Progressive histopathological damage occurring up to one year after experimental traumatic brain injury is associated with cognitive decline and depression-like behavior

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Abstract

Increasing clinical and experimental evidence suggests that traumatic brain injury (TBI) is associated with progressive histopathological damage. The aim of the current study was to characterize the time course of motor function, memory performance, and depression-like behavior up to one year after experimental TBI and to correlate these changes to histopathological outcome.

Male C57BL/6N mice underwent controlled cortical impact (CCI) or sham operation and histopathological outcome was evaluated 15 min, 24 hours, 1 week, 1, 3, 6, or 12 months thereafter (n=12 animals per time point). Motor function, depression-like behavior, and memory function were evaluated concomitantly, MRI imaging repeatedly performed. Naïve mice (n=12) served as an unhandled control group.

Injury volume almost doubled within one year after CCI ($p=0.008$) and the ipsilateral hemisphere became increasingly atrophic ($p<0.0001$). Progressive tissue loss was observed in the corpus callosum ($p=0.007$) and the hippocampus ($p=0.004$) together with hydrocephalus formation ($p<0.0001$). Motor function recovered partially after TBI, but six months after injury progressive depression-like behavior ($p<0.0001$) and loss of memory function ($p<0.0001$) were observed.

The present study demonstrates that delayed histopathological damage which occurs over months after brain injury is followed by progressive depression and memory loss, changes also observed after TBI in humans. Hence, experimental TBI models in mice replicate long-term sequels of brain injury such as post-traumatic dementia and depression.

Keywords: Traumatic brain injury, controlled cortical impact, cognitive function, head trauma, degeneration

Introduction

Traumatic brain injury (TBI) is a major cause of death and permanent disability worldwide, especially in children and young adults [1, 2]. According to a WHO forecast, the incidence of severe traumatic brain injury will significantly increase until 2030 further aggravating the huge socio-economic burden caused by TBI [3].

Due to improved emergency and critical care medicine mortality after severe TBI is constantly declining in industrialized countries [4-6]. However, it is increasingly recognized that TBI does not only acutely damage the brain, but has also long-term neurological and neuro-psychiatric sequelae that sustainably affect the quality of life of TBI survivors [7, 8]. Symptom onset may occur years after the initial trauma making correct diagnosis challenging and sometimes impeding patients from getting adequate medical support [9]. Common symptoms are impaired fine motor skills, cognitive decline, and mood disorders [10, 11]. Furthermore it has been suggested that TBI predisposes patients for cognitive decline or even dementia [12-15]. Mechanisms underlying these functional deficits in TBI patients may be progressive brain atrophy [16, 17] and hydrocephalus formation [18-21] as a consequence of ongoing neuroinflammation [22, 23]. Post-mortem studies in athletes that suffered repetitive TBIs during their careers suggest that also extensive microfibrillary tangle formation due to tau-protein aggregates and amyloid beta deposits may be involved in this process. So far, it has been established, that inflammatory changes are one important factor for the development of chronic posttraumatic changes [24-26]. The exact cellular and molecular mechanisms of chronic TBI are, however, not fully understood.

In contrast to research on humans, animal models of TBI are particularly suited to study the mechanisms of chronic TBI. Injury severity can be tightly controlled, and injury progression can

be studied not only in autopsy material, but also during the time course of the disease thereby allowing mechanistic insight into the underlying pathophysiology. In fact, progressive neurodegeneration and tissue loss were elegantly demonstrated by magnetic resonance imaging and by histopathology after controlled cortical injury (CCI), one of the most widely used TBI models [26]. However, compared to the extensively studied pathophysiological changes in the early posttraumatic period, comparatively little is known about the long-term functional consequences following experimental TBI. Therefore, the aim of the current study was to investigate whether functional deficits frequently observed in TBI patients months and years after injury, such as motor dysfunction, memory loss, or mood disorders also occur after experimental TBI in mice.

Material and Methods

All surgical procedures were carried out in accordance with the guidelines of the animal care institutions of the University of Munich and approved by the Bavarian government (protocol number 55.2-1-2532-44-2017). Results are reported according to the ARRIVE guidelines (Kilkenny et al., 2010). Animal care, husbandry, and health checks were performed according to the FELASA recommendations (Guillen, 2012). Mice were randomly assigned to experimental groups by drawing lots. Surgical preparation, trauma, neurological testing, MRI scanning and data analysis were performed by investigators blinded to the treatment of the animals. The following experimental groups and time-points were examined: naïve animals (non-traumatized; n=12), sham surgery (n=12), post-traumatic groups evaluated at 15 minutes, 1, 7, 30, 90, 180, and 360 days after CCI (n=12 each). **Figure 1A** shows a graphic summary of the experimental groups.

Controlled cortical impact (CCI)

Traumatic brain injury was induced by the previously described Controlled Cortical Impact method (Engel et al., 2008;Terpolilli et al., 2009;Terpolilli et al., 2013). Anesthesia was induced after injection of Buprenorphin (100 mg/kg i.p., 30 min prior to surgery) by 1.2% isoflurane in 30% oxygen/70% air in spontaneously breathing animals. Briefly, after right parietal craniotomy (4 x 5 mm), the impact (tip size 3mm) of our custom made CCI device (L. Kopacz, University of Mainz, Germany) was directly applied to the dura with 8 m/s velocity, 1 mm impact depth, and 150 ms contact time (see **Figure 1B** for schematic drawing). The craniotomy was closed immediately afterwards using histoacrylic glue. All surgical procedures were performed on a feedback-controlled heating pad (FHC, Bowdoinham, USA) that maintained core body temperature 37°C.

In order to avoid postoperative hypothermia, animals were kept in an incubator heated to 32°C for two hours. In the sham group, craniotomy was performed without applying the impact.

Outcome measures

Body weight

Body weight was measured immediately before CCI and every day after trauma for the first 7 days, then every month after trauma. Body weight is expressed in percent of pre-trauma body weight.

Beam walk assessment

As previously reported, the beam walking test is an adequate tool to test motor function after CCI, either alone(Zweckberger et al., 2003) or as part to the Neurological Severity Score.(Terpolilli et al., 2009;Terpolilli et al., 2013) The number of missteps of the hind paw and the time needed to cross the one meter beam were recorded starting 3 days before trauma, at 1,3, and 7days after trauma; subsequently, mice were tested every month until the end of the observation period.

Tail suspension test

The tail suspension test has been widely used to study depression-like behavior in mice [27, 28]. The animal is suspended head-down by the tail for three minutes under continuous video-monitoring (EthoVision®XT, Noldus Information Technology, Netherlands). The time the mouse moves in order to recover balance (mobility time) and the time the mouse does not move and more (immobility time) are recorded. Longer immobility time indicates depression-like behavior. The test was performed 90, 180, 270, and 360 days after CCI.

Barnes maze test

The Barnes maze test is a paradigm to study spatial learning and memory in rodents [29, 30]. The test is performed on a brightly lit round platform (diameter 100 cm) that contains 20 identical holes (diameter 10 cm) spaced evenly around the perimeter of the platform. Below one of these apertures there is a box (20 X 5 X 3 cm) where the mouse can hide. The mouse learns where the box is located and the time the mouse needs to find the right aperture within the test duration of 180 seconds is evaluated by video monitoring (EthoVision®XT, Noldus Information Technology, Netherlands). Animals are trained daily for 5 days before the actual experiment [30]. The goal box location was the same in all testing runs. After CCI the test is performed at days 90, 180, 270, and 360 after CCI.

Magnetic resonance imaging (MRI)

MRI was performed in a small animal scanner (3T nanoScan® PET/MR, Mediso, Münster Germany with 25 mm internal diameter quadrature mouse head coil) at 15 min, 24 h, 7 days, 1, 3, 6, 9, and 12 months after CCI. For scanning, mice were anesthetized with 1.2% isoflurane in 30% oxygen/70% air applied via face mask. Respiratory rate and body temperature (37 +/- 0,5 °C) were continuously monitored via an abdominal pressure-sensitive pad and rectal probe and anesthesia adjusted to keep them in a physiological range. The following sequences were obtained: coronal T2-weighted imaging (2D fast-spin echo (FSE), TR/TE = 3000/57.1 ms, averages 14, resolution 167 x 100 x 500 um3), coronal T1-weighted imaging (2D fast-spin echo (FSE), TR/TE = 610/28,6 ms, averages 14, resolution 167 x 100 x 500 um3), and DWI (2D spin echo (SE), TR/TE = 1439/50 ms , averages 4, resolution 167 x 100 x 700 um3). MRI data was then post-processed post-processing using Image J; 14 sections surrounding the lesion were chosen

for each dataset and the lesion area measured using the polygon tool. Volume was then calculated using the following equation, V = d*(A1/2 + A2 + A3…+ An/2), with d being the distance between sections in mm 0., and A being the measured area. Hemispheric volume was calculated by measuring the area of each hemisphere separately in all sections obtained; volume for each hemisphere was then calculated according the above-mentioned formula. Volume of the traumatized hemisphere is expressed as percentage of ipsilateral total hemispheric volume.

