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Single mild traumatic brain injury results in transiently impaired spatial long-term memory and altered search strategies

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Highlights

- A mouse model for weak, single mild traumatic brain injury (minimal TBI) is used. **(84)**
- Minimal TBI results in transient deficits in long-term memory reconsolidation. **(81)**
- Minimal TBI causes relapse to less effective search strategies in the water maze. **(84)**
- Minimal TBI leads to a transient increase in freezing during fear conditioning. **(81)**
- A transient rise in hippocampal GFAP after minimal TBI indicates astrogliosis. **(80)**

Abstract

Mild traumatic brain injury (mTBI) can lead to diffuse neurophysical damage as well as cognitive and affective alterations. The nature and extent of behavioral changes after mTBI are still poorly understood and how strong an impact force has to be to cause long-term behavioral changes is not yet known. Here, we examined spatial learning acquisition, retention and reversal in a Morris water maze, and assessed search strategies during task performance after a single, mild, closed-skull traumatic impact referred to as "minimal" TBI. Additionally, we investigated changes in conditioned learning in a contextual fear-conditioning paradigm. Results show transient deficits in spatial memory retention, which, although limited, are indicative of deficits in long-term memory reconsolidation. Interestingly, minimal TBI causes animals to relapse to less effective search strategies, affecting performance after a retention pause. Apart from cognitive deficits, results yielded a sub-acute, transient increase in freezing response after fear conditioning, with no increase in baseline behavior, an indication of a stronger affective reaction to aversive stimuli after minimal TBI or greater susceptibility to stress. Furthermore, western blot analysis showed a short-term increase in hippocampal GFAP expression, most likely indicating astrogliosis, which is typically related to injuries of the central nervous system. Our findings provide

evidence that even a very mild impact to the skull can have detectable consequences on the molecular, cognitive and affective-like level. However, these effects seemed to be very transient and reversible.

Abbreviations: CFC, contextual fear conditioning; CS, conditioned stimulus; GFAP, glial fibrillary acidic protein; GSK3β, glycogen synthase kinase 3β; LTM, long-term memory; mTBI, mild traumatic brain injury; NMDA, N-methyl-D-aspartate; PFA, paraformaldehyde; PSD-95, postsynaptic density protein 95; RM-ANOVA, repeated measures analysis of variance; SEM, standard error of mean; TBI, traumatic brain injury; US, unconditioned stimulus.

KEYWORDS: Concussion, Spatial learning, Morris water maze, Contextual fear conditioning, Immunoblotting

1. Introduction

Traumatic brain injury (TBI) typically occurs when the head hits or is struck by an external object, leading to structural or functional brain alterations from brain acceleration and deceleration or the penetration of an object into the brain [1]. Although 80-90% of all mTBIs resolve spontaneously within a couple of weeks, mTBI may initiate a complex condition called post-concussion syndrome [2]. Patients suffering from the aftermath of concussive brain injury may develop persistent symptoms, including cognitive and psychological complaints [3]. There is, however, some controversy about the prognosis of long-term symptoms and there is a lack of clear evidence of cognitive symptoms attributable to mTBI [4], which complicates the development of new lead compounds and therapeutic targets. Furthermore, the neuropathology of mTBI is characterized by its heterogeneous nature [5,6] with a lack of consensus on how much, or how little, it takes to initiate long-term behavioural consequences [7]. As a result, mTBI represents not a single neuropathological

event but a complex disease process. Among all brain structures, the hippocampus has been reported to be particularly susceptible to TBI in general and mTBI specifically [8–10]. Animal models can help to elucidate the functional and structural alterations caused by mTBI and ultimately support the identification of potential therapeutic targets. However, while a number of experimental animal studies reported short- and long-term behavioural alterations reflecting cognitive and affective deficits after mTBI [8,11–14], results across studies are diverse and inconsistent. As such, the modelling and replication of key features typically seen in humans after concussion remains a major but important challenge (for review see Dewitt *et al.* [15]).

Given the current lack of knowledge regarding the relation between the severity of a single mild head impact and the resulting transient or longer-term behavioural and biochemical consequences, we examined the effects of very mild single closed-skull mTBI (herein referred to as minimal TBI) on performance in established paradigms of hippocampus-dependent spatial and associative learning (Morris water maze and contextual fear conditioning, respectively) and on the expression of several key proteins of synaptic function.

2. Methods

2.1 Animals

63 male and female C57/Bl6J mice, aged 8-12 weeks at the time of surgery, were randomized into a sham control group (n = 31) and minimal TBI group (n = 32). All animals were group-housed in standard animal cages under conventional laboratory conditions (12h/12h light-dark cycle, ~22°C), with *ad libitum* access to food and water. Animal

husbandry and experiments were conducted in accordance with the KU Leuven Ethical Committee (P097/2014) and the European Directive (2010/63/EU).

2.2 Surgery

Experimental mild traumatic brain injury was induced using the closed-skull trauma device described by Flierl *et al.* [16]. Surgery was performed under anaesthesia by chloral hydrate (400 mg/kg i.p.). Analgesia was provided by injection of buprenorphine (0.1 mg/kg s.c.). Additionally, local anaesthesia over the incision site was provided by lidocaine. A mid-line longitudinal incision was performed to expose the skull. A metal rod weighing 333 g was dropped from a height of 1.5 cm onto the skull over the sagittal suture, anterior to the lambdoid suture and posterior to the coronal suture. The rod was retracted immediately to prevent an unwarranted secondary impact. Surgical sutures were used to seal the skin wound rapidly after impact. Sham-operated control mice were subjected to anaesthesia, analgesia, scalp incision, and suturing only.

