Traumatic brain injury in late adolescent rats: effects on adulthood memory and anxiety

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ABSTRACT

The consequences of traumatic brain injury (TBI) sustained during late adolescence (7 weeks-old) on spontaneous object recognition memory and on anxiety-like behaviors in the elevated plus maze were tested in rats during adulthood. Testing took place at two different post injury times, in separate groups: three and six weeks, when animals were 10 and 13 weeks old, respectively. The rats were either submitted to controlled cortical impact injury, an experimental model of focal TBI with contusion, or were sham-operated. TBI animals failed to remember the familiar object and had a significantly lower performance than sham-operated animals, indicating memory disruption, when the retention delay was 24 h, but not when it was 3 h. TBI did not have any significant effect on the main anxiety-related behaviors, but it reduced time in the central platform of the elevated plus maze. The effects of TBI on memory and on anxiety-like behaviors were similar at the two post injury times. In both TBI and sham-operated groups, animals tested six weeks after surgery had lower anxiety-related indices than those tested at three weeks, an effect that might be indicative of reduced anxiety levels with increasing age. In summary, focal TBI with contusion sustained during late adolescence led to object recognition memory deficits in a 24-h test during adulthood, but did not have a major impact on anxiety-like behaviors. Memory deficits persisted for at least six weeks after injury, indicating that spontaneous modifications of these functional disturbances did not take place along this time span.

Keywords

Traumatic brain injury

Controlled cortical impact

Object recognition memory

Emotional reactivity

Rat

Running head: TBI, object recognition memory and anxiety

Introduction

Traumatic brain injury (TBI) afflicts millions of people worldwide. In contrast to other kinds of acquired brain damage, that show their highest prevalence in old populations, TBI is the leading source of acquired brain damage in children and youngsters. It causes a large range of deficits, which can be maintained or even aggravated over time, leading to subsequent long-term personal, social and economical burdens (Thurman, 2014). In children and adolescents the prevalence of single and repeated events of mild TBI leading to concussive damage is higher than that of more severe injuries up to the point that they have been considered a silent epidemic that should be more fully characterized (Petraglia, Dashnaw, Turner, & Bailes, 2014). However, a review of epidemiological research of pediatric TBI carried out in North America, Europe, Australia and New Zealand indicated that the incidence of hospitalized (and thus often moderate or severe) brain injuries, as well as fatal injuries, consistently peaked among late adolescents (compared to younger ages), and that males had a higher risk of injury than females (Thurman, 2014).

TBI can be roughly classified as either focal or diffuse. Focal damage results from a direct impact to the skull, and produces focal contusions as well as hematomas, while diffuse damage, which causes widespread axonal injury, is the result of rapid acceleration-deceleration of the head. It is estimated, though, that 50% or more patients with moderate-to-severe TBI exhibit a combination of focal and diffuse damage (Andriessen, Jacobs, & Vos, 2010). In both focal and diffuse TBI the mechanisms of injury can be classified as either primary or secondary. Primary injury is a direct consequence of mechanical deformation of brain tissue occurring immediately after being exposed to an external force, and giving rise to contusion, bleeding and axonal rupture, with subsequent necrotic neuron and glial cell death. Secondary injury, which evolves over a period of hours to days, and even months, after the primary insult, is the

result of biochemical and physiological events, such as ischemia, inflammation, altered neuronal and glial functions, loss of membrane integrity, etc, that ultimately lead to neuronal cell death (O'Connor, Smyth, & Gilchrist, 2011). In parallel with neurodegenerative changes, other phenomena with potential reparative capacity, such as cell proliferation, neurogenesis, and a wide array of plasticity-related mechanisms, can also be observed (Saha, Jaber, & Gaillard, 2012). The conjunction of long-term neurodegenerative and neuroreparative phenomena leads to dynamic histological and functional changes over time.