Histopathology

For histological evaluation 15 min, 24 h, 7 days, 1, 3, 6, and 12 months after CCI, coronal floating sections of the brain were prepared as previously described [31]. After transcardial perfusion with 0.9% NaCl followed by 4% PFA using a pressure-controlled perfusion system (Perfusion One, Leica Biosystems, Richmond, VA, USA), brains were removed and stored in 4% PFA at 4°C for 12-16 hours for post-fixation. Brains were stored in phosphate buffered saline (PBS) until further use. Fifty micrometer thick coronal sections were prepared using a vibratome (Leica VT1200S, Nussloch, Germany) starting 1000 µm behind the olfactory bulb; a total of 12 sequential coronal 50 µm thick sections were collected every 600 µm and stained with cresyl violet.

Evaluation of Defect Volume

Gross examination revealed that tissue is lost in the contused area seven days after CCI. Therefore, we did not determine contusion volume as previously described (Terpolilli et al., 2009;Zweckberger et al., 2003), but the defect area (**Figure 1C**)(Zweckberger et al., 2003;Zweckberger et al., 2006) according to the following formula:

Defect Area (DA) = A (area contralateral hemisphere) – C (area contralateral ventricle) – B (area ipsilateral hemisphere).

The defect volume was then determined using the following formula:

V = d*(DA1/2 + DA2 + DA3…+ DA/2) with d = 0.6 (distance between two sections in mm).

Determination of grey and white matter atrophy, hydrocephalus, and hippocampal volume

Quantification of white matter/corpus callosum atrophy and hydrocephalus was performed as previously described [32]. Maximum corpus callosum thickness was assessed in a coronal section at Bregma level by histomorphometry (see **Fig. 1D** for exemplary picture). Hemispheric atrophy was determined using one coronal section (bregma +1.0 mm) and expressing the area of the ipsilateral hemisphere as percentage of contralateral hemisphere area. For assessment of hippocampal damage underneath the contusion one coronal brain section (bregma -2.0 mm) was used to measure the total hippocampal area (see **Fig. 1E** for exemplary picture). Ventricle enlargement was assessed at the level of the lateral ventricles by histomorphometry (see **Fig. 3A** for exemplary pictures at six and 12 months after TBI).

Statistical Analysis

Sample size calculations were performed using SigmaPlot (Version 13.0, Jandel Scientific, Erkrath, Germany) with the following parameters: alpha error = 0.05, beta error = 0.2, standard deviation 22%, and a target effect size of 30%. Due to the group size non-parametrical tests were used for further analysis. Hence, the Mann-Whitney rank sum test was used for comparisons between two groups and the Kruskal-Wallis test with Student-Newman-Keuls post hoc test was performed for multi group comparisons. Differences between groups were

considered statistically significant at $p < 0.05$. All data is expressed as mean ± standard deviation (SD).

Results

Histopathological changes

Brains sampled 15 min and 24 hours TBI showed a hemorrhagic contusion upon macroscopic inspection, while brains from naïve or sham-operated mice (data not shown) had no damage (**Fig. 2A**). Already seven days after TBI the contused tissue was removed and replaced by a cyst filled with CSF. The volume of this cyst was significantly smaller than the initial contusion and the contusion observed seven days after injury (**Fig. 2A**). This finding is well in line with previous reports from our laboratory and the literature (Loane et al., 2014;Zweckberger et al., 2006). With time, the volume of the cyst increased and one year after TBI the volume and the depth of the cyst increased so much that white matter structures became visible at the bottom of the cavity (**Fig. 2A**). When quantified by histological analysis, the volume of the defect was found to significantly increase in an almost linear fashion until one year after trauma (**Fig. 2B**, see **Supplemental Fig. 1** for exemplary coronal sections; 7d vs. 30d, 90d, 180, 360 d: $p<0.05$; 180d vs. 7, 30d, 90d: $p<0.05$). While the cyst increased in size, we observed a pronounced atrophy of the whole traumatized hemisphere over time (**Fig. 2C**) at 7 days after trauma (83 ± 1% of the contralateral hemisphere) which continued to 74 ± 5% 180 days and 68 ± 3% 360 days after TBI (day 7 vs. all later time points: $p < 0.05$; 30d, 90d, 180d vs. 360 d: $p<0.05$). At the same time the size of the ipsilateral ventricle enlarged significantly (**Fig. 3A**). At 7 days after TBI there was asymmetrical hydrocephalus resulting in enlargement of the ventricle area ipsilateral to trauma (**Fig. 3B**). The thickness of the corpus callosum ipsilateral to the injury also decreased significantly over time (**Fig. 3C**). Ninety days after trauma only a slight asymmetry between the left and the right corpus callosum was visible, but at later time-points the ipsilateral corpus

callosum became significantly narrower than the contralateral one (180 d after CCI: p = 0.024, 360 d: p = 0.007). Lastly, we evaluated hippocampal damage as a potential morphological correlate for memory deficits (**Fig. 4**). While in most cases the hippocampus is well preserved within the first 24 hours after TBI (data not shown), the ipsilateral hippocampus was completely absent 360 days after TBI (**Fig. 4A**). This process was initiated within the first week after trauma, since already at this early time point the area of the ipsilateral hippocampus was significantly reduced by about 75% ($p < 0.0001$). Hippocampal atrophy aggravated over time until no ipsilateral hippocampal tissue could be detected 360 days after CCI (7 d vs. 360 d, p = 0.007, **Fig. 4B**). Concomitantly, also the contralateral hippocampus became atrophic, but to a far lesser degree as compared to the ipsilateral side (p = 0.004 7 vs. 360 days).

MRI imaging

MRI scanning closely mirrored the results obtained by histology. The exemplary timeline of T2 weighed scans obtained from 15 minutes until 12 months after CCI displayed in **Fig. 5A** shows an increasing defect formation over time. Quantification (Fig. 5B) reveals differences compared to histological examination. While at earlier time points lesion quantification is complicated due to tissue necrosis, edema, and hemorrhagisation resulting in volumes higher than those in histology, from one month after TBI on, the same progression of defect size was evident (1 months vs. 6,9,12 months: $P < 0.01$). Atrophy of the traumatized hemisphere was also visible in MRI scanning. After initial brain edema formation with subsequent swelling which lead to an increase of the ipsilateral versus the non-traumatized hemisphere, there was progressive atrophy to approximately the same level observed in histology (71±2.6% of contralateral hemisphere volume, 7d, 1 month, 3 months vs. 360 d: $p < 0.01$).

Functional Outcome

No epileptic seizures were observed during routine check-ups, handling, or neurological testing of traumatized, sham-operated, or naïve animals. All animals survived until the end of the respective observation period.

Naïve animals continuously gained weight during the observation period of one year (**Supplemental Fig. 2**). Sham-operated and traumatized mice, however, gained significantly less weight and only sham-operated mice started to recover their body weight 270 days after TBI and reached the level of naïve mice at the end of the observation period; traumatized animals had a flatter weight gain curve and did not reach the body weight of naïve or sham-operated mice (naïve: 41.3 ± 1.9 g, sham: 40.4 ± 2.3 g, CCI: 34.4 ± 1.5 g for the CCI group; $p < 0.0001$ vs. naïve, $p < 0.0001$ vs. sham).

Motor function as assessed by the beam walk paradigm was not different between groups before surgery (**Fig. 6A and B**). After TBI the time needed to cross the beam (**Fig. 6A**) as well as the number of missteps (**Fig. 6B**) increased massively in traumatized animals, while naïve mice and sham-operated animals performed as before trauma. The performance of traumatized animals improved substantially within one month after CCI, however, motor function was still significantly impaired as compared to pre-trauma levels and to both control groups. Thirty days after TBI the initial phase of improvement ended and traumatized animals suffered a residual and stable motor dysfunction until the end of the observation period (CCI vs. own pre-trauma baseline, $p < 0.0001$; CCI 360 d vs. naïve 360 d, $p < 0.0001$; CCI 360 d vs. sham 360 d, $p < 0.0001$). In order to detect and quantify depression-like behavior we used the Tail Hanging test (**Fig. 7**). Before trauma there was no difference in mobility time among groups (naïve: 33.0 ± 22.2 s,

sham: 31.0 ± 9.7 s, CCI: 32.6 ± 7.4 s). Over the course of the experiment, mobility time declined in all groups, most probably due to habituation and/or weight gain, however, sham-operated as well as traumatized animals displayed shorter mobility times 90 days after TBI (sham vs. naïve, $p = 0.05$; CCI vs. naïve, $p = 0.05$). Like with body weight, sham-operated animals recovered to the level of naïve mice one year after injury, but mobility time of mice subjected to TBI further declined over time and 360 d after trauma all traumatized mice remained completely immobile when hanged head-down by the tail (CCI vs. sham, $p < 0.0001$; CCI vs. naïve, $p = 0.0002$).

