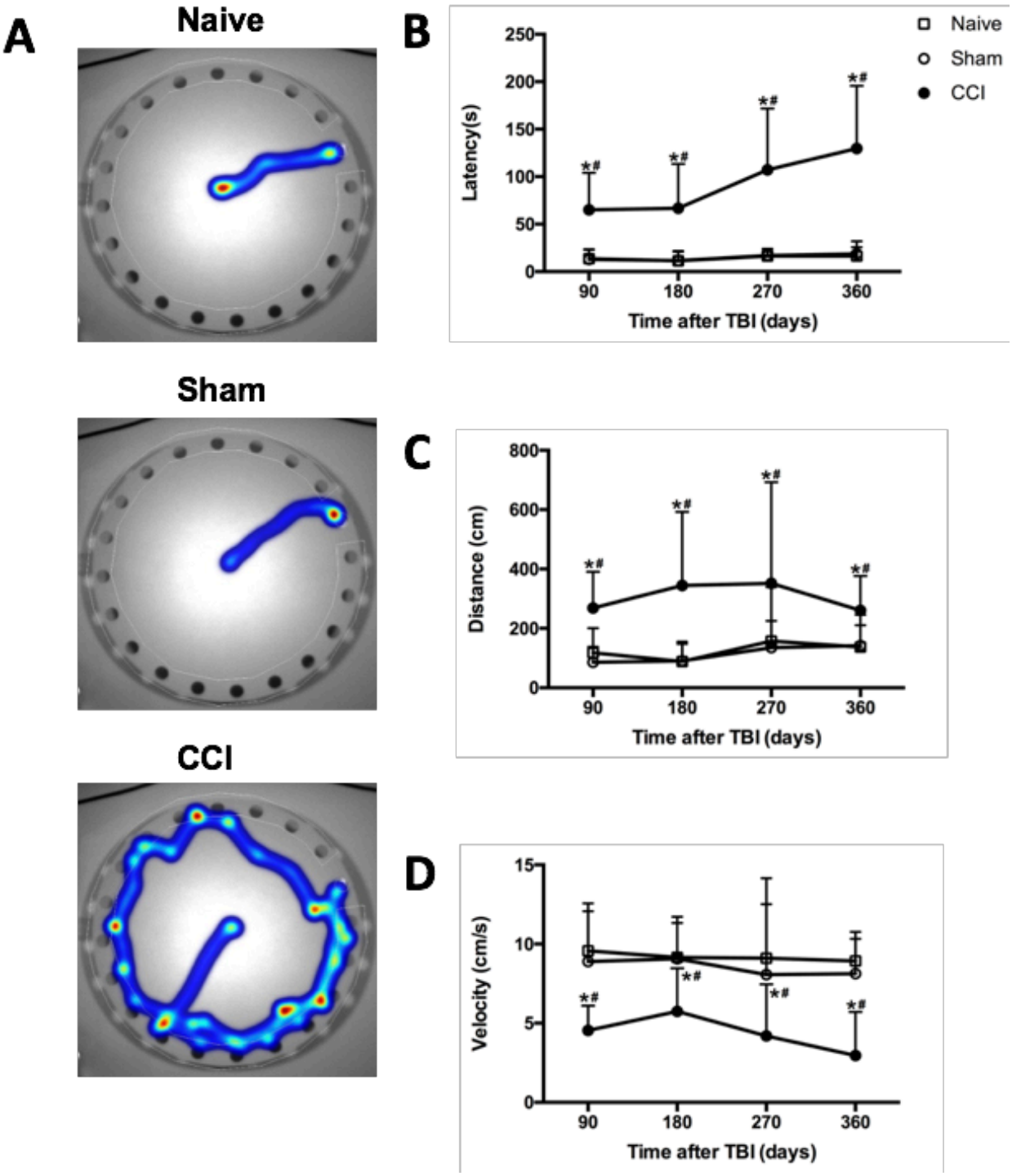


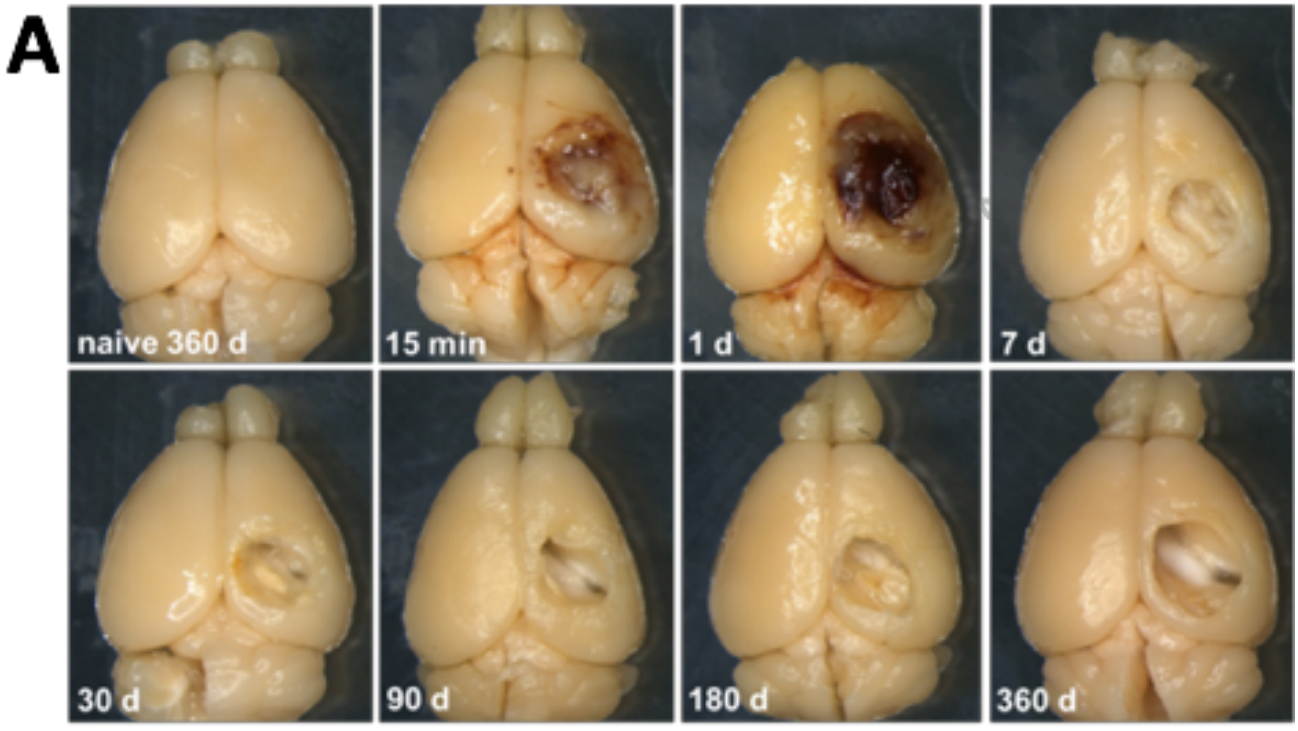
Figure 21 Barnes Maze Test. A. Summary heat maps for all groups obtained at t=360 d; B. Quantification of latency: CCI animals need significantly more time to correctly identify the home cage; C. Quantification of distance: CCI animals need significantly more

distance to correctly identify the home cage; D. Quantification of mean velocity: CCI animals showed significantly less velocity to correctly identify the home cage (n=10; *, CCI vs. naïve, $p<0.01$; #, CCI vs. sham, $p<0.01$).

3.2 Histopathological Changes

3.2.1 Defect Volume

Gross histopathological exam showed that the posttraumatic lesion significantly enlarged over time (Figure 22A). At 7, 30, 90, 180 or 360 days after CCI the defect volume significantly expanded from 16.28 ± 1.417 cm^3, 19.67 ± 5.825 cm^3, 21.44 ± 2.752 cm^3, 27.62 ± 4.586 cm^3 , and 33.31 ± 2.614 cm^3 on day 7, 30, 90, 180, and 360 respectively (Figure 22B, 7 d vs. 360 d, $p=0.0079$).