2.3 Morris water maze

Spatial memory was assessed in an allocentric place-learning task in the standard hidden-platform version of the Morris water maze. A circular pool (150 cm diameter) was filled with water, opacified with nontoxic white paint, and kept at ~26°C as described previously [17]. A round platform (15 cm diameter) was hidden 1 cm beneath the water surface at a fixed position. Four equidistant positions around the rim of the pool were used as starting positions and arbitrarily assigned numbers 1 through 4 (clockwise), thereby dividing the tank into four quadrants: target, adjacent 1, opposite and adjacent 2. Testing began three days post-surgery (sham, n = 9; minimal TBI, n = 9). Each mouse received four swimming trials per day at a 10 min inter-trial interval for 5 consecutive days. The start position was pseudo-randomized across trials. Mice that did not find the submerged platform within 2 min were guided to the platform, where they remained for 15 s, and were returned to their cages

thereafter. The distance travelled (path length) and swim speed (velocity) were determined. 72 hours after the last acquisition trial, a probe trial was conducted, during which the platform was removed and the search patterns of the mice were recorded for 100 s. Thereafter, all mice received a second block of acquisition training according to the same protocol, followed by a second probe trial 72 hours later. In the following reversal phase, the escape platform was placed in the opposite quadrant and the training was continued with four trials per day. During acquisition and probe trials, the Ethovision XT (Noldus Information Technology, Wageningen, The Netherlands) video tracking system was used to record and analyse behaviour. See also Fig. 1 for a schema and timeline of all experimental procedures.

Search strategies were analysed as explained previously [17]. Briefly, the type of search strategy used on each trial was determined using an automatic classification algorithm implemented in Matlab 8. The strategies were defined in a similar manner as previously described by Garthe *et al.* [18] and included the following: "thigmotaxis" describing swimming close to the wall of the pool; "chaining" referring to the preference for swimming within an annulus that contains the platform location; "perseverance" as a steady persistence of swimming in one spot not in the platform vicinity and without any focal tendencies; "random search" comprising all random swimming while covering almost the entire pool area; "scanning" referring to a preference for the central pool area (enabling animals to scan the environment for distal visual cues). Both random search and scanning are devoid of any spatial or directional preference. "Focal incorrect" describes the preference for a specific well-circumscribed position irrespective of the current platform location. "Directed search'' delineating a more direct swimming between the starting position and the platform area; "focal correct", where the search is predominantly in the vicinity of the platform position and "direct swim" where the animal swims directly towards the platform. These behavioural strategies were assigned into three categories: (1) repetitive (thigmotaxis, chaining, perseverance); (2) non-spatial (random search, scanning, focal incorrect) and (3) spatial

(directed search, focal correct, direct swim). Tracks that could not be classified as any of these search strategies were pooled under "error".

2.4 Contextual fear conditioning

The contextual fear conditioning experiment was based on the protocol used by Paradee *et al*. [19]. For one group, testing started three days post-surgery (sham = 7, minimal TBI = 8) and for a second group five weeks post-surgery (sham = 9, minimal TBI = 9). A plexiglas test chamber (26 x 22 x 18 cm) was placed inside a dark, sound-attenuated box. A constant current shocker (MED Associates, USA) was used to apply electric foot shocks (2 seconds, 0.3 mA) through a metal grid floor. The experiment was conducted over three consecutive days. On day one, animals were placed individually into the test chamber and allowed to habituate for 5 min. On day two, animals were again placed into the same test chamber. They were monitored for two minutes (baseline score) and subsequently a non-aversive tone (4 kHz, 80 dB) was presented for 30 s. This auditory stimulus, the conditioned stimulus (CS), was immediately followed by a foot shock, the unconditioned stimulus (US). After a 60 s rest-period, a second CS-US stimulus pairing was presented, followed by another 60 s rest-period. Twenty-four hours later, the animals were again placed in the test chamber and monitored for 5 min (context score) before being removed to the holding cage. Ninety minutes later, the animals were returned to the test chamber and exposed to altered contextual cues: a white plastic insert was placed in the chamber to cover the grid floor and alter the colour. In addition, a different scent (mint extract) was used to alter the smell and lights were turned on. After 3 min, during which animals could explore the new context (pre-CS), the auditory stimulus was presented for 3 min (post-CS). Freezing behaviour was recorded in intervals of 10 s during each trial block using standard interval sampling procedures.

2.5. Immunoblotting

For immunoblotting experiments, a separate batch of animals (n = 24; minimal TBI = 12, sham = 12) was sacrificed three days (n = 12; minimal TBI = 6, sham = 6) and 5 weeks post-surgery (n = 12; minimal TBI = 6, sham = 6). The brains were removed and their hippocampi and remaining cortical tissue were dissected out. The tissue was gently homogenized in 200 µl of the following buffer on ice: 10 mM Tris.HCl (pH 7.4), 320 mM sucrose, 2 mM EDTA, 200 mM ammonium acetate, 10 mM of 0.001% v/v sodium azide and phosphatase inhibitor cocktail 1 (Roche Diagnostic, Germany). Then, tissues were snap-frozen in dry ice/ethanol and stored at −80°C until analysis. Total protein concentrations were determined by the bicinchoninic acid assay Kit (Pierce, France) and total protein extracts were normalized to 1 µg/µl in reducing LDS Sample Buffer (Invitrogen, USA) and denaturated at 95°C for 10 minutes. Then, 10 µg of proteins were loaded on 10 % Tris-Glycine gels (Anamed, Germany) and transferred onto nitrocellulose membranes and, after blocking, probed overnight (4°C). Membranes were rinsed in TBS-Tween 0.1% and incubated with appropriate HRP-Labeled secondary antibody diluted in blocking buffer. The following antibodies were used: Z0334 for analysis of glial fibrillary acidic protein (GFAP; Agilent Technologies, USA), total glycogen synthase kinase 3 ß (GSK3ß; Thermo Fisher Scientific, USA), GSK3ß phosphorylation at serine 9 (Ser9; Cell Signaling Technology, USA); GSK3ß phosphorylation at tyrosine 216 (Tyr216; Thermo Fisher Scientific, USA), postsynaptic density protein 95 (PSD-95), GluN2A, GluN2B and CD68 (all from Abcam, UK). ß-actin (Sigma-Aldrich, Germany) was used as internal loading controls. Reactions were developed by chemiluminescence (Western Lightning ECL Pro, Perkin-Elmer, USA) followed by digital picture acquisition and analysis (LAS 4000; ImageQuant v7.0; GE Healthcare). Final blot pictures were equally adjusted to enhance visibility using Adobe Photoshop (version 7.0) (Adobe Systems, USA).