Multiple animal models have been designed in order to replicate the diverse physiopathological and functional consequences of human TBI. The most widely used are lateral fluid percussion (LFP), controlled cortical impact (CCI), and weight-drop. In LFP a fluid wave impacts dura and fills into insterstitial spaces, causing focal brain injury, as well as a certain degree of diffuse injury. In CCI a pneumatic device causes a piston to impact on the dura at predetermined speed and depth. CCI is considered a model of focal contusion, but it also involves widespread damage to both gray and white matter regions. In weight-drop models, the skull is exposed (with or without a craniotomy) to a free falling weight. Variations of these models have also been designed in order to model mild concussion and more diffuse patterns of injury. These variations include medial fluid percussion and closed head injury by means of a piston impacting on the skull (and not the dura) or by dropping a weight on a disk cemented onto the skull (Gold et al., 2013; O'Connor et al., 2011; Xiong, Mahmood, & Chopp, 2013).

Using animal models, long-term evolution (from several weeks and up to one year) of TBI has been characterized both post-mortem and *in vivo* (neuroimaging) mainly after LFP (Bouilleret et al., 2009; Bramlett & Dietrich, 2002; Immonen et al., 2009; Liu et al., 2010; Pierce, Smith, Trojanowski, & McIntosh, 1998), but also after

CCI (Ajao et al., 2012; Chen, Pickard, & Harris, 2003; Kamper et al., 2013; Park et al., 2014; Turtzo et al., 2012), as well as after models of diffuse TBI (Adelson et al., 2001). In general terms, these studies indicate progressive enhancement of lesion severity over time, although evolution profiles may vary depending on the outcome measure targeted (Osier, Carlson, DeSana, & Dixon, 2014).

Temporal evolution of functional deficits has also been studied with several TBI animal models by comparing memory performance at different post injury times. In rodents submitted to TBI at adult ages, persistence of spatial memory deficits has been reported after LFP over periods of weeks (Bramlett, Green, & Dietrich, 1997), and even up to one year (Pierce et al., 1998), but there is one report indicating that the memory deficits found in the first month after the initial damage were no longer present at a later time; this functional recovery might be related to increased neurogenesis and survival of new neurons (Sun et al., 2007). Using a non-spatial memory task, object recognition memory (ORM), persistence of deficits has been also described after closed head injury (Chen et al., 2013; Siopi et al., 2012) , and CCI (Darwish et al., 2014). However, there are also instances of partial recovery over time. For example, Tsenter and colleagues (Tsenter et al., 2008) found that ORM deficits induced by closed head injury in mice were lower 28 days after lesion than 3 days post injury. Finally, some reports indicate that memory deficits can show a delayed appearance. For example, Milman and colleagues (Milman, Rosenberg, Weizman, & Pick, 2005) found memory deficits in the water T-maze and passive avoidance in mice submitted to weight drop injury when testing took place 30 and 90 days post injury, but not 7 days post injury.

Besides memory and other cognitive functions, there is also an elevated prevalence of emotional alterations in TBI patients (Malkesman, Tucker, Ozl, & McCabe, 2013), which can significantly impair the quality of life. Animal studies on

this topic have led to inconsistent results, perhaps as a consequence of differences in TBI models and severity, as well as in other methodological considerations (specific tests used, post injury testing times, etc). For example, in adult rodents increased anxiety has been reported after LFP (Liu et al., 2010), closed head injury (Meyer, Davies, Barr, Manzerra, & Forster, 2012), and an impact acceleration variation of weight drop injury (Pandey, Yadav, Mahesh, & Rajkumar, 2009), while decreased anxiety levels have been found after CCI (Washington et al., 2012). Finally, lack of changes in anxiety and emotional reactivity have also been described after closed head injury (Siopi et al., 2012). Anyhow, changes in emotional processes can interfere with performance in memory tasks, and this fact underscores the importance of taking measures of emotional reactivity when assessing learning and memory functions.