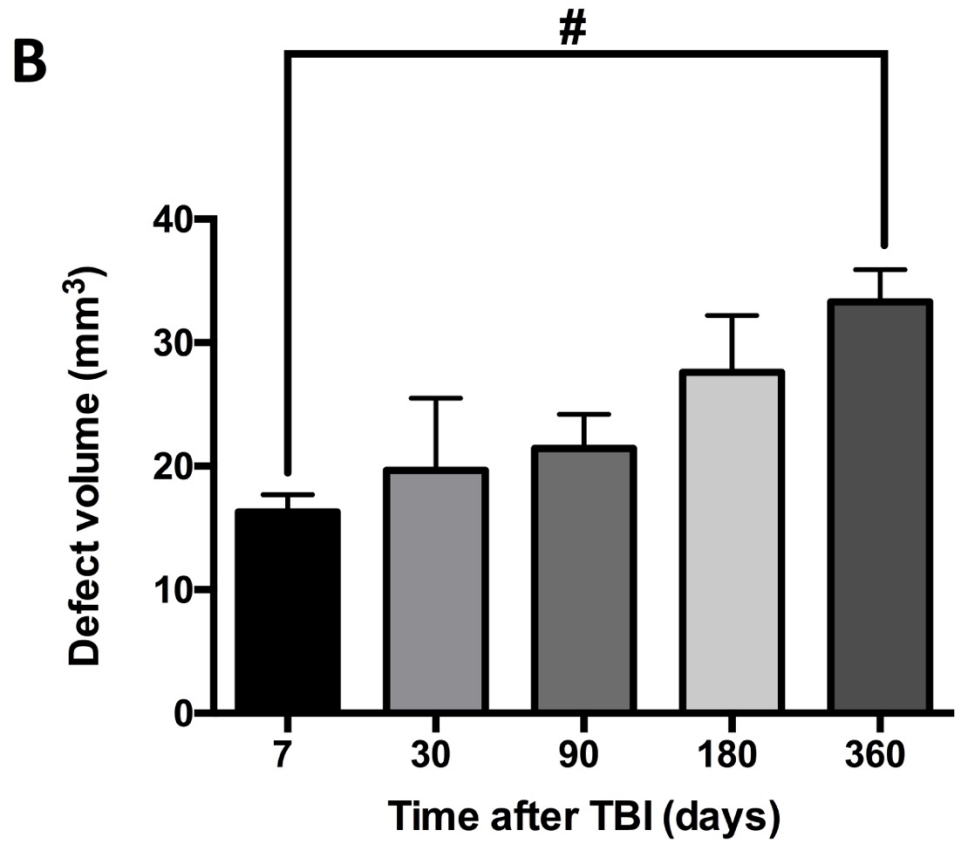


Figure 22: A. Gross histopathology in different groups and different time points; B. Defect Volume. Defect volume significantly increased over time reaching a maximum at 360 d (n=5; #, $p<0.01$).

3.2.2 Hemispheric Atrophy

The atrophy was described by the ratio of traumatized hemispheric volume / non-traumatized hemispheric volume (%). The traumatized hemisphere undergoing significant atrophy over time decreased to 83.16 ± 1.308 %, 77.88 ± 5.949 % , 78.22 ± 2.325 %, 74.30 ± 4.860 %, and 67.69 ± 2.946 % on day 7, 30, 90, 180, and 360 respectively (Figure 23, 7 d vs. 360 d, $p<0.0001$). There was no change in naïve and sham group till 360 d.

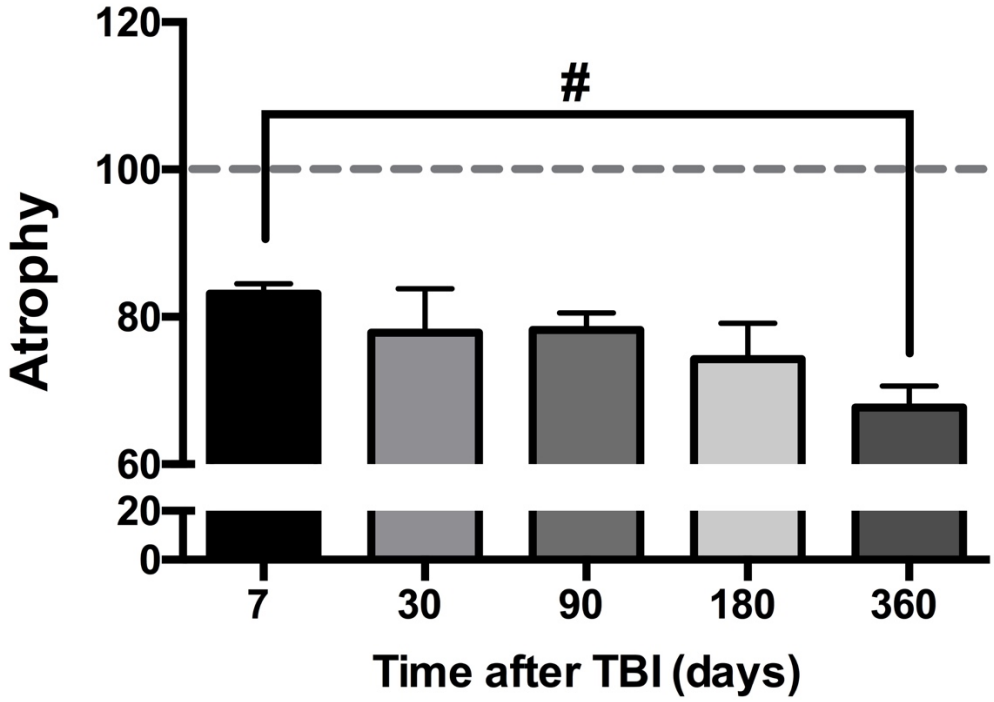


Figure 23: Hemispheric Atrophy. The traumatized hemisphere was undergoing significant atrophy over time (n=5; #, $p<0.01$).

3.2.3 White Matter Injury – Corpus Callosum Thickness

Thickness of the corpus callosum ipsilateral to the traumatic lesion progressively decreased over time (see exemplary section in Figure 24A) and was significantly reduced compared to the contralateral hemisphere on 180 and 360 days after CCI ($p=0.024$, $p=0.007$, respectively, Figure 24B).