Finally, we assessed orientation, memory, and learning using the Barnes Maze paradigm. Exemplary heat-maps of the movement of single mice obtained 270 days after TBI, illustrate that naïve (top panel) and sham-operated animals (middle panel) were able to find the home-box quickly and with no delay. In contrast, the traumatized mouse (bottom panel) found the home box only after extensive, random searching (**Fig. 8A**). This loss of memory function is also represented by the quantitative analysis: naïve and sham-operated mice showed normal memory function in terms of the time needed to find the home-box (latency), the distance travelled to find the home-box (distance travelled) and the search seed (velocity), while traumatized mice showed a significantly reduced performance of all investigated parameters already three months after injury (**Fig. 8B-D**). Despite the fact that the distance travelled remained almost stable over time, the latency to find the home-box increased 180 days after TBI and reached a maximum one year after injury, suggesting progressive loss of memory function in brain injured mice starting six months after trauma.

Discussion

In the current study we assessed the behavioral and histopathological effects of a single cortical contusion up to one year after experimental traumatic brain injury in mice. On the histopathological level, we described the time course of gray and white matter atrophy, the loss of hippocampal tissue, and the formation of hydrocephalus. This was corroborated by MR imaging; the differences observed, especially in the early phase after TBI, most probably are due to residual brain swelling, possible hemorrhagic tissue within the contusion, and the fact that MRI scans were performed on many more levels than histology. On the functional level, we demonstrated improvement of motor dysfunction over time, but loss of memory and depression-like behavior beginning six months after and progressing up to one year after TBI. Since brain atrophy, progressive loss of memory function, and delayed depression are also features of TBI in humans, the current study underpins the clinical and translational value of experimental TBI models in rodents, provided sufficiently long observation periods are used.

For decades, acute pathophysiological processes were the main focus of clinical and experimental TBI research as it was believed that acute traumatic brain damage is the main determinant of clinical outcome. In recent years, however, there is increasing experimental and clinical evidence that an acute injury to the brain may also be the trigger for delayed processes which may result in additional and long-lasting sequelae. The most common neurological manifestations after TBI are motor and cognitive deficits [33-37]. Neuropsychological disorders after TBI are more common when TBI occurred in early adulthood and seems to depend on trauma severity [36-38] . Furthermore, a single history of TBI seems to be a predisposing factor for neurodegenerative disorders such as Parkinson's Disease and dementia [7, 13, 14]. In some cases

post-traumatic dementia was linked to tau and β-amyloid deposits [12, 39, 40], specifically in contact-sports athletes [41-43] and military veterans [44, 45], however, amyloid-plaques were also found in a third of long-term survivors of a single severe TBI [40].

Despite this clear epidemiological and clinical evidence, most knowledge about chronic traumatic encephalopathy (CTE), chronic posttraumatic brain damage, and related long-term sequels of TBI derives from human autopsy material which, however, allows only limited insight into pathophysiological mechanisms. Hence, specifically when trying to establish novel therapeutic approaches, experimental studies are the only possible approach [46]. Of the more than 1400 experimental studies assessing outcome listed in Pubmed, however, only a minority evaluated post-traumatic brain damage long-term (more than 7-30 days after insult), i.e. over periods of time covering a significant proportion of the animal's live span [26, 47-62]. Recent promising studies already detected important roles for inflammatory processes [48, 57, 58] and cis-phosphorylated tau [55, 59-61, 63] and identified potential target pathways for a possible chronic TBI treatment.

In order to expand our knowledge about the chronic sequels of TBI, the current study was therefore not only designed to evaluate histopathological parameters up to one year after TBI, but also to assess behavioral changes in traumatized, sham-operated and age-matched naive animals. This approach allowed us to correlate the time course of histopathological and behavioral changes after TBI and to dissect these changes from findings which occurred only due to surgery or anesthesia.

The most pronounced histopathological observation we made was progressive loss of brain tissue at the lesion, but also in brain areas further away from the contusion, such as the rostral

corpus callosum. Atrophy is a well-known feature of chronic posttraumatic brain damage in patients [16, 17] and its severity correlates with the development and severity of cognitive deficits [16, 37, 64], as also shown in the current study. The same seems to be true for white matter damage, which also correlates with the occurrence and severity of cognitive defects [65]. In our study we detected not only atrophy of the ipsilateral hemisphere, but also atrophy of trans-hemispheric white matter tracts as evidenced by corpus callosum shrinkage. This finding is in line with radiomorphological and histopathological findings in humans and is also supported by previous experimental series [48, 53, 54]. Hemispheric atrophy, which was detected in the present study from day 7 on, seems to precede corpus callosum/white matter defects which became evident only later than six months after the insult. A similar time course of brain tissue atrophy was also reported after mild experimental TBI [55] and recently by diffusion tensor imaging (DTI) after CCI [48].

Ventricular enlargement/hydrocephalus is a common finding after in TBI patients [18, 19]. In the early phase after TBI hydrocephalus occurs mainly due to obstruction of CSF passage or impaired absorption caused by hemorrhage. Later after TBI, ventricular enlargement is most often considered to be an e vacuo phenomenon due to tissue atrophy. This is the most likely reason why late hydrocephalus after TBI is associated with adverse neurological outcome [21, 64]. In our model we detected ventricular asymmetry starting as early as one week after TBI and progressing until the end of the observation period. In our severe lesion model tissue loss was quite pronounced towards the end of the experiment supporting the e vacuo theory. Furthermore, we did not detect any midline shift indicating increased ICP at any time after TBI.

A very severe loss of brain tissue was also observed in the ipsi- and contralateral hippocampus, a finding also commonly present in TBI survivors [66] and in previous experimental studies [48, 49, 53]. Post-traumatic hippocampal atrophy has been linked to memory deficits [67-70] as well as to the occurrence of mood disorders [71] and may therefore be the (most relevant) pathophysiological correlate for the progressive behavioral abnormalities observed in the Barnes Maze and the Tail Suspension test seen in this study. As cognitive deficits and mood disorders are among the main reasons for TBI patients not to return to their previous occupation [72], it is of utmost importance that an experimental model of chronic TBI reproduces such a deficit.

Memory functions are frequently impaired after TBI in humans [33-35]. Accordingly, cognitive tests are frequently performed in experimental studies investigating long-term outcome after TBI. Among the most widely used and accepted tests to assess memory function in rodents are the Morris Water Maze [73] and the Barnes Maze [74, 75]. Multiple reports described short-term memory impairments 4-6 weeks after CCI, starting as early as two weeks after CCI, but only few studies provide data for time-points later than 6 months or perform repetitive and long-term investigations over time in the same cohort of animals. Albayram et al. [60], Luo et al. [56], Ferguson et al. [63], and Pischiutta [48] found neurocognitive deficits 6 months after TBI, significant memory impairments were reported 205 days after CCI injury [76] and for up to one year after experimental trauma [50, 77, 78]. In a very recent study, cognitive impairment as assessed by contextual fear conditioning was absent at 2 weeks, but present in untreated CCI mice 20 months after TBI compared to sham animals [62]; however, there is no information about the time course of memory decline between these two time points. In the present study, we detected a significantly increased latency to find the target area three months after CCI. In the further

course of the experiment (at 6, 9, and 12 months after CCI) memory function further deteriorated in the same cohort of animals, indicating progressive memory deficits.

Motor function was assessed by the Beam Walk Test, deteriorated most pronounced during the first week after TBI, but recovered quickly thereafter as also observed by others [76, 79, 80]. Recovery was not complete and persistent motor deficits were observed for up to one year after TBI. These motor deficits are in line with observations in patients, where motor deficits are also most pronounced in the early phase after TBI, but may persist at a lesser degree together with postural instability for years [81-84].

Depressive symptoms, mood instability, and lack of impetus are common symptoms after TBI of all severities in humans. Neurobehavioral deficits have been increasingly reported to occur already after mild TBI and may significantly affect quality of life and decrease the frequency of patients returning to work [85, 86]. While quite common in humans, depression and depressive symptoms occurring later than 4 weeks after trauma have been less studied in experimental TBI models than other, more easily quantifiable deficits (e. g. paresis) as depressive symptoms are challenging to assess [87]. So far, depression-like behavior was not consistently found up to three months after TBI [88-95]; as with motor and cognitive symptoms, later time points than three months have been little investigated and yielded conflicting results [93, 96, 97], maybe facilitated by small group sizes and single point investigations. In the present study we found significant depression-like behavior in our mice beginning six months after TBI. Six and nine months after TBI the mobility time was reduced, however, one year after TBI traumatized mice did not try at all to straighten up when suspended by the tail. These results suggest that mice suffer from progressive and severe depression like behavior after TBI. Furthermore, these results stress the

importance and necessity of a long observation period in order to achieve stable and reproducible results and to adequately assess the role of neurobehavioral symptoms.