Age at the time of brain injury is a significant factor affecting long-term functional outcome. On the one hand, it has been described that TBI leads to poorer functional outcome in old rats compared to adult and young rats (Mehan & Strauss, 2012). On the other hand, there is evidence that brain injury in juveniles can lead to increased severity of symptoms, compared to injury in adulthood, probably as a result of disrupted neurodevelopment (Kamper et al., 2013). In spite of that, the number of animal studies on this topic using ages equivalent to human infancy and childhood is relatively scarce (although it has increased in the latter years). This scarcity is even more pronounced for adolescence, a period of high vulnerability to stress and other vital experiences (Lynn & Brown, 2010; Schneider, 2013), so that it has been claimed to be a hole in animal literature concerning the long-term effects of TBI sustained during this period of life (Hartman, 2011). Moreover, the number of studies that have tested emotional and cognitive function after different post injury times in order to analyze the temporal evolution of these functions after TBI is low. These studies have been mainly

carried out with rodents lesioned at ages equivalent to human neonates and toddlers and have used several TBI models, such as CCI (Ajao et al., 2012; Kamper et al., 2013), a modified midline CCI that induces concussion-like injury (Huh, Widing, & Raghupathi, 2008), a closed head CCI (Huh, Widing, & Raghupathi, 2011), or impact acceleration injury by means of weight drop (Adelson et al., 2001). Long-term evolution (up to 12 weeks) of memory deficits has also been examined in rats that sustained CCI injury at an age (4 weeks old) corresponding approximately to late childhood or beginning of adolescence (Park et al., 2014), but, to our knowledge, studies comparing memory performance and/or emotional disturbances after different long-term post injury times in rodents submitted to TBI during late adolescence are lacking.

To sum up, at any age TBI gives rise to a complex conjunction of long-term neurodegenerative and neuroreparative phenomena, but the former tend to win the battle over the latter if no effective treatments are administered, so that pervasive deficits are common, especially after moderate or severe degrees of injury. Moreover, the neurodegenerative and neuroreparative phenomena initiated by TBI can interfere with normal brain development. For these reasons it was hypothesized that TBI in late adolescent animals would lead to persistent deficits of memory and emotional functions during adulthood. To test this hypothesis we have studied, in male rats, the long-term effects of TBI sustained during late adolescence (7 weeks old) on ORM and anxiety-related behaviors during adulthood at two different post injury times: three and six weeks. TBI was induced by means of CCI, which, as stated before, is considered a model of focal brain damage, affecting mainly cortical and subcortical structures proximal to the impact site, but also producing widespread gray and white matter damage (Budde, Janes, Gold, Turtzo, & Frank, 2011; Hall et al., 2005). In addition, CCI reproduces most (although not all) pathophysiological and functional features of human

TBI and allows rather precise control of injury severity (Gold et al., 2013; Xiong et al., 2013). CCI has been widely used in adult rodents, and, to a lower extent, in rodents of pediatric ages, and it has been characterized as a useful model of focal experimental TBI in immature rats (Adelson, Fellows-Mayle, Kochanek, & Dixon, 2013). According to other works using the same or similar parameters (Turtzo et al., 2012; Yu et al., 2009), the degree of injury inflicted by the CCI parameters applied can be considered as moderate or moderate-to-severe.

Materials and Methods

Ethics and animal welfare. All procedures were performed in compliance with the European Community Council Directive for care and use of laboratory animals (86/609/EEC), and with the related directive of the Autonomous Government of Catalonia (DOGC 2073 10/7/1995).

Fifty-two Sprague-Dawley albino rats (Prolabor, Barcelona, Spain), six-weeks old on their arrival to the laboratory, were used. Upon arrival, they were kept in the quarantine room for one week. Thereafter, they were singly housed in 52 x 28 x 18 cm cages.

The age of the animals at the beginning of the experimental procedures was seven weeks, and their mean initial body weight was 262.77 g (SD ± 27.07). Food and water were available ad libitum. The animals were kept under conditions of controlled temperature (20–22°C) and humidity (40–70 %), and maintained on a 12-h light-dark cycle (lights on at 8:00 a.m.).