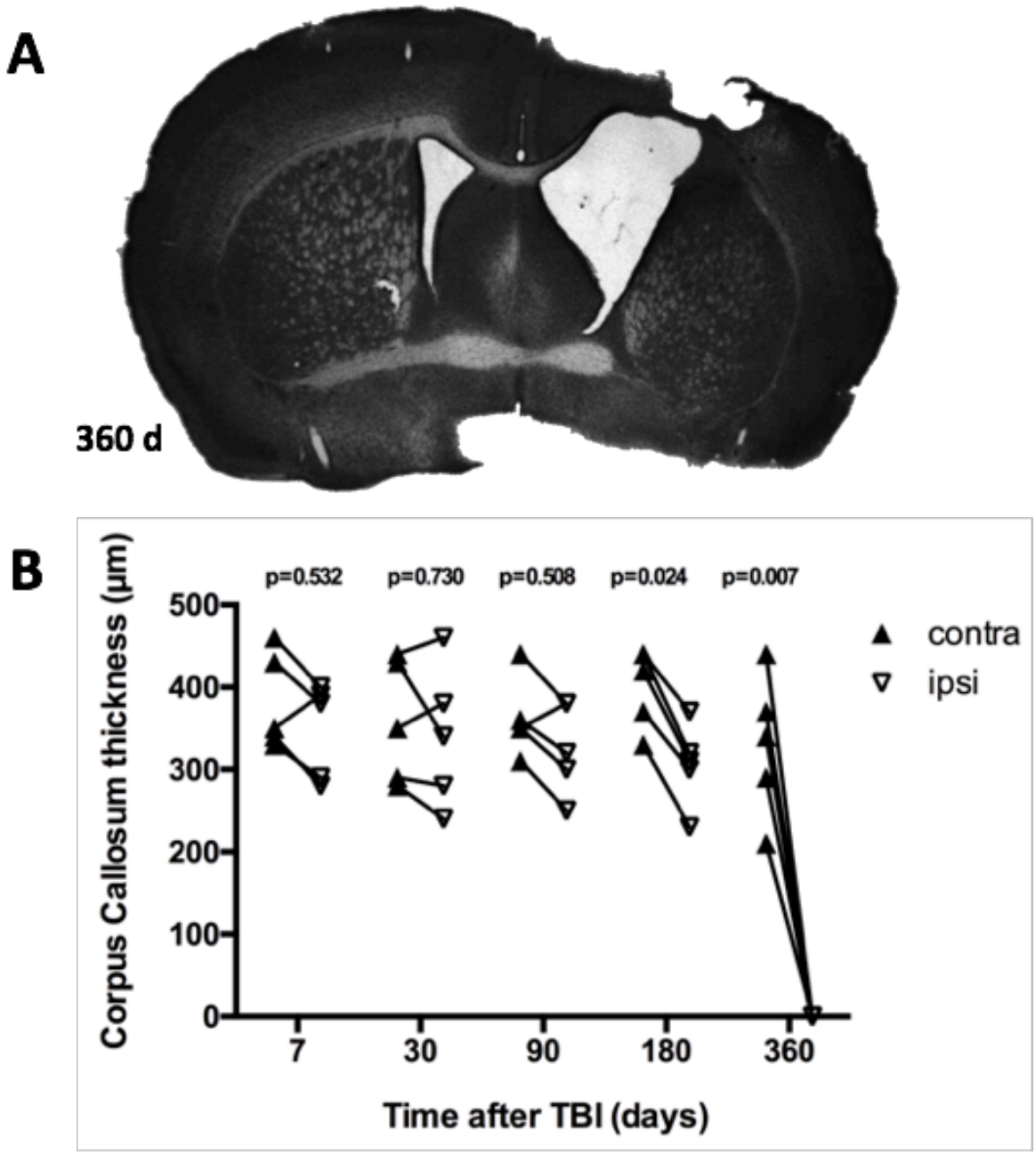


Figure 24: A. Exemplary section obtained 360 d after trauma; B. Quantification. Corpus callosum width decreases over time and this was more pronounced in the traumatized (ipsilateral) hemisphere (n=5).

3.2.4 Hydrocephalus

Ventricle enlargening in the traumatized hemisphere was observable till 360 d after CCI (see Figure 25A for exemplary section). Over time, there was progressive increase of the size of the lateral ventricle of the traumatized hemisphere. The progressive ventricle area ratio (ipsilateral / contralateral) indicates increasing asymmetrical hydrocephalus (Figure 25B).

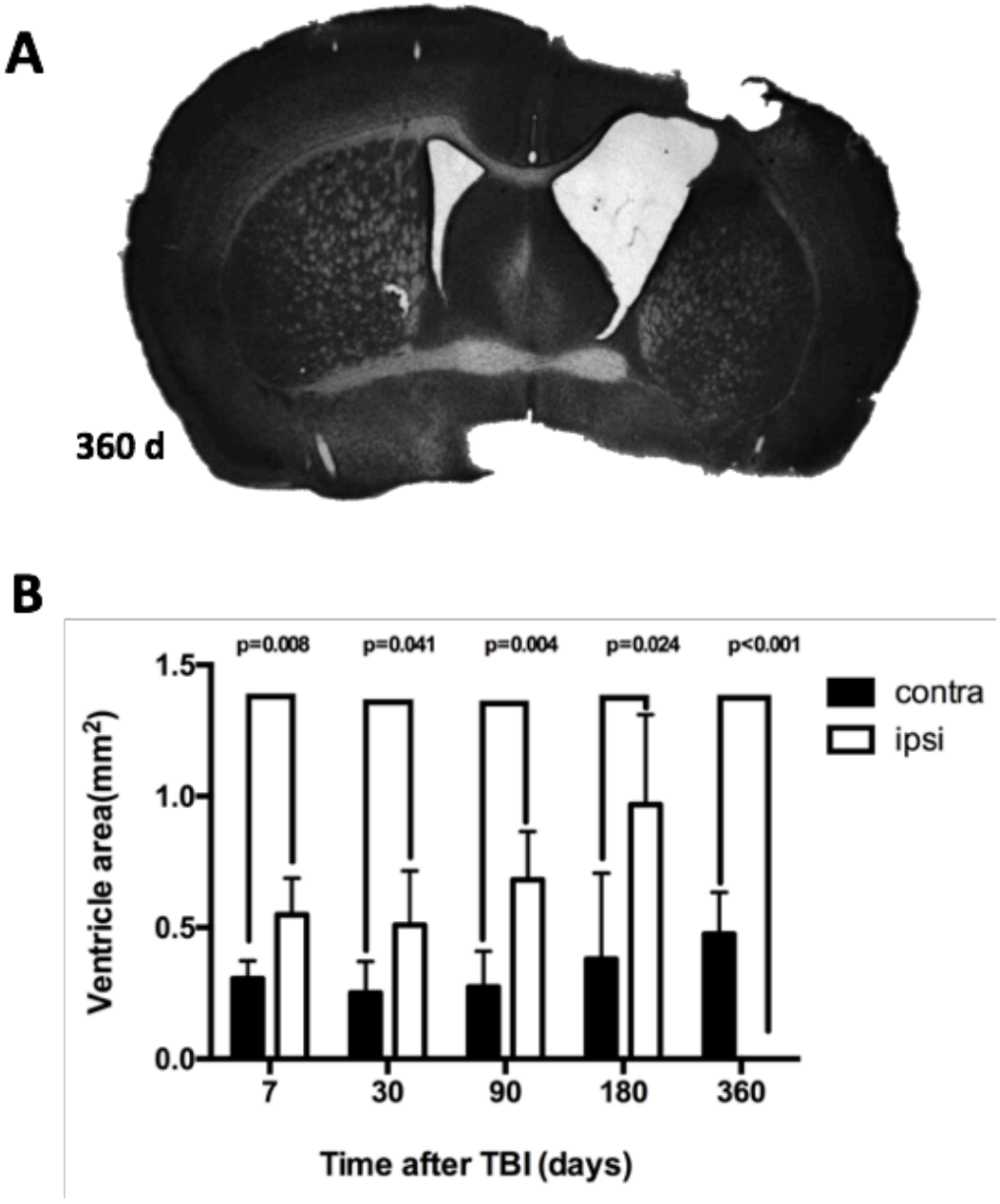
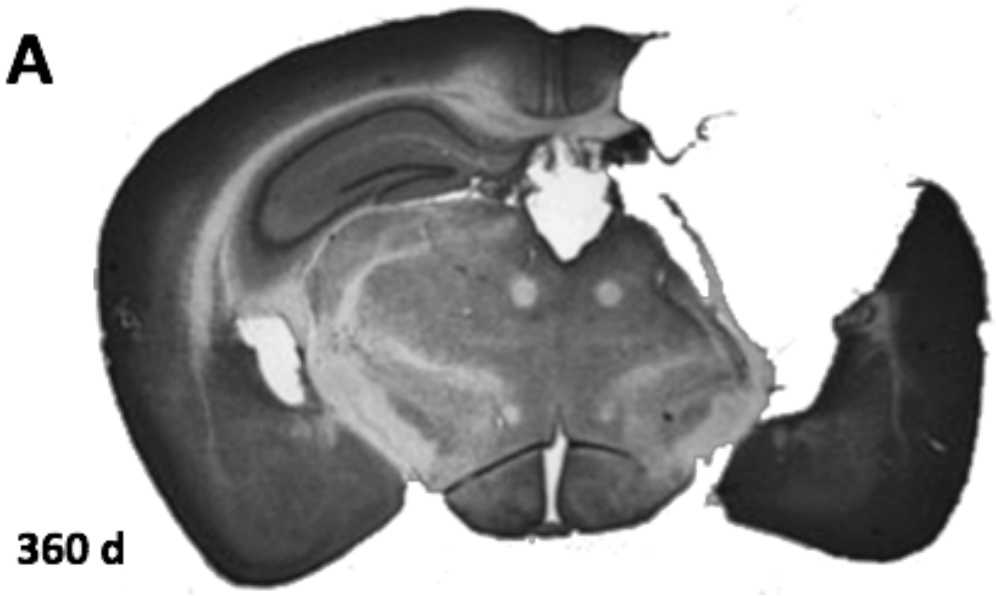


Figure 25: A. Exemplary section at 360 d after trauma; B. Quantification. Over time, there was progressive ventricular enlargening of the lateral ventricle on the traumatized (ipsilateral) hemisphere (n=5).