In conclusion, the present study observing mice up to one year after induction of a cortical contusion adequately reproduces key behavioral and histopathological features of TBI in humans, such as progressive loss of memory function, progressive depression-like behavior, sustained motor deficits, progressive brain and hippocampal atrophy, and progressive white matter damage. For this, we used a big cohort of animals that were assessed repetitively over time; furthermore, each timepoint where neurobehavioral deficits were assessed (15 min until 12 months) is correlated with histopathological findings for each timepoint. Our results suggest that the mechanisms of long-term sequels of TBI so far unrecognized to be present in animal models, such as progressive memory loss and progressive depression, can be investigated experimentally in the future.

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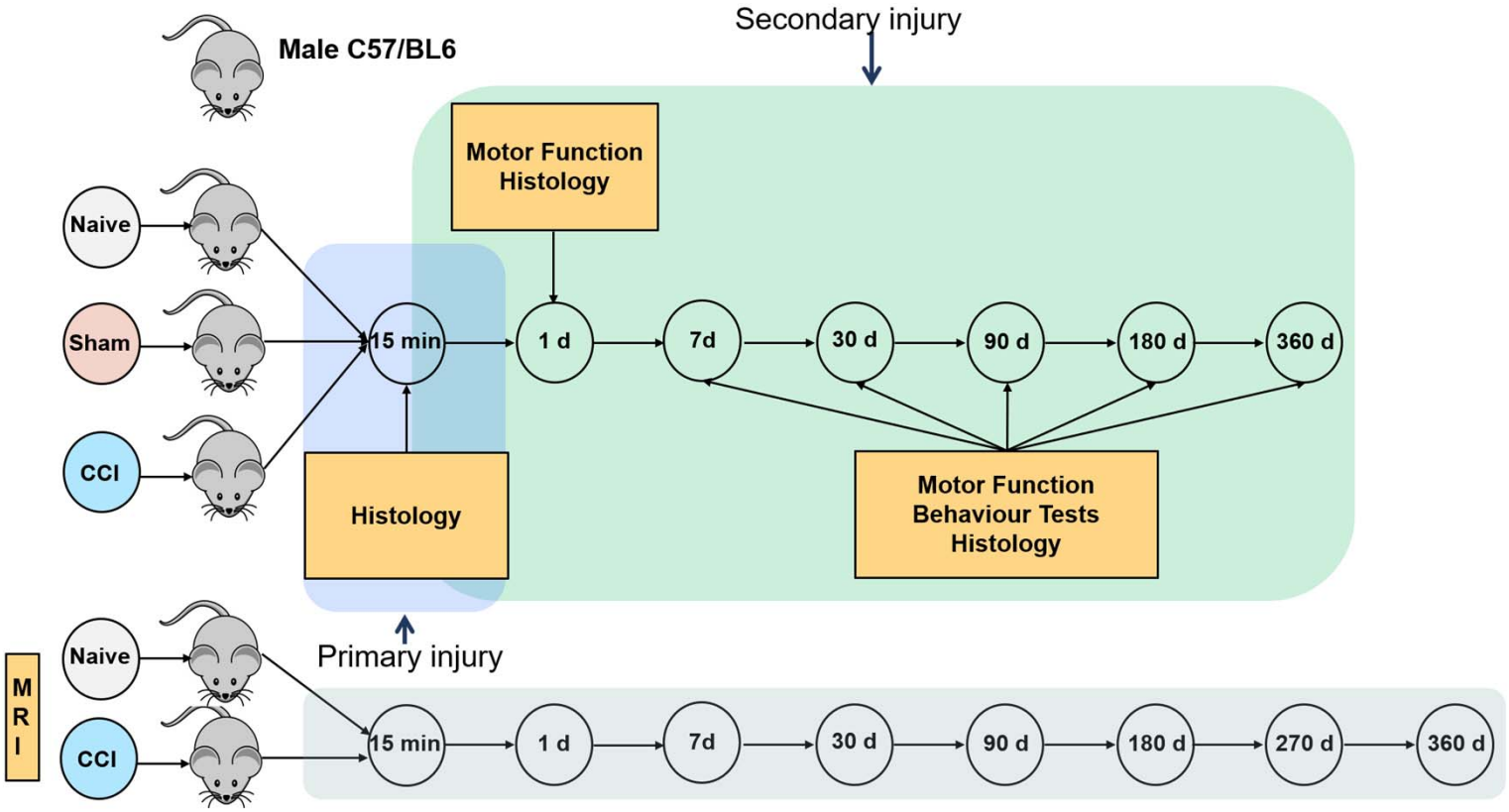
Figure 1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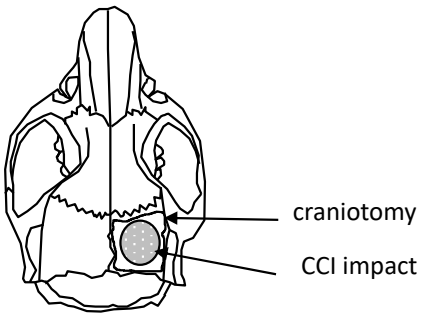
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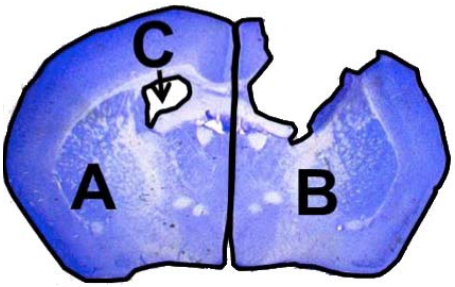
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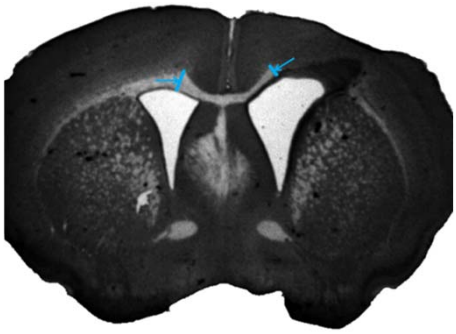
A
B


D

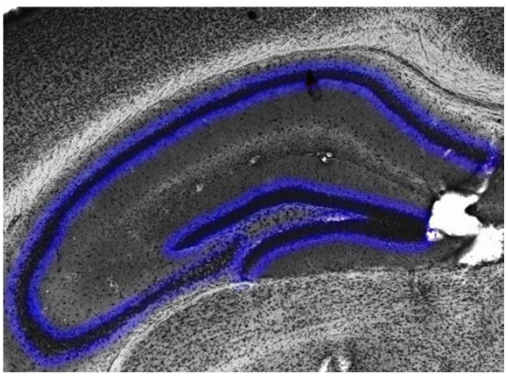
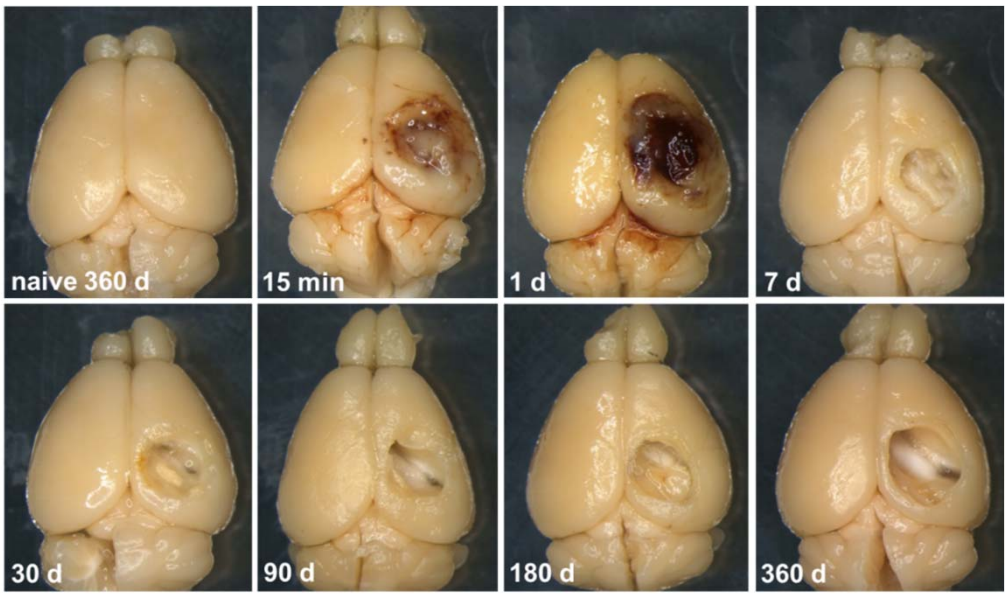
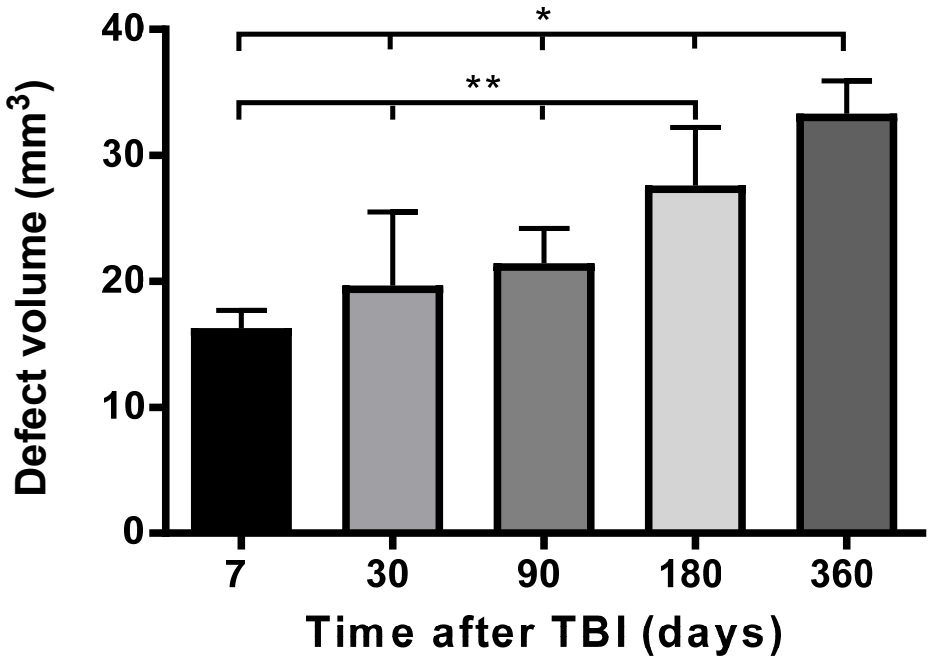