Experimental groups

Four groups of animals were used: TBI-3W, Sh-3W, TBI-6W and Sh-6W. These

four groups were the result of combining the following two conditions: 1) lesion: TBI (TBI groups) or sham operations (Sh groups), and 2) post-surgery delay: three (TBI-3W and Sh-3W groups) or six weeks (TBI-6W and Sh-6W groups). Assignment of the rats to the groups was random.

Stereotaxic surgery and TBI model (CCI)

For stereotaxic surgery, anesthesia was induced with 5% isoflurane (Forane, Abbot Laboratories, SA, Madrid, Spain) in oxygen (2 l/min) in a Plexiglas chamber (20 x 13 x 13cm) for 7 min. The animals were then placed in a stereotaxic frame (David Kopf Instruments, Tujunga, USA) and the anesthesia was continued by delivering 2% isoflurane in oxygen (1 l/min) through a nose mask. The scalp was incised on the midline, and after the skull was exposed, a craniectomy (4 mm diameter) was performed over the right hemisphere (4.5 mm posterior to Bregma and 3 mm from midline). The pneumatically operated TBI device (Pittsburgh Precision Instruments, Inc., USA) with a 3 mm tip diameter impacted the brain at a velocity of 6 m/s reaching a depth of 2 mm below the dura matter layer, and remained in the brain for 150 ms. The impactor rod was angled 15° to the vertical to maintain a perpendicular position in reference to the tangential plane of the brain curvature at the impact surface. A transducer connected to the impactor measured velocity and duration to verify consistency. Thereafter, the scalp was sutured. To control for postoperative pain, a single 0.2 ml subcutaneous injection of buprenorphine (Buprex, Schering-Plough, SA, Madrid, Spain) was administered. Animals of Sh-3W and Sh-6W groups were operated in a similar way, except that no impact was applied.

Elevated plus maze test

The animals were tested in an elevated plus maze (EPM) either three (TBI-3W

and Sh-3W groups) or six (TBI-6W and Sh-6W groups) weeks after being operated.

The EPM (Cibertec S.A., Madrid, Spain) consisted of four black methacrylate arms arranged in the shape of a plus sign. Each arm was 10 cm wide, 49 cm long and elevated 31.5 cm above the ground. The four arms were joined at the centre by a 10 cm x 10 cm square platform. Two of the arms opposite each other had no sides and were open. The other two arms were closed on the sides, with 40 cm high walls, but open on the top. The open arms had 1 cm high edges as a tactile guide to prevent the animals from falling off these arms. The source of light was a light bulb suspended 1.6 m above the centre of the EPM giving illumination of approximately 60 lux on the floor of the central platform, 80 lux on the floor of the open arms, and 30 lux on the floor of the closed arms.

The rats were placed in the centre of the maze, always facing the same open arm. Each animal was tested for 5 min in a single session. An automated system (Test 4B, Cibertec S.A., Madrid, Spain), consisting of ten pairs of photoelectric cells that were strategically located in several parts of the apparatus, enabled us to record exploratory behavior in the EPM. The measurements recorded for all the subjects were: time spent in open arms, closed arms and central platform; number of open, closed, and total arm entries; incursions into the end of the open arms, defecations and micturitions, and grooming and rearing episodes. The open arm entries/total arm entries ratio (entries *ratio*) and the time in open arms/time in all four arms ratio (time *ratio*) were also calculated for all the subjects. During the EPM session a masking noise was provided by an electric fan. Before the first animal and between subjects, the EPM was carefully wiped with a 70% ethanol and dried in order to avoid the presence of olfactory cues.

Object recognition memory (ORM).