3.2.5 Hippocampal Damage

Hippocampus damaged in the traumatized hemisphere was observable after CCI (see Figure 26A for exemplary section). There was a significant loss of hippocampal tissue in the traumatized hemisphere which was progressive over time (Figure 26B). Significant reduction of ipsilateral hippocampal area presented as early 7 days after CCI ($p<0.0001$) and aggravated over time reaching a maximum 360 days after CCI (7 d vs. 360 d, $p=0.0069$). For the non-traumatized hemisphere, the hippocampus also changed from 7 d to 360 d after trauma (7 d vs. 360 d, $p=0.0043$).



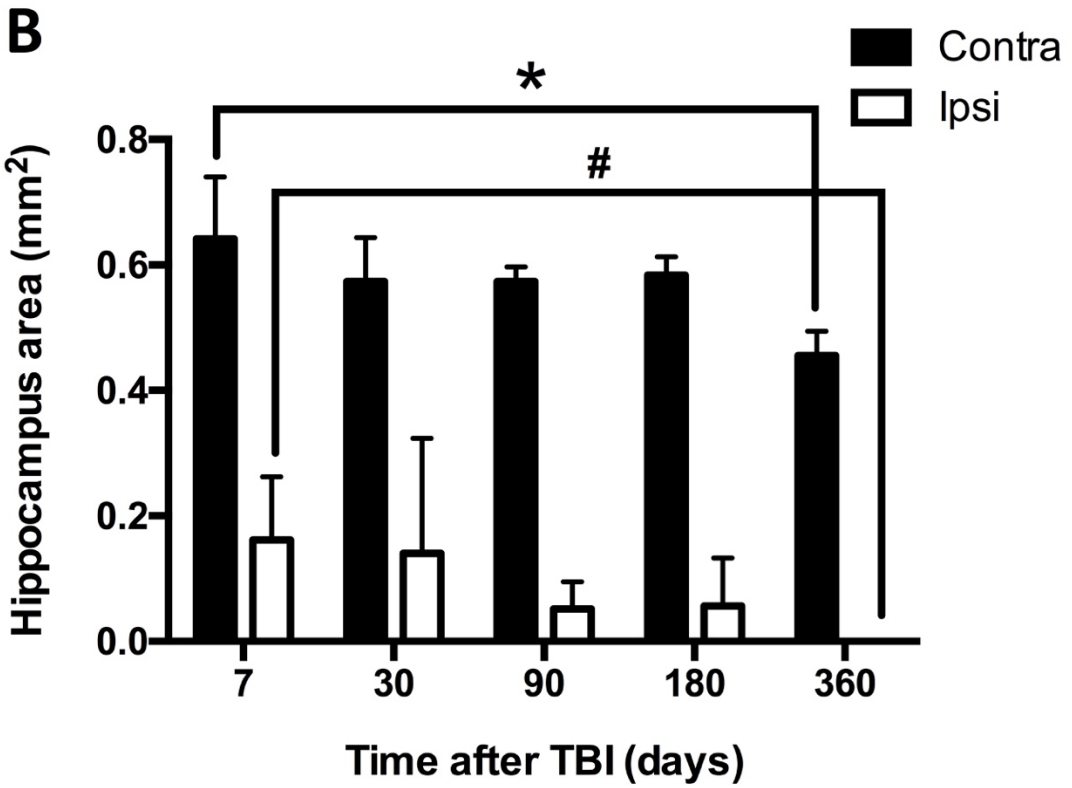


Figure 26: A. Hippocampal damage exemplary slides demonstrating hippocampal defect in the traumatized hemisphere after 360d after CCI; B. Over time, there was progressive hippocampal defect on the traumatized (ipsilateral) hemisphere (n=5).

3.3 Summary of Results

Experimental TBI led to progressive behavioral and histopathological changes. There was a sustained impairment of the general condition as evidenced by stunted weight gain persisting for up to 12 months after single TBI. Motor function was most severely affected in the first few weeks after trauma; animals then recovered quickly but revealed significant deficits up to the end of the observation period. By contrast, depression like symptoms and memory deficits were observable early after trauma but then progressively deteriorated, reaching a maximum at 12 months after TBI. On a histolpathological level, after resorption of acute changes in the contused area, there

was progressive tissue loss leading to an expansion in defect volume, incremental white and grey matter atrophy of the traumatized hemisphere, and increasing ventricular enlargening, consistent with hydrocephalus. These histopathological changes most probably are the pathophysiological correlate for the observed progressive neurocognitive deterioration.

4. Discussion

4.1 Discussion of the Method

4.1.1 Selection of the experimental animal species

The experimental animals chosen for the present study were male C57Bl/6 mice. Mice are the most common experimental animal models used for traumatic brain injury studies because they have a number of advantages when compared to rats: firstly, they can be quickly and inexpensively bred due to their short reproduction cycle, secondly, they offer the possibility of breeding of genetically modified strains. As for the CCI model, our laboratory has vast experience in performing these experiments in mice [97, 105, 110, 111, 113, 138, 194-196, 203-212], therefore it is possible to compare the present data with results obtained by various researchers in the past. We chose to perform this study in young adult male mice as endogenous production of estrogens and progesterone in female mice may influence secondary brain damage and outcome after experimental traumatic brain injury and therefore have a significant impact on our observations [52, 213, 214]. As this is the first study of its kind we primarily wanted to establish the nature and time-course of long-term changes after traumatic brain injury; of course, these findings need to be validated in female and aged animals at a later time point. Another important concern of the mouse model is the animal´s small body size which makes surgical manipulation and preparation more challenging. However, this can be overcome by the intraoperative use of surgical microscopes, microsurgical techniques and adequate surgical training. Also, neuropsychological testing is difficult in mice; this is, however, not restricted on rodents but true for all animal experiments. For this study three well-established paradigms were chosen (see chapter 4.1.4 for a more thorough discussion of this topic). The metabolism of pharmaceuticals in mice can be considerably different from that of humans [215, 216]; as the present study uses a purely observational approach without pharmacological intervention, however, this possible

disadvantage of the mouse model does not apply to the current study. Considering all disadvantages and advantages, we think that the mouse model selected as experimental animal is adequate and suitable for the purpose of the present project.

4.1.2 Selection of the traumatic brain injury model

As traumatic brain injury is a complex disease with many cerebral and extracerebral parameters influencing lesion development and outcome it is unrealistic that one experimental model will adequately reproduce all relevant factors pertaining to TBI pathophysiology. Some patients sustain TBI in the context of multiple/multi-organ trauma [217]; as the aim of the present study was to evaluate the effect of head trauma only we chose a model of isolated head injury. Over the years, many different experimental models for the study of traumatic brain injury pathomechanisms have been developed [218-220], each with specific advantages and disadvantages. When choosing a model for an experimental study it is therefore important to consider each model´s characteristics and its suitability for the intended scientific purpose.