E

Figure 2

A


naive 360 d
15 min
1 d
7 d
30 d
90 d
180 d
360 d

B


Defect volume (mm3)
40
30
20
10
0
*
**
7
30
90
180
360
Time after TBI (days)

C

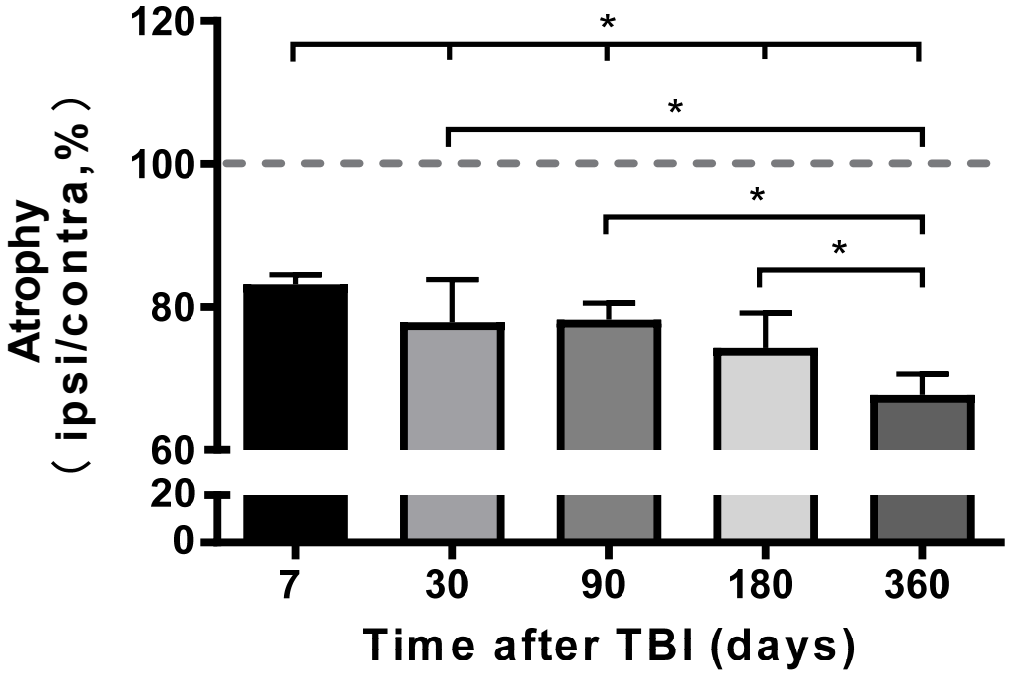
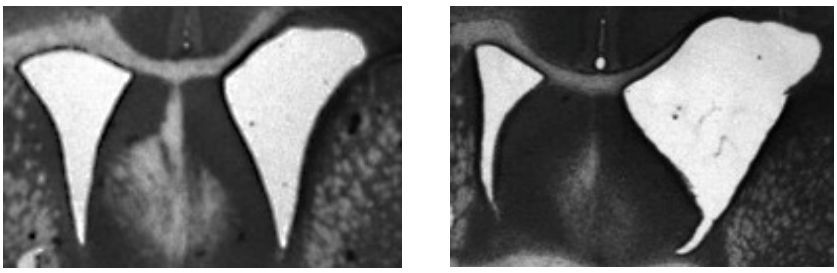

Atrophy
（ipsi/contra,%）
120
100
80
60
20
0
*
*
*
*
7
30
90
180
360
Time after TBI (days)

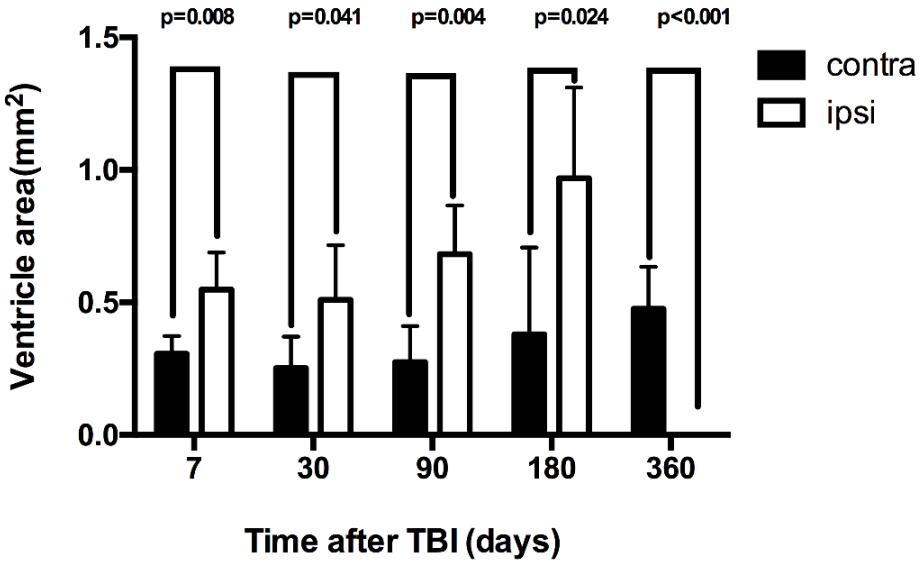
Figure 3

A



180 d 360 d

B



C

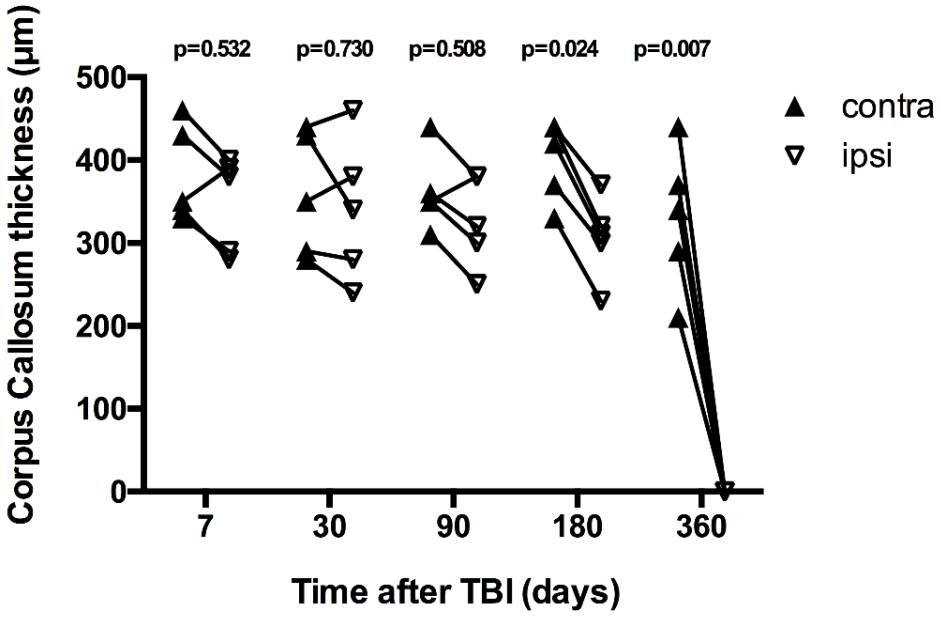
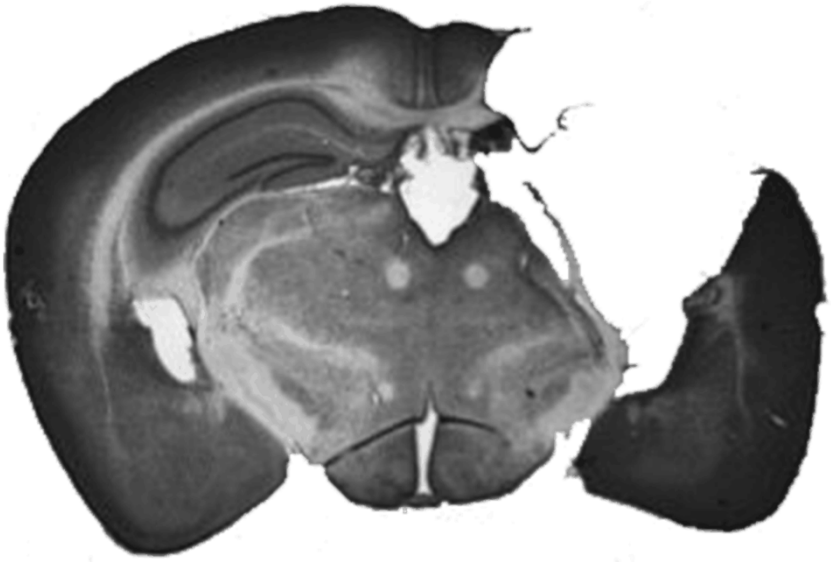