2.6 Histology

For histology, animals (minimal TBI = 3, sham = 3) were transcardially perfused with 4% paraformaldehyde (PFA) three days post-surgery. The brains were removed and post-fixed overnight in PFA. Next, they were cut into 50 μm coronal sections on a vibratome (Leica, VT1000 S, Germany). Every sixth section was mounted onto 2% gelatine-coated slides. The sections were Nissl stained with 5% thionine acetate (Alfa Aesar, Germany) and assessed under a light microscope (Zeiss Axio Imager ZI). Images at 5x magnification were obtained with an AxioCam camera using ZEN software (Carl Zeiss, Benelux). Brightness and contrast were adjusted using Paint.NET software v4.0.9 (available at http://www.getpaint.net).

2.7 Statistical analysis

Statistical calculations were performed with SPSS 23 (IBM, USA) and Graphpad Prism 5 (GraphPad Software, USA). All data are presented as mean ± standard error of the mean (SEM). In all statistical tests, $p < 0.05$ was considered significant.

Overall task performance in the Morris water maze was evaluated by calculating the distance travelled to find the hidden platform (path-length). Repeated measures analysis of variance (RM-ANOVA) was used to test for learning-related changes in the path length of experimental groups over the course of training. Greenhouse-Geisser correction was applied if violations of the sphericity assumption in the ANOVAs were indicated. Differences in path length between experimental groups on day 6 and 7 as well as both probe trials were determined by an unpaired Student's *t*-test. A retention index was calculated as follows: (distance session 5 trial 4 – distance session 6 trial 1) / distance session 5 trial 4. For a further analysis of interaction effects, simple effect analyses and Bonferroni-adjusted post hoc pairwise comparisons were conducted. RM-ANOVAs were used to test for changes in search strategies of the experimental groups over the course of training.

Differences between groups in contextual fear conditioning performance and immunoblotting data were determined by unpaired Student's *t*-tests (with Welch's correction in case of significantly different variances). Outliers were detected and excluded according to the Grubb's test.

3. Results

Fig. 1 presents an overview and timeline of all experimental procedures. Closed-skull injury resulted in a brief and transient period of apnoea ranging from about 10 to 30 sec in the minimal TBI group. Microscopic examination revealed no focal lesions in any of the animals subjected to injury (Fig. 2). There were no skull fractures, cerebral haemorrhages or contusions identified after closed-skull injury.

Statistical analyses of the water maze acquisition data for the two groups yielded no difference between minimal TBI and sham mice in the first acquisition phase, $F(1,17) = 0.041$, $p = 0.843$, $\eta_p^2 = 0.054$ (see Fig. 3A), and also not in the second, $F(1,16) = 2.113$, $p = 0.165$, $\eta_p^2 = 0.117$ (Fig. 3B). The overall distance traversed (path length) was 671.31 ± 49.85 cm for minimal TBI mice and 597.43 ± 47.61 cm for sham mice in the first phase, and 347.745 ± 44.873 cm for minimal TBI mice and 255.489 ± 44.873 for sham mice in the second acquisition phase. However, when we analysed the performance of both groups in more detail by inspecting also the behaviour during single trials, we noticed a drop in performance during the first two-day pause in minimal TBI mice but not in sham animals. Thus, when comparing the distance travelled on the very last trial of the first acquisition phase (training day 5; sham: 441.59 ± 102.92 cm; minimal TBI: 249.07 ± 53.63 cm) and the first trial of the second acquisition phase (training day 6; sham: 412.8 ± 160.69 cm; minimal TBI: 903.18 ± 149.5 cm), a significant trial effect [$F(1,16) = 7.038$, $p = 0.017$, $\eta_p^2 = 0.305$] and interaction effect [$F(1,16) = 8.393$, $p = 0.011$, $\eta_p^2 = 0.344$] was obtained. The different

performance after the weekend pause is further supported by a significantly longer path of minimal TBI compared to sham mice during the first trial on day 6 ($t(16) = 2.372$, $p = 0.033$, paired t-test), but not during the fourth trial on day 5. Hence, minimal TBI mice showed a stronger increase in the travelled distance after the two-day pause than the sham group (sham: ($t(8) = 0.163$, p = 0.874, minimal TBI: $t(8) = 4.193$, $p = 0.003$, paired t-test). The impaired performance of minimal TBI after the 72-hour break is also reflected in the retention index calculated between these trials. The minimal TBI group has a marked negative index of -428.34 ± 162.5 as compared to -29.95 ± 44.11 of sham controls, resulting in a significant difference [$t(16) = 2.326$, $p = 0.033$, t-test].