In the present study, the controlled cortical impact model was used. The CCI model has been routinely performed in our laboratory for more than 15 years [105, 110, 111, 113, 117, 138, 194, 203, 205, 206, 208, 209, 211, 212, 221, 222]. All parameters determining contusion size (e.g. impact depth, impact velocity, and dwelling time) can be chosen and adjusted separately and accurately therefore inducing a highly reproducible lesion, a high level of standardization, and high inter- and intra-observer stability. In the present study, an impact depth of 1 mm, an impact velocity of 8 m/s, and a dwelling time of 150 milliseconds was selected as these parameters result in a midsized to large contusion with a well quantifiable secondary lesion expansion of approximately 60% of the initial tissue lesion [105, 110, 194, 203, 212, 221] which mirrors the situation found in patients with traumatic cortical contusions [26]. Furthermore, animals subjected to CCI show

distinct neurological deficits after trauma that persist (and may therefore be quantified) while having a low mortality rate [194, 212]. CCI has been successfully used not only in rodents (rats [223-225], mice [226-229], ferrets [193, 230]), but also in large animal models (swine [231-233], non-human primates [234]) where pathophysiology was similar and comparable to small animal experiments. CCI mainly leads to contusional brain damage which is a very important component of human TBI pathophysiology [74]. Other damage patterns such as diffuse axonal injury do not occur regularly in CCI. However, CCI leads to a variety of well-defined and well-quantifiable secondary phenomena such as intracranial hypertension [105, 194, 203, 221], cerebral ischemia [138], and brain edema formation [47, 105, 212, 222] which are crucial mechanisms for secondary brain injury in humans [88, 235].

The free weight drop model developed by Marmarou et al. predominantly causes diffuse axonal injury and hippocampal cell death [236, 237]. In this model, brain injury is induced by dropping a defined weight which is enclosed in a glass tube from a certain height onto the exposed skull protected with a metal disk. This model may be adjusted and changed in severity by changing the drop height and the weight used. Disadvantages include: re-hits [238], and lack of fixation [239]. Another widely used model for TBI is the fluid percussion injury (FPI) model. In FPI, the traumatic lesion is induced by a pendulum that impacts a fluid-filled tube placed over the epidural space after craniotomy [240, 241]. The impact may be applied lateral or para-sagittal (central) [242]. The mechanical forces disrupt cell membranes, blood vessels, and neuronal processes, and may also lead to subarachnoid/intraparenchymal hemorrhage. Thus, FPI is widely used in experimental TBI research because it replicates clinical contusion without skull fracture. However, the FPI model also has some limitations, such as restricted biomechanical control and inability to reflect the entire complexity of human TBI. This prompted the search for alternative TBI models [243]. Considering all the points mentioned above, we decided to use the CCI technique because of our interest in the pathophysiology of contusions, the

high reproducibility of the CCI model, and its adequate modelling of a majority of pathophysiological factor relevant to human traumatic brain injury.

4.1.3 Histological analysis (Necrosis Volume, Defect Volume, Hydrocephalus, White Matter Injury, and Hippocampus)

There are various methods for the calculation of lesion volume and other parameters of parenchymal injury in experimental TBI. In the present study, we used a histological approach (using 50 µm floating sections) to determine the extent of tissue damage induced by experimental TBI. This evaluation method is highly precise, can be inexpensively performed and does not require specialized infrastructure. Our group has been working for many years with Nissl staining proving that a very high quality standard in the production of tissue sections, high diagnostic accuracy, and low inter-researcher variation can be achieved [105, 194, 212, 222, 244]. Because infiltrating inflammatory cells harbor intact mitochondria, tetrazolium chloride (TTC), a widely employed and easily to use mitochondrial vital stain should not be used for time points beyond 24 hours after injury because injury volumes may be underestimated [245, 246]. Accordingly, Nissl staining may be the most accurate method for the purpose of the current study.

In recent years, CT or MRI is increasingly used in order to evaluate lesion size. The major advantage of radio-morphological quantification of brain injury is that it may be performed repeatedly in on animal allowing to track the lesion over time while all histological methods require to sacrifice the animal. With MRI, it is furthermore possible to determine not only the lesion volume but it also offers the possibility to evaluate cerebral blood flow and brain edema at the same time [247, 248]. However, the evaluation of the MRI data is hindered by pronounced inter-individual variation. Also, it may be difficult to reach the high resolution necessary for the exact determination of

lesion size – especially in mice - , as technical problems can negatively impact image quality and – thus – the feasibility of exanimation. Furthermore, no standardized examination conditions are available yet, so the comparability of results is difficult [249]. It therefore remains unclear what constitutes necrosis /lesioned tissue, because damage areas in MRI imaging are not exclusively comprised of necrosis alone, but are also influenced by other factors such as brain edema formation [249]. Some studies show progressive T2-alterations at the lesioned hemisphere [168, 250] but it remains unclear what the exact structural correlate of this alterations is. Therefore, the lesion detected by MRI may not be exclusively necrotic, which is a major disadvantage compared to histological evaluation used in the present study. Lastly, animals need to be anesthetized repeatedly for the scans which may influence the development of a chronic lesion [251]. In the present study we therefore used histological methods to quantify structural brain damage as this allowed the most exact determination of tissue damage and other parameters of chronic traumatic brain damage even though this meant to use a higher number of animals.

In the present study, we also evaluated other features commonly occurring after TBI in patients that may significantly influence outcome and quality of life [168, 252-254]: hydrocephalus [255], white matter injury [256], and hippocampal damage [257].

Posttraumatic hydrocephalus (PTH) is defined as an active distention of the ventricular system within the brain related to inadequate passage of CSF from its point of production within the ventricular system to its point of absorption into the systemic circulation [255, 258], and is a frequent pathological finding after TBI [259]; its incidence has been estimated to be as high as 45% [151] of patients examined between 3 and 12 months after TBI [260]. Lateral ventricular enlargement in patients has been associated with memory deficits [261], and is considered a predictor for cognitive decline after brain injury [261]. Ventricular enlargement is an obvious structural biomarker of TBI [253]

which can be precisely quantified in brain sections [167, 202, 262, 263], as previously proven by our laboratory [202] (see chapter 4.2.2.4).

Clinical and experimental data indicate that after TBI progressive white matter damage occurs preferentially in the corpus callosum and the external capsule and is associated with axonal pathology in both hemispheres [167, 168, 191, 262, 264]. Atrophy of the corpus callosum is present in 63–86% of severe TBI patients 5 and 20 months after injury [265, 266]. Previous studies showed that the extent of damaged white matter increases with the severity of TBI [267, 268]. The width of corpus callosum is an indicator for the level of white matter injury [202] and it may be exactly quantified in coronal histological sections [202].

Lastly, we assessed the extent of neuronal damage in the hippocampus. Hippocampal change is one of the most common findings in TBI patients [254, 268-270], and this damage pattern is thought to be associated with memory loss [271, 272], and mood disorders [273]. Previous experimental and clinical studies showed that neurons in the hippocampus are highly vulnerable to TBI [262, 269, 274] and that extent of hippocampal damage increases with injury severity [275]. We therefore decided to quantify hippocampal damage histologically in the present study.

4.1.4 Determination of Neurological Outcome (Beam walk, Tail suspension, Barnes maze)

As mentioned above neurological testing is challenging in all experimental animals models, especially those in mice. In the present study we focused our neurological and behavioral evaluation on three areas that are commonly found after TBI and significantly affect patients´ quality of life: motor/gait disturbances [276], depressive behavior [277] and neurocognitive dysfunction such as memory deficits [278]. For this, we chose three

well established test paradigms: the beam walk test [279-281], the tail suspension test [282], and the Barnes Maze test [283].

The beam walk test has been widely used to evaluate motor balance and coordination in mice [279-281, 284]. The test paradigm is easy to learn, the setup inexpensive, and the evaluation by camera cam be standardized very well. Therefore the beam walk test has been shown to have a high reproducibility and a low inter-observer variability after CCI [110, 194, 285]. The test is very sensitive in diagnosing subtle deficits in motor function that may not be detected by other motor tests, such as the Rotarod test [286], cylinder test [287], and grid walking test [288]. The Beam walk test is more sensitive to detect misplacements of the hindpaw, however, the grid walking test is more sensitive to forelimb deficits [289]. Considering these points we selected the Beam Walk test because our CCI model affects mainly the function of hindpaw in mice.