Object recognition memory procedures were started the day after testing in the EPM. Training was carried out in an open box (65.5 cm width x 65.5 cm length x 35 cm height) made of a conglomerate covered with brown melamine and enclosed in a sound-attenuating cage (72 cm width x 72 cm length x 157 cm height) made from white melamine, and ventilated by an extractor fan. The illumination on the floor of the box was 30 lux. The objects used varied in shape, color and size, and consisted of Lego pieces, a hanger and a drink can. They were fixed to the floor of the box with double-sided adhesive tape so that the rats could not move them. They were not known to have any ethological significance for the rats, and had never been seen by the animals. A prior pilot study had shown that rats of the same strain and age had no spontaneous preference for any of them. The objects for the recognition task were available in duplicate copies. All behavioral sessions were recorded with a video camera mounted above the experimental apparatus and controlled with video tracking software ANY-Maze (Stoelting Europe, Dublin, Ireland). All the measures were acquired through ANY-Maze software, except for object exploration, which was scored off-line by a trained observer who was unaware of the treatment condition and position of novel and familiar objects. To avoid the presence of olfactory cues, the apparatus and objects were thoroughly cleaned with a solution of 70% alcohol in distilled water and dried before the first rat, and after each animal.

To habituate the animals to the experimental box, three habituation sessions were carried out (two on the same day, separated by a 2-hr interval, and the third one on the following day). The animals were introduced into the recognition memory box, under the same lighting and sound conditions as during training but without any objects, and were allowed to explore it for 12 min. Total distance moved and number of defecations were recorded.

Neophobia test. In order to habituate the animals to the presence of unknown objects, a so-called neophobia test was carried out 2 h after the last habituation session. An unfamiliar object was exposed in the center of the open box. The animals were placed in the box facing away from the object and allowed to explore for 10 min. Latency of first object exploration, total time exploring the object, and total distance moved, were recorded. Throughout the experiment, exploration of an object was defined as directing the nose to the object at a distance ≤ 2 cm or touching it with the nose. Turning around or sitting on the object was not considered exploratory behavior.

Acquisition trial and memory tests. ORM training began the day after the neophobia test. During the acquisition session two identical objects were placed near two adjacent corners of the cage. The rat was placed in the experimental apparatus, facing the center of the opposite wall, and was allowed to explore for 15 min. Two memory tests were carried out, the first one 3 h after the acquisition trial, and the second one 24 h after it (that is, 21 h after the first memory test). In the first retention test, one copy of the object used on the acquisition session (familiar object), as well as a novel object were placed in the same two corners of the cage as in the acquisition trial. The novel object was presented in the left corner for half of the animals, and in the right corner for the other half. In the second retention test, one copy of the object used on the acquisition session (familiar object) and a novel object (different to the one presented in the first retention test) were also presented. The position of the familiar object was exchanged between the first and the second retention tests. The specific objects used as either familiar or novel were balanced, so all the possible combinations were present in each group. These procedures were intended to reduce potential biases due to preferences for particular location or for a particular object. Both retention tests had a duration of 5 min. The variables that were recorded were: time spent exploring each object, latency to first

object exploration, total object exploration times, and total distance moved. The identity (novel *vs*. familiar) of the object that was visited in the first place was also recorded in the retention trials, while the time exploring the object in the left and right corners was also recorded in the acquisition session. To determine the possible existence of a side bias, a left-right ratio [(time exploring the object in the left corner – time exploring the object in the right corner)/total object exploration time] was calculated for acquisition session. A ratio significantly different from 0 indicates a preference for either the left (when values are positive) or the right (when values are negative) object, while a ratio not differing significantly from 0 indicates a lack of preference for any corner.

Two measures were used to analyze cognitive performance: Percent novel object exploration time [(time exploring the novel object / total exploration time) x 100], and discrimination index [(time exploring the novel object – time exploring the familiar object)/ total time spent on both objects]. A value significantly higher than chance (50%) for percent novel object exploration time, and higher than 0 for discrimination index indicates that the animal devotes a significantly higher amount of time to explore the novel object than the familiar one. Thus, and since ORM is based on the natural tendency of rats to explore novelty, values significantly higher than 50% and 0, respectively, are considered a good recall of the familiar object, whereas values close to 50% or 0, respectively, (i.e., animals exploring both objects similarly) are considered to reflect a lack of recall (Akkerman et al., 2012). Both indices of relative exploration make it possible to adjust for any differences in total exploration time (Akkerman et al., 2012).