The performance deficit of minimal TBI mice became also overt in the first probe trial, performed on training day 6 before the continuation of acquisition sessions (Fig. 3D). Minimal TBI animals crossed the area over the former platform position significantly less often than sham animals [$t(16) = 2.326$, $p = 0.033$; unpaired Student's t-test; Fig. 3D]. No significant difference was found; however, between groups on the second probe trial on day 11, i.e. 72 hours after the second block of acquisition trials.

Subsequent to the second probe trial, cognitive flexibility was tested by moving the platform to the opposite quadrant (reversal learning). Under these conditions, no significant difference was found [minimal TBI: 429.34 ± 32.25 cm, sham: 365.19 ± 32.25 cm, $F(1,16) = 1.979$, $p = 0.179$, $\eta_p^2 = 0.263$; Fig. 3C]. Further, no differences in velocity were found in either of the training phases.

To analyse water maze performance in more detail, the animals' search strategies were classified into 9 different types by an automatic algorithm as described before [17] and assigned to one of the three categories: spatial, non-spatial and repetitive (Fig. 4). While we could not detect overall group differences in the use of these categories for either of the acquisition phases or the reversal phase (two-way RM-ANOVA), we found a significant

difference for the use of spatial strategies on session 6 [sham: 33.33 ± 10.2 %, minimal TBI: 2.78 ± 2.78 %, $t(9,179) = 2.889$, $p = 0.018$]. Minimal TBI animals used a greater proportion of non-spatial strategies after the 72 hour break [minimal TBI: 71.22 ± 15.11 %; sham: 33.33 ± 9.32 %, $t(16) = -3.577$, $p = 0.003$; Fig. 4].

To evaluate whether minimal TBI affects another established category of hippocampus-dependent learning, we took advantage of the contextual fear-conditioning paradigm. Three days post-surgery, both groups displayed strong conditioned responses to the introduction of the CS (tone) – US (shock) pairing, as measured by a significantly higher amount of freezing (immobility) compared to the pre-US baseline [$F(1,13) = 119.933$, $p < 0.001$, $\eta_p^2 = 0.902$] (Fig. 5A). Minimal TBI mice, however, displayed a significantly stronger freezing response after the presentation of CS-US [$F(1,13) = 6.042$, $p = 0.029$, $\eta_p^2 = 0.317$] (Fig. 5A). No group differences were observed when exposed to the old context and to a new context in the next trial, neither before nor after presenting the CS. Fear-conditioning at five weeks post-surgery resulted in a strong conditioned response for both sham and minimal TBI mice [$F(1,17) = 83.275$, $p < 0.001$, $\eta_p^2 = 0.830$], but no between-group differences were found in any of the following phases (Fig. 5B).

Next we investigated whether the performance deficits in the Morris water maze were accompanied by changes in molecular parameters that have been reported to be affected by TBI [20,21]. Hereto, we used western blotting and focused on the expression of GFAP, commonly used as a marker for traumatic brain injury [22–27], and of proteins involved in synaptic function and signalling such as PSD-95 [28,29], GluN2A and GluN2B subunits of the *N*-methyl-D-aspartate (NMDA) receptor [30], GSK3ß [21,31,32] and phosphorylation of GSK3ß at its Ser9 and Tyr216 residue. The selection of synaptic proteins was supported by pilot experiments in our laboratory which indicated a severe impairment of long-term potentiation in the hippocampal CA1 region 3 days post-injury, in agreement with other

animal models of mTBI [8,33–35]. As depicted in Fig. 6A-B, minimal TBI caused a significant increase in hippocampal [$t(10) = 2.618$, $p = 0.026$] but not in cortical [$t(10) = 0.237$, $p = 0.817$] GFAP at 3 days post-injury. At 5 weeks, no differences between groups could be detected in the hippocampus [$t(9) = 0.149$, $p = 0.885$] or cortex [$t(5) = 0.274$, $p = 0.795$]. Furthermore, no effects of minimal TBI were found at either 3 days or 5 weeks post-injury for PSD-95, total GSK3ß, GSK3ß phosphorylated at Ser9 or Tyr216, nor NMDA receptor subunits GluN2A and GluN2B (Fig. 6C-H). Although GFAP is often used in TBI studies as a marker of glial cell damage and reactive astrogliosis in regions of neuronal damage [36,37], it does not indicate whether or not this is accompanied by neuroinflammation. The transient increase in GFAP levels in the hippocampus at 3 days post-injury tempted us to further evaluate whether this was accompanied by changes in neuroinflammation. Hereto, we performed additional western blots targeting the microglia and macrophage marker CD68, which is linked to inflammation [38–41]. While minimal TBI mice showed a trend towards elevated CD68 levels, this difference was not significant [$t(10) = 0.997$, $p = 0.342$] (Fig. 7).

4. Discussion

The nature and extent of behavioural changes seen after mTBI are still poorly understood. In the present study, we induced a very mild single, closed-skull mTBI (minimal TBI) using the weight-drop device described by Flierl *et al.* [16]. This non-repetitive protocol induces a very mild head impact. In agreement with the latter and the definition of mTBI, histological examination did not reveal any gross anatomical abnormalities. Immunoblotting experiments revealed a transiently increased expression of GFAP in the hippocampus, a marker of astrocyte activation [24,27]. This increase was significant at 3 days post-trauma, but no longer present at 5 weeks. GFAP is increasingly considered as a specific biomarker for TBI [22–27] based on reports that patients with a history of brain injury show increased

GFAP expression as a function of the severity of brain injury [see Patterson and Holahan [27] for detailed discussion and further references]. However, Metting *et al.* [42] came to the conclusion that despite this correlation, GFAP is not suitable for prediction of individual patient outcome. In agreement with a limited predictive value of GFAP expression, the expression of CD68 was not significantly enhanced at three days post-trauma, indicating the absence of neuroinflammation [41] and thus, a rather confined glial reaction.