Figure 4

A



360 d

B

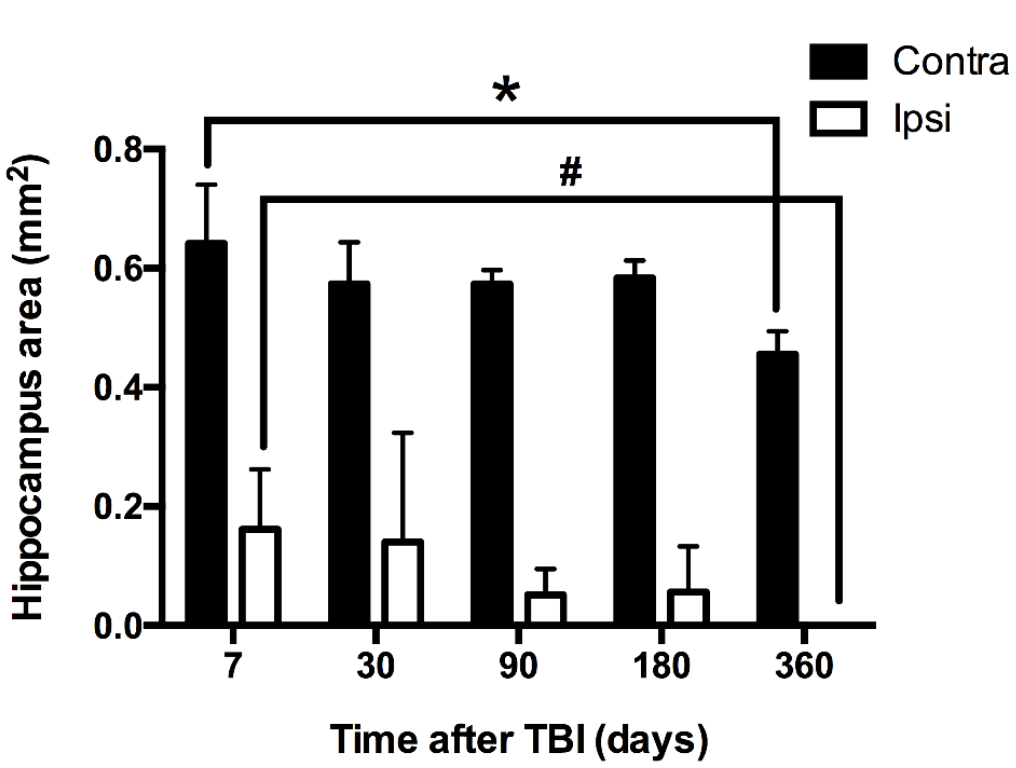
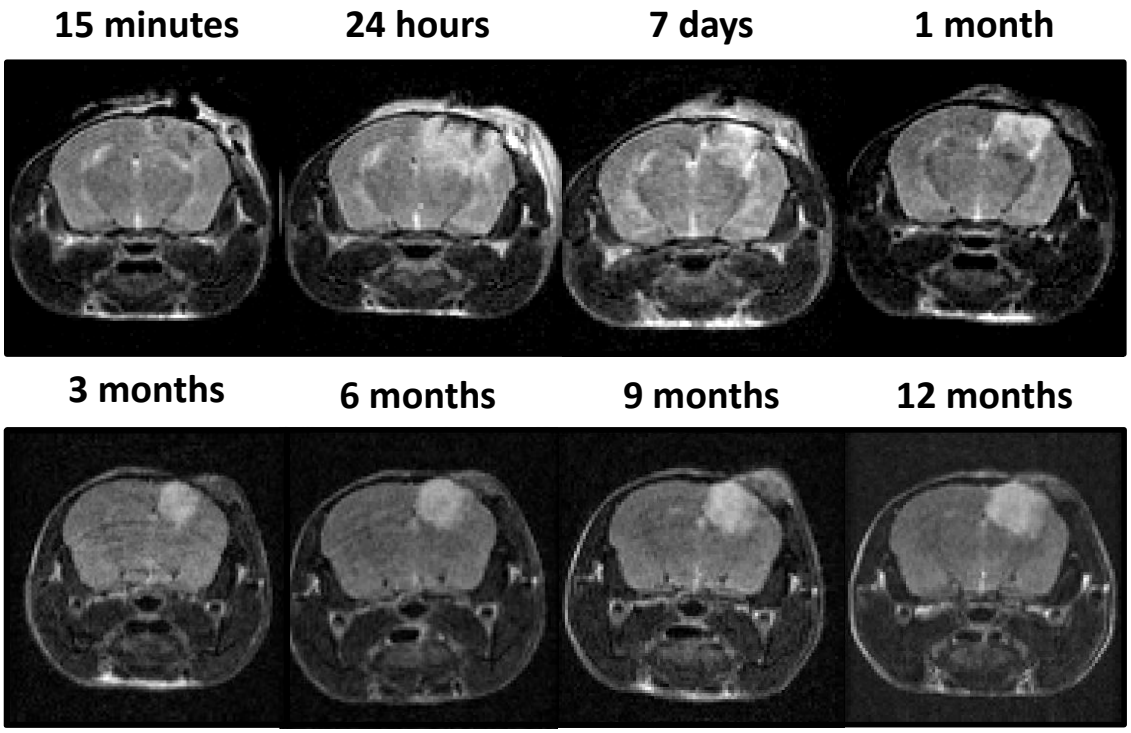
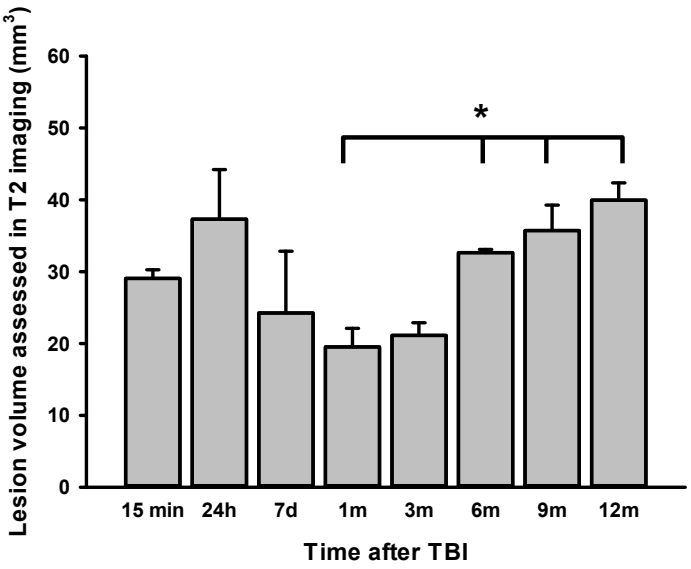


Figure 5

A


15 minutes
24 hours
7 days
1 month
3 months
6 months
9 months
12 months

B


Lesion volume assessed in T2 imaging (mm3)
60
50
40
30
20
10
0
*
15 min
24h
7d
1m
3m
6m
9m
12m
Time after TBI