A criterion of ≥10 s of exploration during the acquisition session was established for animals to be included in the statistical analyses of ORM performance, since low exploration times may distort encoding processes in this task. This criterion was

selected because a methodological study found that the minimal amount of exploration that was required for reliable discrimination performance was 9-10 s (Akkerman et al., 2012).

Brain processing.

Twenty-four h after the second memory test, the animals were euthanized with an overdose of sodium pentobarbital (Dolethal, 200 mg/kg; Vetoquinol SA; Madrid, Spain) and intracardially perfused with 4% paraformaldehyde (PFA; Sigma-Aldrich, Madrid; Spain) in phosphate buffer saline. The brains were then extracted and submerged in a 4% PFA solution, rinsed with phosphate buffer, and submerged into a cryoprotective solution (sucrose 30% in phosphate buffer) for 3-4 days at 4ºC. Finally they were stored at -80ºC.

Nissl staining. Coronal slices, 40 μm width, were obtained using a cryostat (Shandon Cryotome FSE, Thermo electron corporation, Waltham, USA), and mounted on gelatin coated slides. In order to examine the macroscopic effects of TBI, one out of every ten coronal sections throughout the extent of brain tissue where the lesion cavity was visible were stained with cresyl violet in the animals of TBI-3W and TBI-6W. These sections were digitalized with a scanner (HP Scanjet G4050). Using Fiji image analysis software digital images were calibrated, and the areas of the following regions in the hemispheres ipsilateral and contralateral to the cortical impact were measured: lesion cavity, hippocampal formation, and lateral ventricle. For volume calculations, the areas obtained in each slice were multiplied by 0.04 mm (slices width) and by 10 (number of sections until the next slice analyzed).

In each section, an interhemispheric ratio score was computed [(ipsilateral area / contralateral area) x 100] for the hippocampal formation and the lateral ventricle. The mean ratio scores for all the sections in each rat were used for a more standardized

comparison between the two TBI groups. Given that these ratio scores are expected to be significantly similar to 100 if there is no volume change due to brain damage, and significantly different to 100 if otherwise, one sample t-tests were also used to determine whether interhemispheric ratio scores for each group were statistical different to 100.

Statistical analyses.

The statistical analyses were carried out with the statistical programming language R (R Development Core Team, 2011) and the support of the graphical user interface Deducer (Fellow, 2012).

Most of the behavioral data were analyzed by means of a linear model analysis of variance (ANOVA) with a full factorial 2x2 design. The two independent variables (factors) were lesion (two categories: TBI and sham), and post-surgery delay (two categories: three and six weeks). For the analyses of variables recorded during the habituation tests, repeated measures linear model ANOVA was used, with three repeated measures (one per each habituation session) for the dependent variable. When the conditions for application of linear model ANOVA were not fulfilled non parametric tests (Kruskal-Wallis rank sum test, comparing the four experimental groups) were used.

Two-sample *t*-tests were applied for the comparison between the histological data of the two TBI groups, as well as for any other comparisons between two conditions. One-sample *t*-tests were used when it was required to determine whether mean values per group were statistically different to a given reference value.

Finally, contingency table tests were used, for each retention test, to determine whether there was any significant relationship between the experimental condition and

the distribution of proportions of animals visiting the novel or the familiar object in the first place.

Statistical significance was set at the level of $P<.05$.

Results

Three animals died during surgery. Therefore, the final sample was composed of 49 rats, distributed as follows: Sh-3W (n=12), TBI-3W (n=10), Sh-6W (n=13) and TBI-6W (n=14).

Elevated plus maze

Table 1 indicates the mean (and SD) values per group for each of the measurements taken in the EPM.

A significant main effect of post-surgery delay was found on open arm entries [$F(1,45)=5.26$; $P=.026$], and on entries ratio [$F(1,45)= 4.619$; $P= .037$], while neither the main factor lesion nor the interaction between the two factors were significant. Specifically, both open arm entries and entries ratios were higher in the animals in the 6 week condition compared to rats in the 3 week condition, regardless of whether they had sustained TBI or had been sham-operated.