In contrast to GFAP, parameters that are indicative of synaptic disturbances and remodelling, like the expression of NMDA receptor subunits GluN2A and GluR2B, PSD-95, and GSK3ß as well as the phosphorylation of Ser9 and Tyr216 residues of the latter, did not show any alterations at 3 days or 5 weeks post-trauma. Other studies have, however, reported changes in the expression of some synaptic proteins after mTBI [21,28–32]. For example, hippocampal expression of GluR2B was reduced after repeated mTBI (7 injuries) in mice [30]. Taken together, the histological and immunoblotting data confirm the mild and transient nature of the minimal TBI used in the current study.

Accordingly, we found only transient deficits in spatial memory retention. Although the increase in distance travelled that minimal TBI mice showed on days 6 and 7 (Fig. 3B) may seem rather minor or random on first sight, the evaluation of other measures supports the view that this increase is indicative of a long-term memory deficit:

(i) Minimal TBI mice showed an increase in spatial search strategies during acquisition days 3-5 similar to that of controls (and to other control animals that we analysed before [17]). However, on day 6, after a 72-hour long break, the proportion of spatial strategies suddenly collapsed to similar values as observed during the first day of training. Sham animals, in contrast, showed a similar proportion as on day 5.

(ii) After the 72-hour training break, only minimal TBI but not sham animals showed a worse performance during the probe trial, i.e. under different training conditions without an escape platform.

Together this points to an impaired reconsolidation [43–45] of long-term memory (LTM) [46–48] as a pathophysiological consequence of minimal TBI. The deficit seems to be specific for longer retention intervals, because it appeared after 3 days (72 hours) but not during the first five days of training where the day-to-day (re)consolidation of spatial memory was unaffected by minimal TBI. Furthermore, the deficit was transient and compensated by further training because it was not detectable during the second probe trial and the subsequent reversal training.

The transiently impaired performance is most likely a consequence of temporary functional disturbances in the hippocampus, the major brain structure involved in spatial water maze learning, which has been described to be compromised in mTBI [8,10,12–14,49]. Repetition of the spatial learning task seemed to strengthen the spatial memory in the minimal TBI group, possibly due to a "compensational" process allowing the minimal TBI animals to compensate the deficit and again reach similar task performance as the sham group with further training. This compensation might be mediated by the medial prefrontal cortex, which is known to be involved in reconsolidation of spatial memory [43,44] and to show hyperconnectivity after mTBI [49–51].

A comparison of our results, obtained after minimal TBI to previous studies is difficult because of the big variability of experimental methods and conditions reported in the literature. Using an open-skull mTBI rat model, Aungst *et al.* [8] observed increased escape latencies (i.e. reduced performance) in a Morris water maze task, 17 days post-injury, solely in response to repeated but not to single mTBI. Likewise, Dawish *et al.* [14] failed to find significant differences between sham and mTBI in escape latency 20 days post open-skull

mTBI in rats. Thus, it seems that after mTBI, impaired performance in this task can hardly be observed after more than 2 weeks of recovery (in rats). This is corroborated by findings of Petraglia *et al.* [12] who reported significantly higher escape latencies after single and repeated closed-skull mTBI in mice during the first 5 days post-injury. Thereafter, the performance difference between the experimental and the sham control group declined. Probe trial performance on day 6 post-injury, however, was solely reduced in repeated mTBI mice but not in single mTBI animals. Another closed-skull mTBI study in mice examined spatial learning in the Morris water maze at 7 days post-injury and found impairments of performance that were correlated to the severity of the impact [13]. Similar deficits were noticed in the probe trial, conducted one day after completion of acquisition trials. Taken together, these studies support our conclusion that our mice were impaired in LTM reconsolidation, as our probe trial was measured 72 hours and not 24 hours after acquisition as in the other studies. Thus, it appears reasonable to assume that such deficits in LTM develop gradually over time in the absence of learning trials. They are, however, masked by the subsequent acquisition trials during the second week of training which initiate a new cascade of reconsolidation events.

No differences between groups were found during the reversal-learning phase where the platform was moved to the opposite quadrant. Platform reversal forces the animals to adapt their spatial map acquired during the 10 training days before. This adaptation process led to, or was accompanied by, a transient decline in the use of spatial search strategies. However, both sham and minimal TBI mice improved their performance during the subsequent days to similar values as before the reversal, making the overt performance of both groups very similar.

In the contextual fear-conditioning task, a hippocampus-dependent “one-trial” non-repetitive type of learning, minimal TBI animals showed a significantly stronger conditioned

response after the first pairing of tone and shock at four days, but not five weeks post-surgery. Nevertheless, no group differences in the conditioned memory tests were found, leading to the hypothesis that the observed changes in freezing behaviour during conditioning could be related to an increase in anxiety-like behaviour after an aversive stimulus in concussed animals and not to cognitive changes. This pattern falls in line with a number of clinical and experimental studies showing impairments in affective states after concussive brain injury. Patients sustaining mTBI typically report emotional in addition to cognitive symptoms; particularly anxiety, irritability and depression [52]. Furthermore, Meyer *et al.* [49] reported increased conditioned fear and anxiety-like behaviour after experimental mTBI. Petraglia *et al.* [12], who investigated anxiety-like behaviour after mTBI utilizing an elevated-plus maze, described initially pronounced anxiety-like behaviour after mTBI at two weeks post-surgery, as indicated by less time spent in the open arms. However, at one to six months post-surgery, risk-taking was more pronounced in mTBI animals than in sham animals [12]. In the present study, we found no differences in baseline freezing behaviour but a specific transient increase in freezing behaviour after the context was paired with the shock. This suggests that anxiety-like behaviour is pronounced only after presenting an aversive stimulus, indicating amplification of an affective reaction rather than an overall increase in anxiety-like behaviour. However, the exact mechanisms behind this process remain to be further experimentally examined.