C

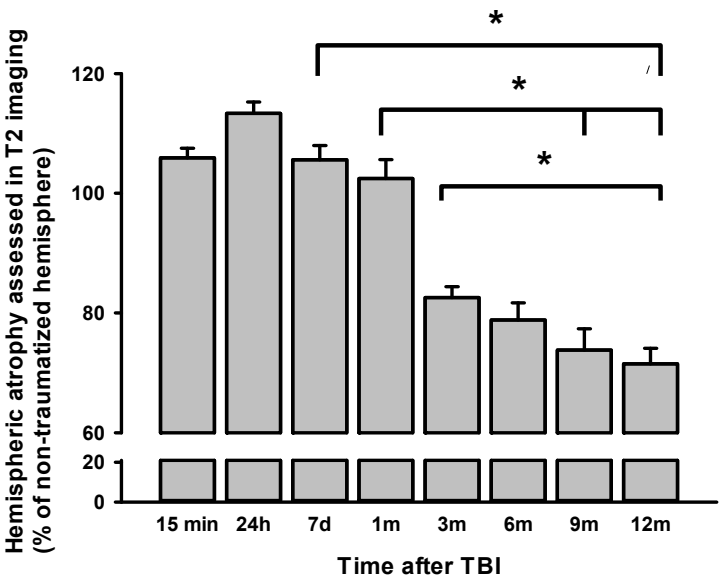
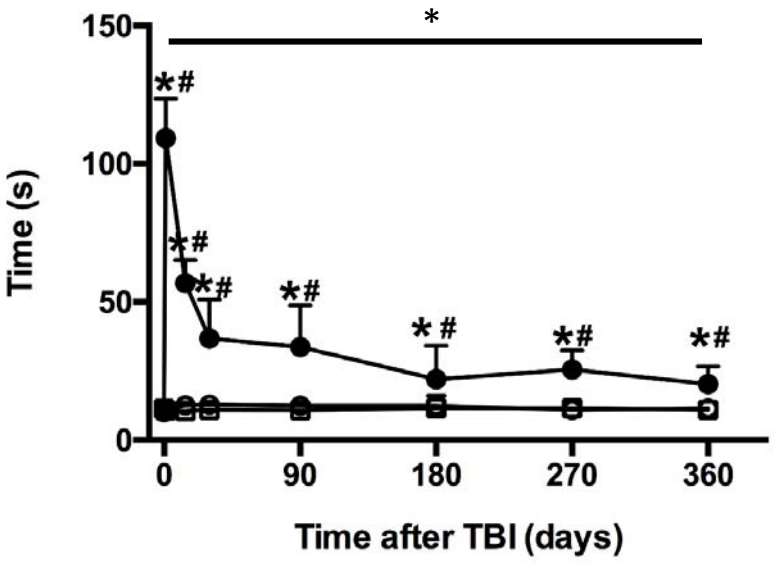

Hemispheric atrophy assessed in T2 imaging
(% of non-traumatized hemisphere)
120
100
80
60
20
0
*
*
*
15 min
24h
7d
1m
3m
6m
9m
12m
Time after TBI

Figure 6

A


*
150
100
50
0
Time (s)
*#
*#
*#
*#
*#
*#
*#
0
90
180
270
360
Time after TBI (days)

B

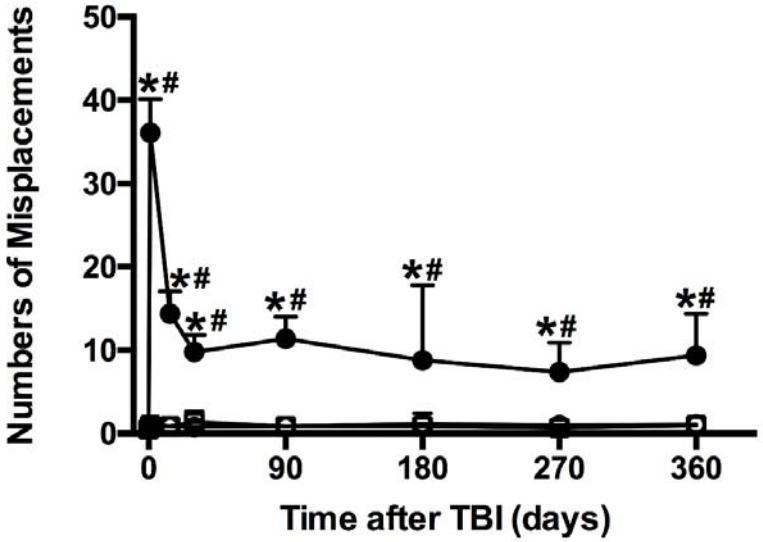

50
40
30
20
10
0
Numbers of Misplacements
*#
*#
*#
*#
*#
*#
*#
0
90
180
270
360
Time after TBI (days)

Figure 7

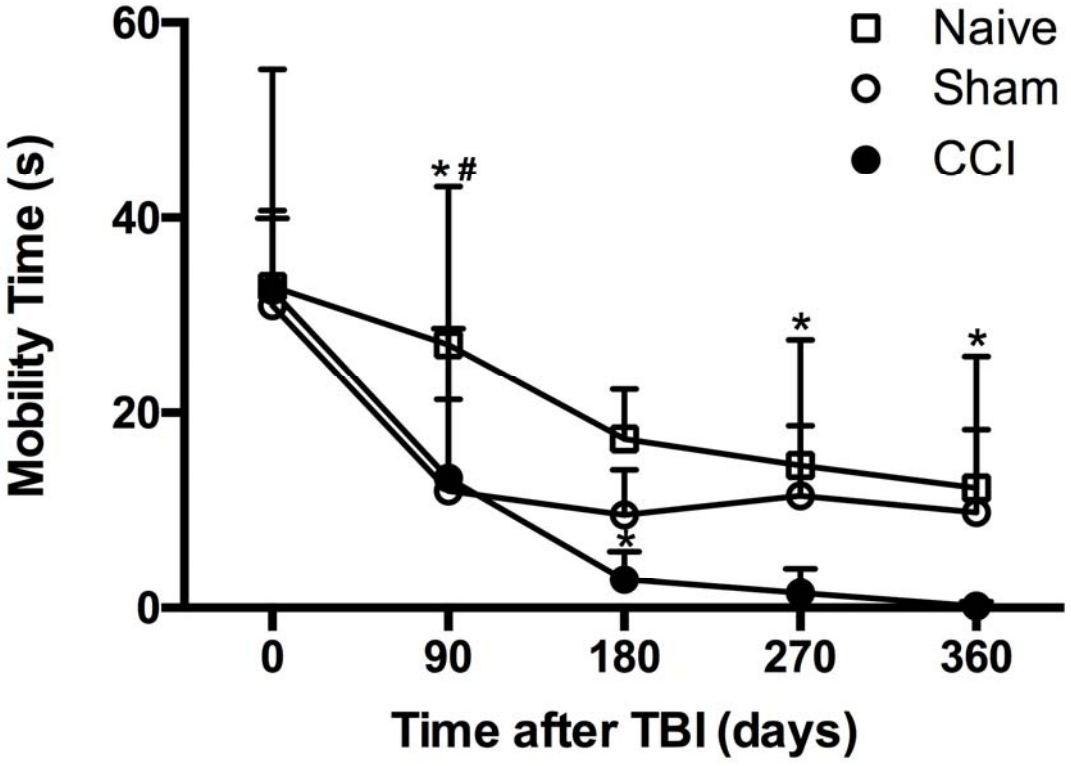
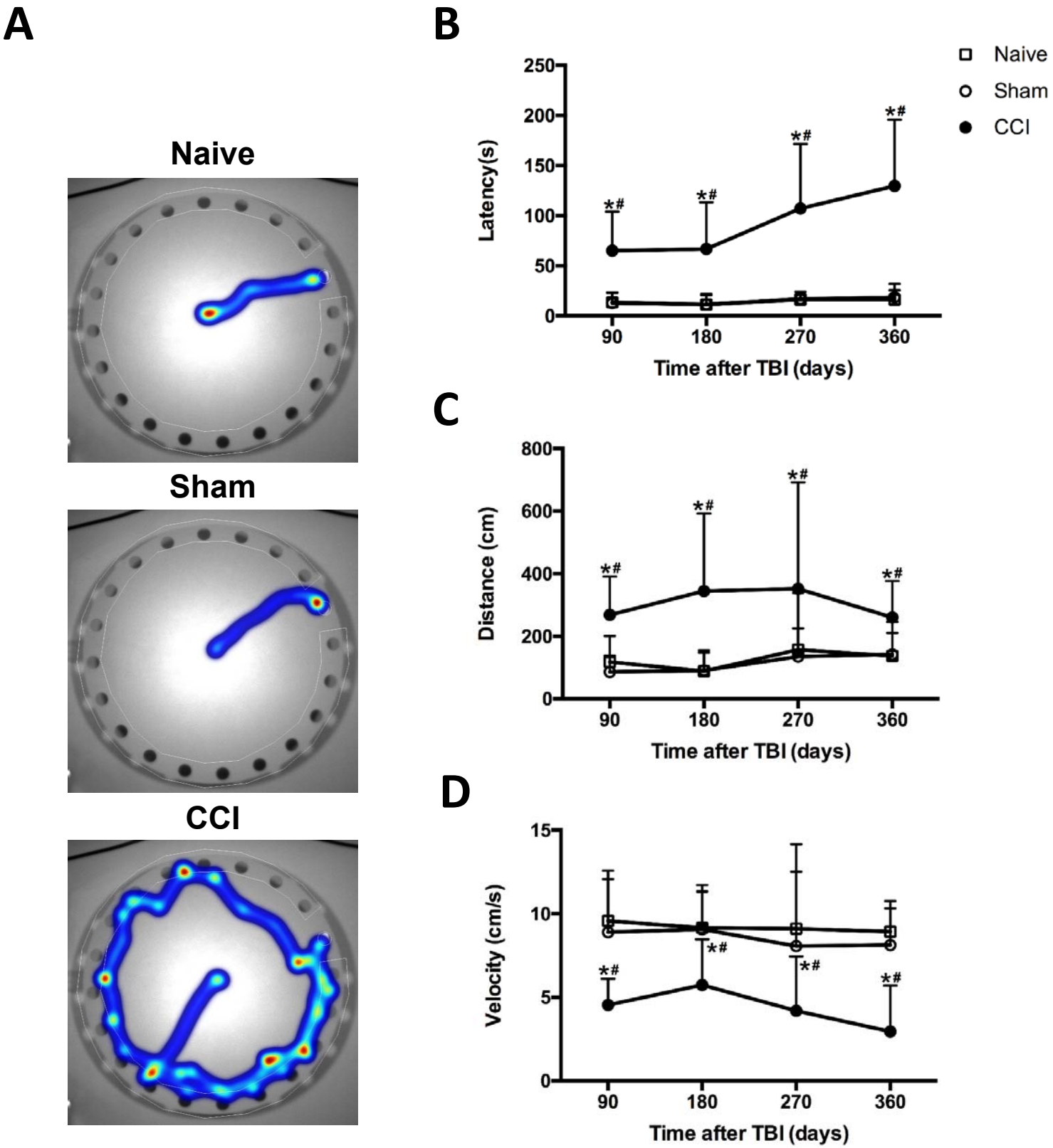
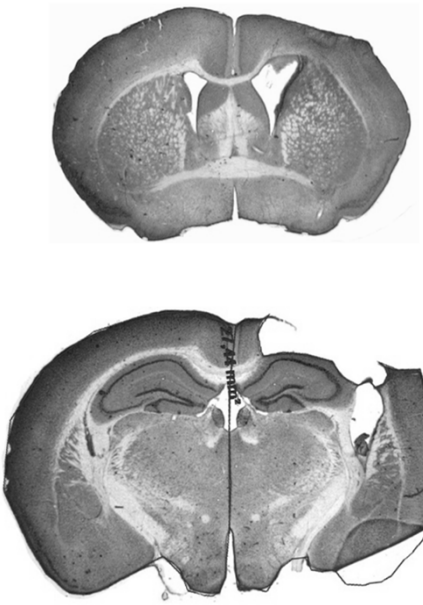