No effect of the main factor lesion was found, except for time in the central platform [$F(1,45)=6.92$; $P=.011$]. Specifically, TBI animals remained less time in this EPM location than sham rats. No other significant main effects or interactions were found on the EPM measures.

Object Recognition Memory

Habituation trials. A significant main effect of session was found for total distances

moved [F(2,90)=47.07; *P*<.001], while lesion, post-surgery delay, and their interaction, were not significant. Polynomial contrasts indicated that the evolution of distances moved fitted a quadratic function (*t*=4.39; *P*<.001), with a sharp decrease from the first to the second habituation sessions.

Exploration and locomotor activity in the neophobia test. One animal in Sh-6W group was excluded from the analyses of object exploration latency in the neophobia test because it had null object exploration.

No significant main effects or interaction were found for object exploration time and object exploration latency, while a significant lesion x post-surgery delay interaction was found for total distance moved [F(1,45)=5.50; *P*=.023]. Analyses of nested effects indicated that, in sham groups, animals tested three weeks after surgery moved longer distances in the neophobia test than rats tested six weeks after surgery [F(1,46)=5.72. P=.021]. In contrast, post-surgery delay had no effect on distances moved by TBI animals.

Object exploration and locomotor activity in the acquisition and retention trials. No significant effect of the main factors and their interaction was found for total exploration time, latency of first object exploration, and total distance moved in the acquisition session. One sample *t*-test (bilateral) indicated that the left-right side ratio of the acquisition session did not differ significantly from 0 in any of the groups; thus, no side bias was found for any of the groups.

With regard to the retention tests, no significant effects were found for distance moved, but there was a significant main effect of lesion on total object exploration during the 3-h retention test, indicating that TBI groups explored significantly less than sham groups [F(1,45)=4.50; *P*=.039]. TBI animals also tended to explore less than sham rats on the 24-h retention test, but this difference only approached significance

(*P*=.061).

In the 3-h and 24-h retention tests, contingency table tests indicated that there was no significant relationship between the experimental condition and the proportions of animals that first visited either the novel or the familiar object; i.e., these proportions were not statistically different across groups.

Discrimination indices and percent novel object exploration in the retention trials. According to the established criterion (a minimum of 10 s exploration time in the acquisition session), five subjects were excluded from the analyses of discrimination index and percent novel object exploration time (1 in TBI-3W, 2 in TBI-6W and 2 in Sh-6W groups). The final sample for memory analyses was thus composed of 44 subjects, 9 in TBI-3W, 12 in TBI-6W, 12 in Sh-3W, and 11 in Sh-6W.

Figure 1 depicts the mean values of percent time exploring the novel object for each of the four experimental groups in the 3-h and 24-h retention tests. With regard to the 3-h retention test, one-sample *t*-test showed that in all the groups percent time exploring the novel object was significantly higher than chance (50%) [TBI-3W: $t(8)=4.28$; $P=.001$; Sh-3W: $t(11)=6.14$; $P<.001$; TBI-6W: $t(11)=5.92$; $P<.001$; Sh-6W: $t(10)=5.12$; $P<.001$], and discrimination index was significantly higher than 0 [TBI-3W: $t(8)=4.30$; $P=.001$; Sh-3W: $t(11)=6.11$; $P<.001$; TBI-6W: $t(11)=5.90$; $P<.001$; Sh-6W: $t(10)=5.12$; $P<.001$]**,** indicating a good recall of the familiar object. Lineal model ANOVA indicated that the two main factors and their interaction were not statistically significant for either of these two measures. Thus, percent times of novel object exploration and discrimination indices were similar in TBI and sham rats and in both post-surgery delays.

With regard to the second (24-h) retention test, one-sample *t*-test showed that in the two sham groups percent time exploring the novel object was significantly higher

than chance (50%) [Sh-3W: t(11)=8.67; *P*<.001; Sh-6W: t(10)=4.27; *P*<.001], and discrimination index was significantly higher than 0 [Sh-3W: t(11)=8.61; *P*<.001; Sh-6W: t(10)=4.25; *P*<.001], indicating a good recall of the familiar object. In contrast, these values did not differ significantly from chance reference values in both TBI groups (TBI-3W and TBI-6W), indicating a lack of recall.