5. Conclusions

Our results demonstrate that exposure to a very mild single closed-skull mTBI (minimal TBI) causes behavioural abnormalities that manifest as temporary deficits in long-term memory during the early phase of spatial learning in the water maze, accompanied by a higher proportion of non-spatial search strategies and compensated by further training. These

deficits were accompanied by an increase of GFAP in the hippocampus. In addition, minimal TBI results in an increased anxiety-like response to an aversive stimulus. This spectrum of behaviour falls in line with clinical data implicating mild TBI in the development of sub-acute and in some cases even chronic neurological sequelae. Our findings provide substantial evidence that even a single-episode mTBI leads to cognitive and affective impairments that are, however, confined to specific phases and parameters of spatial learning and contextual fear conditioning. Although a number of experimental studies have investigated functional impairments after mTBI, the specific experimental conditions and animal models have been very different, complicating comprehensive conclusions. The transient long-term memory deficits observed in our study turned out to be paralleled by an increased expression of GFAP, but no changes in CD68 expression, indicating astrocyte activation without major neuroinflammation. It will be relevant for future studies to undertake a further characterization of the cellular and molecular mechanisms underlying such mild forms of mTBI, and to examine whether they increase the risk for the development of cognitive disorders in concert with other detrimental environmental factors.

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Figure Captions

Fig. 1. Schema and timeline (in days) of all experimental procedures. Numbers in brackets refer to the amount of sham and minimal TBI animals used, respectively. Abbreviations: CFC: contextual fear conditioning, MWM: Morris water maze, acquis. = acquisition, WB: western blot.

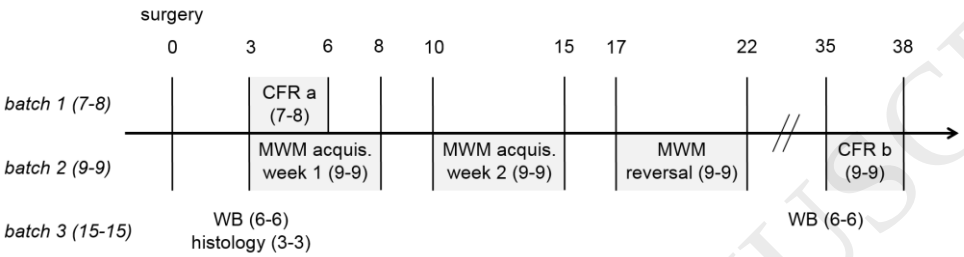

Fig. 2. Representative Nissl-stained, 50 μm coronal brain sections of a sham (left) and minimal TBI (right) mouse, 3 days post-surgery. Insets focus on cortex and hippocampus, indicating no overt gross anatomical abnormalities. Top scale bar: 2 mm, inset scale bars: 0.5 mm.