Depression is a heterogeneous, multifaceted disorder. Many of the diagnostic features used in humans (e.g. recurring thoughts of death or suicide), however, can for obvious reasons not be investigated in mice. In order to assess depressive behavior in rodents, several test paradigms were developed. The forced swim test (FST) is probably the most widely used method in preclinical depression research [290-292]. For this test, the mouse is put in a tank filled with water and observed for a certain period of time; the physiological response is vigorous swimming. Immobility is considered a sign of reduced impetus and depressive behavior, the time spent immobile has been shown to correspond to the level of depression. However, immobility in this test may be influenced by body cooling which is dependent on the temperature of the water [293]. While well established, the test is seen critical by some ethics´ committees and judged to be very stressful for the animals, also by the government of Upper Bavaria, the agency responsible for approval of all animal experiments in our university. Therefore, alternatives were developed. The tail suspension test was introduced as a new model for testing anti-depressant compounds in 1985 [198]. For this test, animals are suspended

by the tail, then, as in the FST, mobility and immobility time are assessed over a period of 2 minutes. Immobility/mobility time conforms to depression like behavior. The test is well validated and the correlation with depression-like behavior is solid. Compared to the FST, it is easier to perform, may be done in up to three animals at a time, test conditions can be better controlled, and there is no risk of hypothermia due to submersion in water [294]. Given these advantages this test paradigm was chosen for the present study.

Analogous to depressive behavior, cognitive deficits are difficult to detect and quantify in experimental animals, especially in rodents [105, 203, 295-297]. Maze tests like the Morris Water Maze (MWM) or Barnes Maze test evaluate whether the experimental animals is able to memorize the location of its home-cage. In case of the water maze, the home-cage is an elevated platform that is placed in a basin filled with opaque water. The MWM was designed by Richard G. Morris in 1981 [298] and later adapted to evaluate spatial learning [299]. The animal is put in the middle of the tank and - after an adequate training phase – is quickly able to swim to the platform. After cerebral insults animals take longer to reach the platform as they have problems remembering its location; the distance travelled and the speed of swimming are tracked with a camera, then assessed automatically and are correlated to memory loss [300]. Ennaceur and Delacour subsequently developed the Object Recognition Test (ORT) which is based on the natural propensity of rats to explore novel objects [301]. For this test, animals are placed in a cage with two identical objects; subsequently one of the objects is replaced with a new object in a second session. The ORT is a method to assesses whether an object is recognized or it is considered "new" by the animals [301]; the time spent exploring the changed/new object is considered a measure for recognition and memory function. Different species react differently in the same test, therefore results of the ORT in rats cannot be considered to equal those obtained in mice [302]. Compared to the MWM, the ORT does not require training or food restriction [303]. The disadvantages of the ORT

include non-specific and confounding factors that can affect object exploration times [304]. The Barnes Maze was developed by Dr. Carol Barnes in 1979 [283] and is also regarded as a dry-land version of MWM. As described in chapter 2.6.3, animals were put onto a disk (diameter 100 cm) with 20 identical holes (diameter 10 cm). Scent cues left on the maze by the previous animal may influence the performance of subsequent animals. This, however, can be easily corrected by cleaning the maze after each trial. Various parameters may be analyzed in order to assess the spatial memory such as latency, mean velocity and total distance [305]. Latency, path length to escape box, and number of errors to the first nose poke at the escape hole have been used as measures previously [306, 307]. Thus, measurement of multiple parameters, including latency, error rate, velocity, total distance, and search strategy, may collectively provide a better indicator of each subject's spatial navigational learning and memory ability [308, 309]. Because the path to target is in no way constrained, the escape latency is the most important parameter which is dependent on different coping strategies used to overcome memory deficits [305, 307, 310]. The mice were repeatedly exposed to the test which may lead to habituation and affect the test performance, therefore the test was performed every 90 days only. A major problem of all cognitive tests is that the observed endpoint in these test paradigms is not entirely dependent on neurological functions alone but may also be influenced by motor deficits in the case of Maze tests. In order to better standardize the test and make it more reliable, automated evaluation systems are applied. In the present study, we used the Noldus EthoVision®XT tracking system, which is the most widely used video tracking software that tracks and analyzes behavior, movement, and activity of animals [311]. It is an effective solution for all standard behavioral tests such as the MWM and open field testing and can be combined with sophisticated test-protocols.

Memory tests results may be influenced by many factors such as hypothermia after the MWM test [312]. The most frequently disadvantage of the MWM is that it is overly

stressful which may negatively impact test performance [313] and therefore be avoided [314]. The Barnes maze is considered less stressful and less energy-consuming for mice than the MWM [315] even though stress and/or anxiety cannot be completely prevented [316]. However, considering all factors, the Barnes maze seems the most adequate testing method for the present study.

4.2 Discussion of the Results

4.2.1 Neurological and behavioral changes after TBI

4.2.1.1 Motor/ physical deficits/ body weight after traumatic brain injury

In TBI patients motor deficits are common after severe TBI [317]. Severe motor function often improve until sixth weeks after TBI, however, deficits may persist for years [318]. In the present study, severe motor deficit persisted for up to 12 months after trauma in TBI animals compared to non-traumatized and sham-operated animals. As previously shown, severe motor deficits were present on the acute phase of TBI, and recovered over time after experimental TBI [110, 319, 320]. Interestingly, while the massive increase in missteps observed after CCI was quickly declining within 7 days, traumatized animals then reached a plateau phase with a slight, but significant level of motor impairment/gait disturbance which then persisted throughout the observation period and did not recover even one year after trauma. In clinical studies, almost 25% of patients with TBI suffered from motor problems [321] within the first year after injury [322]. Our results were similar to those of Pischiutta et al. who showed that for the first three months after trauma the number of missteps increased between 3 and 12 months after TBI, however, the authors argued that motor deficits were artefactual due to weight gain of the animals [168]. Recent studies reported that in the chronic stage after TBI the increase in body weight and longer inter-test intervals may be related to a

worsening in performance in the beam walk test [168, 323-325]. However, in our study, body weight was recorded over time in all groups and we did not detect any correlation between motor function and body weight in traumatized, non-traumatized or sham-operated animals. Therefore we think that weight gain is not a confounding factor in our analysis.

Also aging was discussed to be a possible reason for declining long-term motor function due to declining muscular strength and/or neuromuscular function [326, 327] and may therefore be a reason for deteriorating performance in the beam walk test over time [326, 328]. However, we did not detect changes in the control groups so this seems to be a negligible confounder in our study.

In summary, we did not find any significant aging or body weight effect in our control groups so we think weight gain and/or a significant aging effect may be excluded as causes for the differences observed in motor function over time after TBI and that the changes detected are solely due to traumatic brain injury.

4.2.1.2 Behavioral changes after TBI

Psychiatric disorders such as depression [329], mania [329], and posttraumatic stress disorder [330] occur frequently after TBI and significantly impact patients´ quality of life [329, 331, 332]. Depression is one of the most common psychiatric disorders observed after TBI [333]; it affects more than half (52%) of patients within the first year after head injury [334]. More than one third (33–42%) of all depressive episodes after TBI occur within the first year after injury [277, 335], and almost two thirds (61%) are diagnosed within the first seven years [336]. The risk of developing depression is not only associated with moderate and severe TBI, but is increasingly recognized to also correlate with mild TBI [331, 337]. On an anatomical and pathophysiological level, development of depressive symptoms is correlated with disturbances/lesions in frontal lobe, basal

ganglia, and anterior ascending monoaminergic pathways [338, 339]. Focal and diffuse traumatic injury in the dorsolateral frontal, temporal, and/or left basal ganglia have been associated with onset of depression [277, 340-342]. The lesion in our model was increasingly affecting these brain regions over time therefore providing an anatomical explanation for the progressive depressive behavior observed in our experimental series. In experimental TBI, previous studies showed that CCI or closed head injury of varying intensity and severity induced depression-like behavior (as measured with the forced swimming test) till day 90 after trauma [343-346]. Interestingly, the development of symptoms was independent of the severity of TBI [343]. Bajwa et al. reported no difference between sham-operated and CCI mice 90 days after trauma [347], however, a non-traumatized control group was missing. In our study, we did also not detect a difference between traumatized and sham-operated mice until 90 days after TBI. Later sham operated mice recovered and reached the levels observed in the non-traumatized animals 180 days after surgery. A similar behavior was also observed by others using diffuse TBI models [285, 348, 349].