Naive
Sham
CCI
Mobility Time (s)
60
40
20
0
*#
*
*
*
0
90
180
270
360
Time after TBI (days)

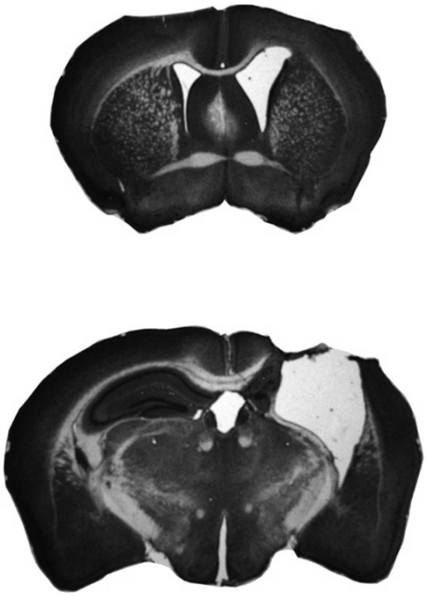
Figure 8


A
Naive
Sham
CCI
B
Naive
Sham
CCI
Latency(s)
250
200
150
100
50
0
90
180
270
360
Time after TBI (days)
*#
C
Distance (cm)
800
600
400
200
0
Time after TBI (days)
D
Velocity (cm/s)
15
10
5
0
Time after TBI (days)

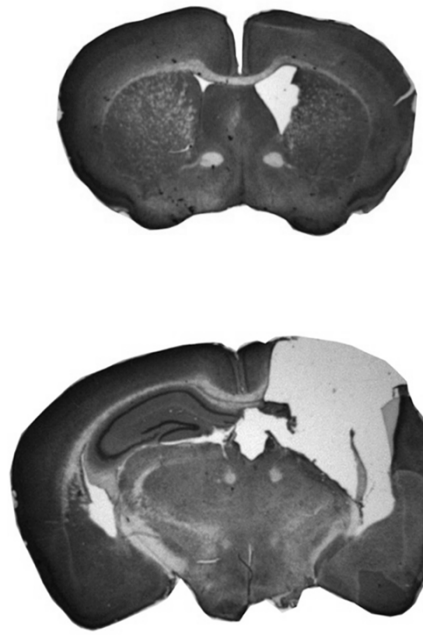
Supp. Figure 1



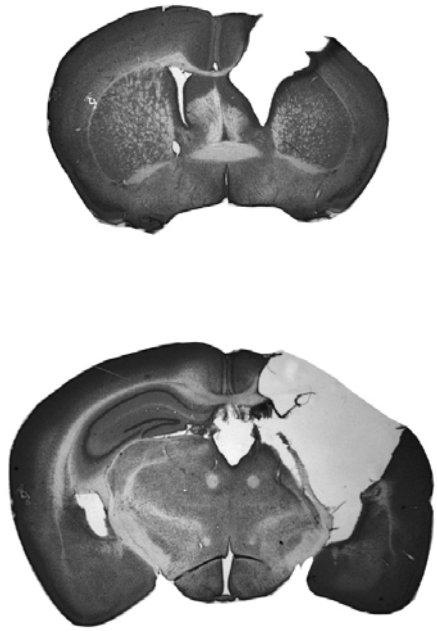
1 month



3 months



6 months



12 months

Supp. Figure 2

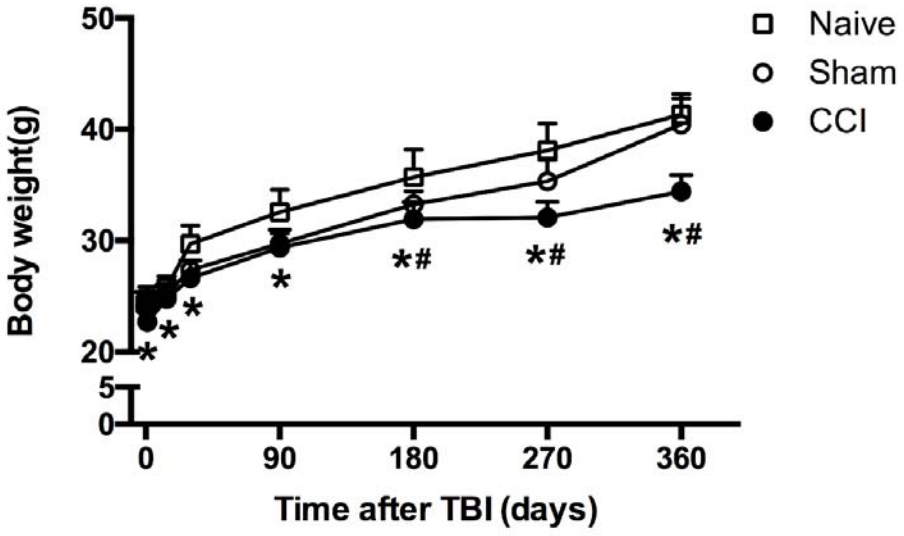


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Figure 7: Depression-like behavior after CCI as assessed by the Tail suspension test. Mobility time, the surrogate for depressive behavior, is reduced over time in all groups (most probably a habituation phenomenon). All operated animals spend less time mobile than the naïve control 3 months after surgery; however, while sham-operated mice recover and reach the levels of non-traumatized animals by day 180 after trauma, CCI mice further deteriorate to significantly worse levels than the sham and naïve controls. (n= 12 each, mean ± SD; * $p<0.05$ vs. naive, # $p<0.05$ v. sham group)

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Supplemental Figure 2: Weight loss after CCI. After surgery, animals gain weight significantly slower than non-operated mice. While sham-operated animals, however, recover and match the naïve controls by day 270 after surgery, CCI mice were found to have a lower bodyweight throughout the observation period (n= 12 each, mean ± SD; * $p<0.05$ vs. naive, # $p<0.05$ v. sham group).

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