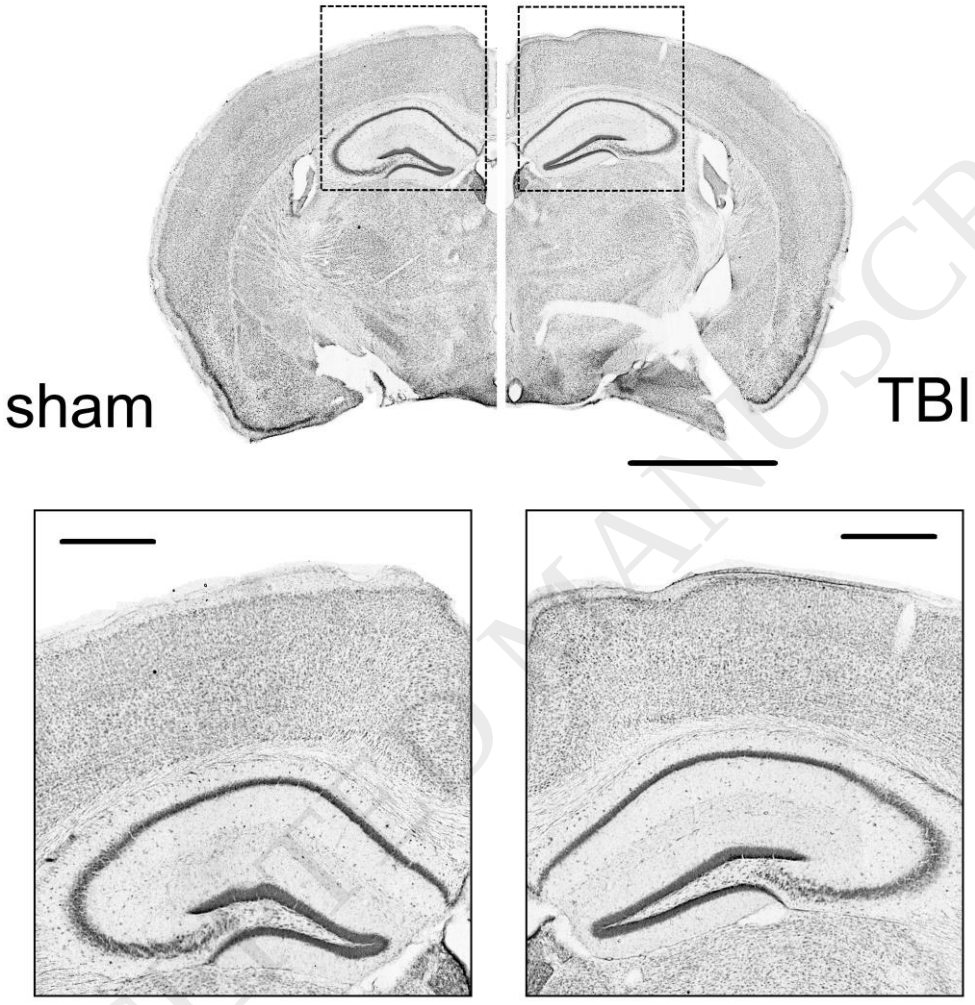

Fig. 3. Spatial learning in the Morris water maze in mTBI mice. Total swimming distance of minimal TBI and sham control mice during (A) first week of acquisition, (B) second week of acquisition and (C) reversal learning. (D) 72 hours after each of the two acquisition phases, a probe trial was performed. Frequency in target crossing is plotted. (E) Comparison of the spatial performance during the fourth trial of training day 5 with the first trial of training day 6 reveals a long-term memory deficit after the 72-hour weekend pause. Asterisks indicate significant intergroup differences between the two groups (*p < 0.05), hash tags significant intragroup differences (## p< 0.01). Values are given as means with SEM.

Figure 3

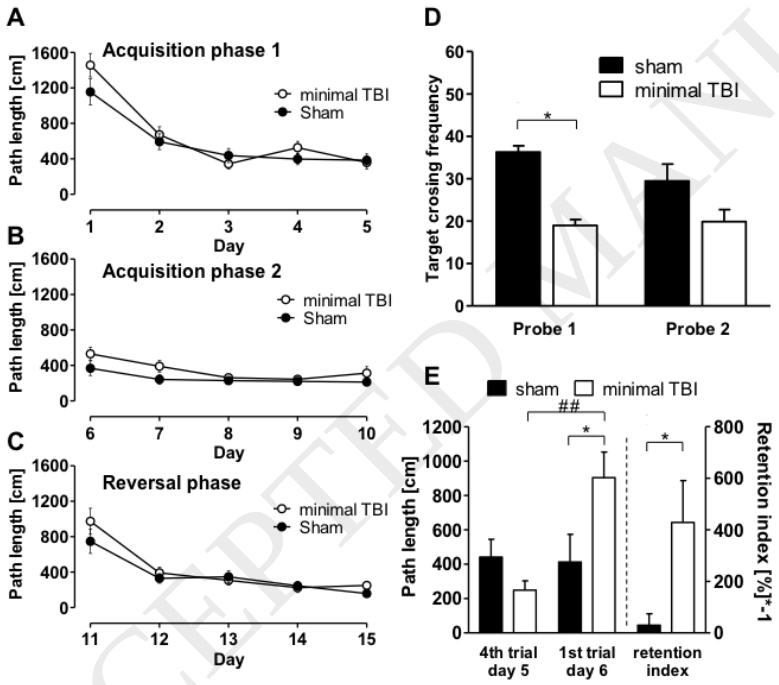

Fig. 4. Search strategies assigned to Morris water maze tracks by automatic classification during the two acquisition phases and reversal learning for (A) sham and (B) minimal TBI. Tracks that could not be classified as either search strategy are pooled under *error*.

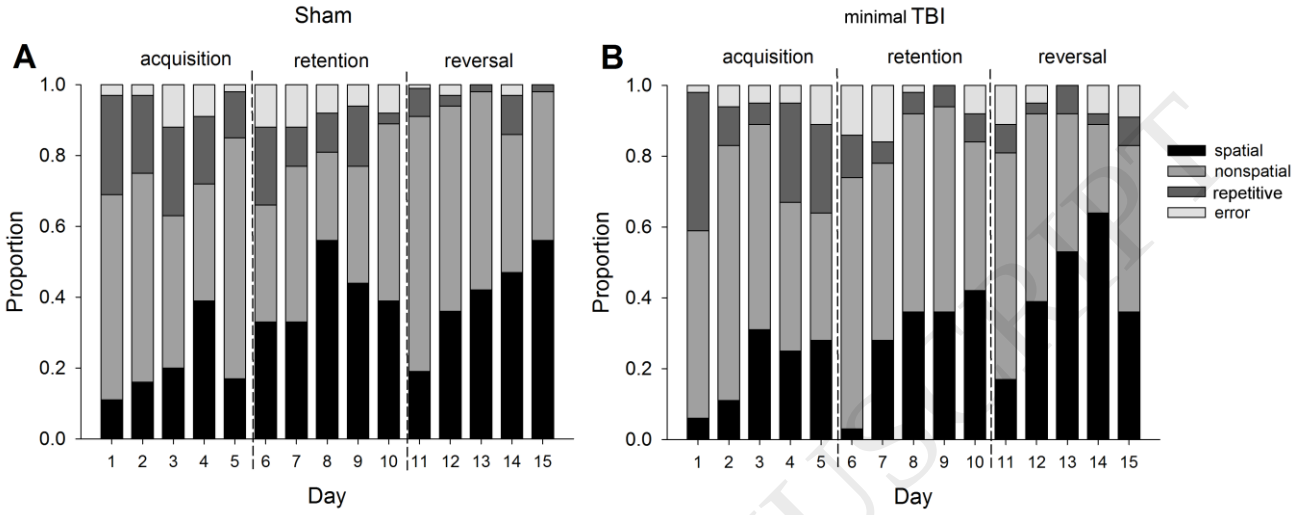

Fig. 5. Contextual fear-conditioning three days (A) and five weeks (B) post-surgery. Both minimal TBI and sham mice show a significant freezing response to the unconditioned stimulus (US; foot shock) at 3 days and 5 weeks post-surgery. Note that the minimal TBI group displays a stronger conditioned response to the US at 3 days post-injury. Nevertheless, both groups show a similar freezing response when re-placed in the old context, and before and after the conditioned stimulus (CS; tone) in a new context. Asterisks indicate significant differences between the two groups (*p < 0.05, ***p < 0.001). Values are given as means with SEM.