In our experiments the mobility time declined in all groups over time. This may be caused by habituation [350], however, since all traumatized mice were completely immobile at the end of the observation period 360 days after TBI as compared to naïve and sham-operated animals , we conclude that depression-like behavior was indeed caused by the expanding traumatic lesion and cannot be solely ascribed to habituation.

4.2.1.3 Neurocognitive disorders after traumatic brain injury

Cognitive deficits such as memory impairment [167, 351], reduced attention span, and working memory deficits [352] are the most common neurological manifestations observed after TBI and are significantly more frequent than physical impairments [353]. These symptoms are often long-lasting and increasing in severity and have a significant

impact on the quality of life of TBI patients [353, 354]. 42% of patients who suffered severe TBI report cognitive deficits as early as three weeks after injury [354]. A systematic review of 33 studies shows that moderate-to-severe TBI is associated with cognitive deficits that last six months or longer post-injury [355]. After experimental TBI, cognitive deficits are most commonly evaluated by MWM or Barnes maze [199, 356]. Using these test paradigms, it was shown that there is significant acute impairment of spatial learning and memory in animals subjected to traumatic brain injury two days after trauma [356]. These deficits may persist for at least one year after trauma in rodents [168, 263, 357-360]. We chose the Barnes maze testz to evaluate memory functions after trauma (see chapter 4.1.4). Significant deficits were observed as early as 90 days after TBI compared to non-traumatized and sham-operated animals; later on performance continuously and progressively deteriorated. This is consistent with other reports that evaluated mice at 12 months after experimental TBI [167, 263]. Others reported that mice showed an improvement of learning ability over time after TBI, however, memory retention deficits persisted for at least 6 months after injury [168]. While this may be due to declining stress/anxiety responses with habituation [316], authors speculate that this partial recovery may be related to remodeling of surviving neurons [361].

In our experiments incremental neurocognitive changes were associated with significant histopathological changes, namely progressive brain atrophy and hippocampal damage. We assume that general loss of brain tissue and progressive hippocampal damage are the most probable correlates for the progressive cognitive changes we observed. As previously shown in patients, a history of TBI represents a high risk for the development of dementia [362]. More and more clinical studies show that (even a single) trauma may produce progressive histopathological changes in the brains of affected patients who may deteriorate over a long period of time. This phenomenon was mainly described in athletes with a long history of repetitive brain injury such as American football players,

and boxers [363]. Damage to and atrophy of prefrontal cortex and temporal lobe are related to the development of cognitive deficits after TBI [364]. In patients symptoms vary with lesion extent and location, however, in experimental studies, where lesion are much more homogenous, memory deficits clearly correlated with injury severity [365]. Especially hippocampal atrophy (which may also occur during normal aging) is associated with the development of mild memory deficits [366]. Hippocampal damage may therefore be a significant risk factor for cognitive deficits [367, 368]. This is also supported by our results which show that progressive deterioration of memory function up to 12 months after trauma is associated with progressive hippocampal damage.

4.2.2 Morphological and Histopathological Changes after traumatic brain injury

4.2.2.1 Lesion/ defect volume

In the present study, we show that a single TBI triggers progressive neuropathological changes mainly in the traumatized hemisphere. We evaluated several parameters of chronic brain damage, defect volume, hemispheric atrophy, corpus callosum thickness, ventricular enlargement, and hippocampal size in the ipsilateral hemisphere to investigate and characterize these neuropathological changes. Our current model produces a substantial focal lesion, with a defect volume that doubles over the course of the experiment, continuously increasing from about 16 mm^3 on day seven to 33 mm^3 12 months after trauma. These results are well in line with results obtained by Dixon et al. in rats, who compared histopathological outcome three weeks and one year after CCI [263]. Loane et al. demonstrated expanding lesion volumes over time in mice using repetitive MRI scans up to one year after CCI. The significance of these findings, however, remained unclear since contusions evaluated by histological analysis 12 months after TBI were much smaller than the lesion quantified by MRI [250]. In our study, the traumatic

tissue defect is deeper and more extensive, mainly because it comprises not only contused cortex, but also white matter [191, 263]. This may be the explanation why in our study progressive loss of brain tissue was associated with progressive functional deficits.

4.2.2.2 Hippocampal damage

A decrease of hippocampal volume correlates with the occurrence of memory deficits [261], and hippocampal damage is one of the most common consequences of TBI [369]. While hippocampal lesions can impair both spatial and non-spatial tasks, spatial memory is almost exclusively dependent on proper hippocampal function [367]. MRI data following experimental TBI showed that the reduction of ipsilateral hippocampal volume occurred from one day until one year after CCI [168]. In the present study, there was a significant loss of hippocampal tissue in the traumatized hemisphere which was progressive over time. Interestingly, also the hippocampal volume in the non-traumatized hemisphere decreased from 7 d to 12 months after trauma. These changes resulted in memory deficits as also shown by others [366]. Hippocampal damage may therefore be a significant risk factor for cognitive deficits in TBI patients [367, 368].

4.2.2.3 Ventricular enlargement/posttraumatic hydrocephalus (PTH)

PTH is defined as an active distention of the ventricular system within the brain related to inadequate passage of CSF from its point of production within the ventricular system to its point of absorption into the systemic circulation [255, 258] and is a frequent pathological finding after TBI [259]. The incidence of PTH has been estimated to occur in

up to 45% of TBI patients [151, 260]. Possible reasons are reduced CSF resorption (Hydrocephalus aresorptivus), blockage of CSF pathways (Hydrocephalus occlusus), or cerebral atrophy [151, 370]. Lateral ventricular enlargement in patients has been associated with memory deficits [261] and is considered a predictor for bad cognitive outcome after brain injury [261]. Ventricular enlargement is also common in experimental TBI and has been detected up to one year after injury [191, 250, 263, 270]. Experimental studies observed enlargement of the ipsilateral lateral ventricle between one month and one year after injury [191, 262, 263]. In the current study we were able to show that progressive ventricular enlarging of the lateral ventricle on the traumatized (ipsilateral) hemisphere started as early as 7 days after injury and continued for 12 months, the time when the ventricle merged with the traumatic lesion (at 1.0 mm posterior the Bregma level). Together with brain atrophy and hippocampal damage progressive PTH may be another reason for progressive cognitive loss after TBI since PTH is well known to cause such changes in patients [150, 253].

4.2.2.4 Hemispheric atrophy

In TBI patients parenchymal atrophy starts three weeks after moderate to severe brain injury and reaches its maximum 8–12 months later [371-373], findings which were confirmed by experimental studies [191, 263, 270]. The most pronounced atrophy was observed in cortex, hippocampus, thalamus, and septum [191]. Smith et al. showed that progressive post-traumatic atrophy is found up to one year after trauma [191]. In keeping with previous similar results up to one year after FPI [191, 270], the present study demonstrates that the traumatized hemisphere underwent significant atrophy over time. Some studies demonstrated relationships between cognitive outcome and degrees of atrophy after TBI [188, 374]. However, the mechanisms of persistent

cognitive deficits and their relationship to progressive tissue atrophy after TBI are unclear and merit further investigation [263].

4.2.2.5 White matter atrophy/ corpus callosum degeneration

Six weeks after TBI 30% of patients display degeneration of white matter tracts [372, 375]. White matter seems to be particularly vulnerable to chronic degeneration after [353, 376] since progressive white matter volume loss has been shown to continue up to 4 years after TBI [263, 377]. Further on white matter damage is an important determinant of cognitive deficits after TBI [378]. Little is known about the temporal dynamics of white matter damage after single experimental TBI. One recent study using repetitive MRI (diffusion tensor imaging, DTI) in a mouse model of single CCI trauma showed evidence for progressive white matter damage spreading from the ipsilateral to the contralateral hemisphere starting 6 months after a single focal CCI injury and increasing up to 12 months after TBI [168].

In our study, thickness of the corpus callosum ipsilateral to the traumatic lesion progressively decreased over time and was significantly reduced compared to the contralateral hemisphere 180 and 360 days after CCI, but the corpus callosum of the contralateral hemisphere was not affected up to 360 days after CCI. This is not in line with the results of the study of Pischiutta et al., however, in their study white matter changes were investigated by MRI, a method which may overestimate tissue damage (see above).

4.2.3 Conclusion

CCI led to a significant and progressive reduction of body weight, motor function, and memory and caused depressive-like behavior. The volume of tissue loss increased

continuously over time and reached a maximum 360 days after CCI. Histopathology revealed progressive atrophy of the ipsilateral hemisphere, of ipsilateral white matter tracts, and of the ipsi- and contralateral lateral hippocampus. A significant and increasing enlargement of the ipsilateral lateral ventricle was detectable, consistent with progressive hydrocephalus formation. Hence, the present study provides a comprehensive overview of the histological and neurocognitive long-term sequels of a single focal brain injury and may therefore serve as an important basis study for future investigations into the mechanisms of progressive post-traumatic brain injury.

5. Summary and conclusion

5.1 English summary

Traumatic brain injury (TBI) is a major public health problem in developed as well as developing country. In past decades, clinical and pre-clinical research efforts focused on the acute, life-threatening pathophysiological events after TBI. Previous research showed that TBI is a risk factor for cognitive decline. The aim of the present study was to characterize the development of chronic neuropathological and neurobehavioral changes in a widely used mouse model of TBI, controlled cortical impact (CCI) injury, over a period of 360 days. Male C57BL/6N mice (n = 10 per group) underwent CCI or sham operation and were observed together with naïve animals for 15 min, 1, 7, 30, 90, 180, and 360 days. Using Beam Walk, Tail Suspension, and Barnes´ Maze tests, we evaluated motor function, depression like behavior, and spatial learning and memory. Histopathological changes were assessed using immunohistochemistry. CCI led to progressive deterioration of motor and memory function and induced increasing depressive behavior. Injury volume increased continuously and reached its maximum 360 days after CCI while the ipsilateral hemisphere became atrophic, including the white matter and the hippocampus. A significant enlargement of the ipsilateral lateral ventricle was detectable, consistent with hydrocephalus formation. The present study demonstrates that after an initial improvement TBI causes progressive chronic neuropathological and behavioral deficits up to 12 months after injury. Hence, a second, chronic wave of secondary brain damage occurs after TBI, which may be modulated by pharmacological treatment or neurorehabilitation.

5.2 Zusammenfassung und Ausblick

Schwere Schädel-Hirn-Traumata (SHT) führen oft zu bleibenden Behinderungen und Beeinträchtigungen. Während die Mechanismen der akuten Hirnschädigung nach SHT inzwischen relativ gut erforscht sind, gibt es bisher wenige Erkenntnisse zum Langzeit-Verlauf des posttraumatischen Hirnschadens, der in Zusammenhang mit sich erst im Verlauf der Erkrankung entwickelnden Symptomen wie progredientem neurokognitiven Abbau und neuropsychologischen Defiziten gebracht wird. Zur Untersuchung dieser chronischen Vorgänge sind experimentelle Modelle notwendig, die die humane Pathologie nachbilden. Das Ziel der vorliegenden Studie war die Entwicklung eines Maus-Modells zur Untersuchung des chronischen Hirnschadens; als Modell wurde das weit verbreitete Controlled Cortical Impact - Modell verwendet, das eine gut standardisierbare und quantifizierbare Läsion induziert. Für die Studie wurden männliche C57 BL/6 Mäuse einem experimentellem Controlled Cortical Impact SHT (8m/s Aufprallgeschwindigkeit, 1 mm Eindringtiefe, 150 ms Kontaktzeit, über dem rechten parietalen Kortex) unterzogen und für 15 min, 24 Stunden, 3, 7 Tage oder 1, 2, 6 oder 12 Monate beobachtet (n=10/Gruppe). Als Kontrollgruppe dienten schein-operierte und nicht-traumatisierte (naive) Tiere. Die Tiere wurden regelmäßig mit unterschiedlichen Testverfahren untersucht: zur Evaluation motorischer Defizite mit dem Beam Walk Test, zur Bestimmung depressiver Symptome mit dem Tail Suspension Test sowie zur Quantifizierung der Gedächtnisleistung mit dem Barnes Maze Test. Am Ende der jeweiligen Beobachtungsphase wurden die Gehirn entnommen und histopathologisch untersucht. Das CCI-Trauma führte zu einer signifikanten und progredienten Verschlechterung der motorischen Funktion, der Konzentrations- und Gedächtnisleistungen sowie zu zunehmend depressivem Verhalten. Das Läsionsvolumen nach bis zum Ende der Beobachtungszeit kontinuierlich zu. Histopathologisch zeigte sich eine zunehmende Atrophie der ipsilateralen Hemisphäre, der weißen Substanz (Corpus callosum) und beider Hippocampi. Zudem war eine deutliche Erweiterung des

ipsilateralen Seitenventrikels nachweisbar, vereinbar mit der Ausbildung eines Hydrocephalus.

Die vorliegende Studie liefert erstmals einen umfassenden Überblick über den zeitlichen Verlauf der histologischen und neurokognitiven Veränderungen nach einer einmaligen fokalen Hirnläsion. Die Ergebnisse dieser Studie stellen eine wichtige Basis für nachfolgende Untersuchungen da und ermöglichen die Untersuchung der Mechanismen, die den beobachteten progressiven Langzeitschäden zugrunde liegen.

6. References

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7. List of Abbreviations

TBI	Traumatic Brain Injury
WHO	World Health Organization
GCS	Glasgow Coma Scale
CT	Computed Tomography
MRI	Magnetic Resonance Imaging
DAI	Diffuse Axonal Injury
ICP	Intracranial Pressure
EAAs	Excitatory Amino Acids
AQP-4	Aquaporin-4
AIF	Apoptosis-Inducing Factor
NO	Nitric Oxide
eNOS	endothelial Nitric Oxide Synthase
nNOS	neuronal Nitric Oxide Synthase
iNOS	inducible Nitric Oxide Synthase
ICAM-1	Intracellular Adhesion Molecule-1
BBB	Blood Brain Barrier
MMP	Metallo-Matrix-Proteins
CSF	Cerebrospinal Fluid
CPP	Cerebral Perfusion Pressure
MAP	Mean Arterial Pressure
DC	Decompressive Craniectomy
CTE	Chronic Traumatic Encephalopathy
NFL	National Football League
AD	Alzheimer’s Disease
ptAD	post-traumatic Alzheimer's Disease

CCI	Controlled Cortical Impact
PFA	Paraformaldehyde
PBS	Phosphate Buffered Saline
FST	Forced Swim Test
ORT	Object Recognition Test
MWM	Morris Water Maze
FPI	Fluid Percussion Injury
PTH	Posttraumatic Hydrocephalus
DTI	Diffusion Tensor Imaging

8. Already Published Aspects of the Work

07/2017 *"CHRONIC HISTOPATHOLOGICAL AND FUNCTIONAL OUTCOME AFTER CONTROLLED CORTICAL IMPACT TRAUMA IN MICE"*
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I hereby declare, that the submitted thesis entitled

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