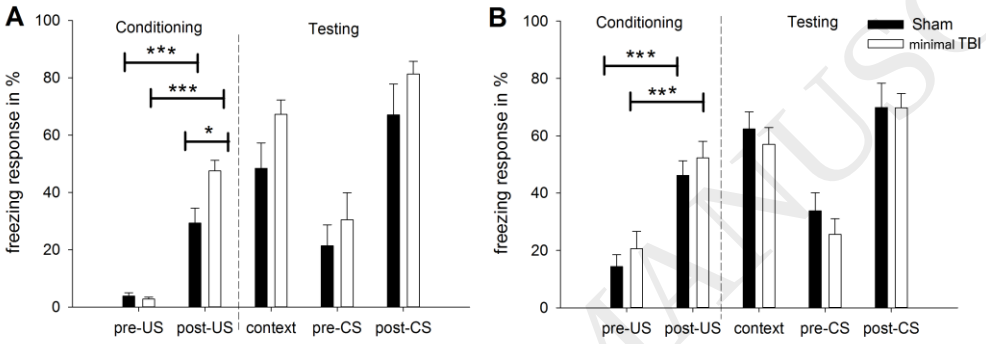

Fig. 6. Immunoblotting results of hippocampus and cortex samples, collected from minimal TBI and sham mice at 3 days or 5 weeks post-surgery. All results were normalized to β-actin as internal gel loading control. For every marker, minimal TBI values were normalized to the average sham value. A. Images of the blots for a representative minimal TBI and sham lane for GFAP. Selected markers: B. GFAP; C. PSD95, D. total GSK3β; E. GSK3β phosphorylated at Ser9; F. GSK3β phosphorylated at Tyr216; G. NMDA receptor subunit GluN2A; H. NMDA receptor subunit GluN2B. Values are given as means with SEM.

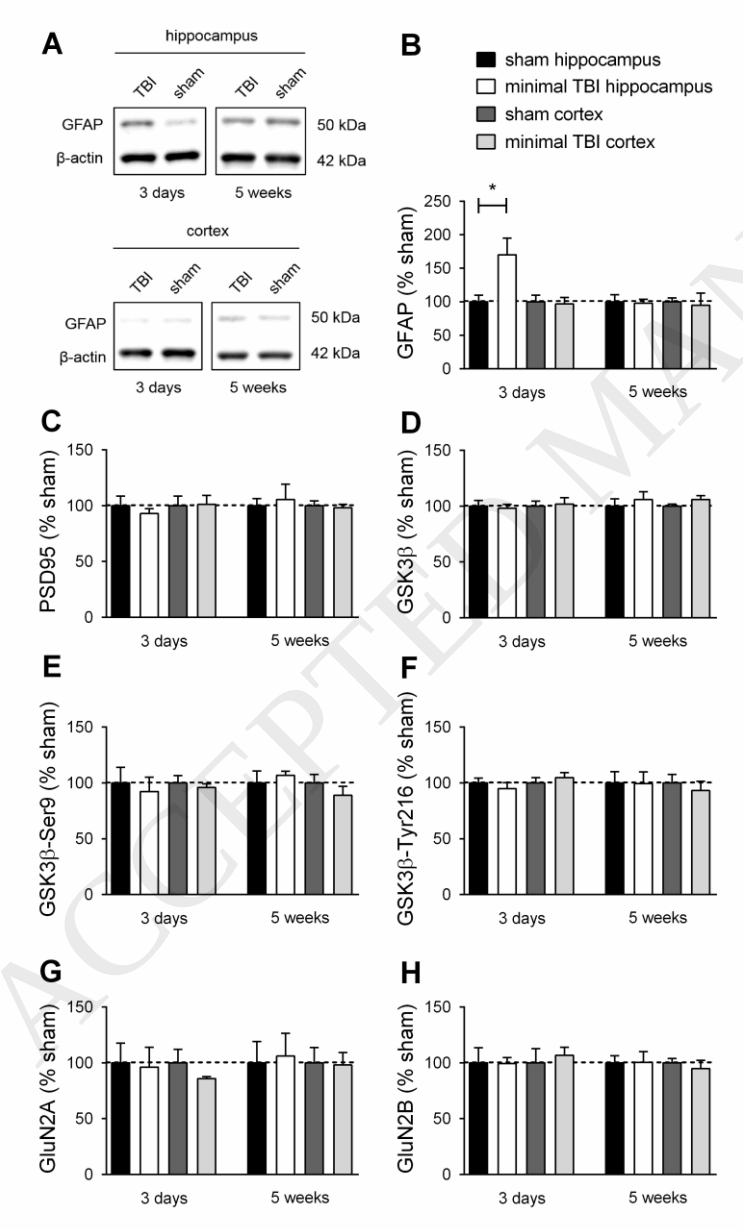

Fig. 7. Immunoblotting results for CD68 in hippocampus samples, collected from minimal TBI and sham mice at 3 days post-surgery. Results were normalized to β-actin as internal gel loading control. A. Images of the blots for a representative minimal TBI and sham lane. B. Quantified immunoblotting results, where minimal TBI values were normalized to the average sham value. Values are given as means with SEM.