Linear model ANOVA showed a significant main effect of lesion on percent time exploring the novel object [F(1,40)=8.97; *P*=.004], and on discrimination index [F(1,40)=8.94; *P*=.004], in the 24-h retention, while neither post-surgery delay or the interaction between the two factors were significant. This indicates that TBI groups spent less time exploring the novel object and had a lower discrimination index than sham groups.

Measures of brain damage.

Figure 2A depicts the mean interhemispheric ratio scores for the volumes of the hippocampal formation and the lateral ventricle in each of the two lesioned groups, while a photograph of one coronal slice stained with cresyl violet in a representative TBI animal is shown in Figure 2B.

One sample *t*-tests (unilateral) indicated that both TBI groups had interhemispheric ratio scores significantly lower than 100 for the hippocampal formation [TBI-3W: *t*(9)=-3.64; *P*= .002; TBI-6W: *t*(13)=-7.26; *P*<.001], and significantly higher than 100 for the lateral ventricle [TBI-3W: *t*(9)=3.41; *P*=004; TBI-6W: *t*(13)=2.97; *P*=.005]. These data indicate that, in both TBI groups, the volume of the hippocampal formation was significantly reduced, and that of the lateral ventricle was significantly expanded, in the hemisphere ipsilateral to the lesion compared to the contralateral hemisphere.

Two-sample *t*-test analyses indicated that there were no significant differences between the two TBI groups in hippocampal and lateral ventricle ratio scores, as well as in the mean volume of lesion cavity.

Discussion

The main results of the present work indicate that, in concordance with our hypothesis, TBI sustained during late adolescence induces severe deficits in ORM during adulthood at two different post injury times (three and six weeks), but only when memory was tested 24 h after the acquisition trial and not when it was tested at 3 h. Specifically, TBI animals failed to remember the familiar object in the 24-h retention test and had a performance in this test that was significantly lower than that of sham-operated rats. Memory deficits were similar in the animals tested three weeks after TBI compared to those tested six weeks post-surgery, suggesting that no spontaneous modifications of TBI-related ORM deficits took place along this time span, which is rather long if we take into account that three weeks of a rat's life during early adulthood are estimated to be roughly equivalent to two years of human life (Sengupta, 2013).

The different outcome of TBI on 3-h vs. 24-h retention may be a consequence of differences in the requirements associated to each test, such as memory load, which is higher in the second retention test, as well as possible differences in the neural circuitry participating in each test. The involvement of the perirhinal cortex in ORM is not under dispute (Brown, Barker, Aggleton, & Warburton, 2012; Winters, Saksida, & Bussey, 2008), but this structure interacts with the hippocampus, and with other regions in and outside the medial temporal lobe to contribute to this memory task (Warburton & Brown, 2014). The specific role of the hippocampal formation within this circuit has not been fully elucidated, but it seems to play a more significant role in ORM when the delay between the sample phase and memory tests is increased (Hammond, Tull, &

Stackman, 2004), and when spatial requirements are emphasized (Warburton & Brown, 2014). Using the same ORM procedures as in the present work, positive correlations have been found between memory performance in a 24-h retention test (but not in a 3-h retention test) and the number of novel immature neurons (cells double labelled for bromodeoxyuridine and doublecortin) in the dentate gyrus, in rats (Jacotte-Simancas et al., 2014). Although correlational analyses do not involve any causal relationship, they nonetheless suggest a higher involvement of the hippocampal formation in the 24-h retention test, at least with the specific procedures used here. However, this is not to say that damage to the hippocampal formation be the solely responsible for the ORM deficits found. Damage to other structures such as cortical areas, thalamus and striatum (Zhao, Loane, Murray, Stoica, & Faden, 2012), reduced number of mature neurons in the perirhinal cortex (Jacotte-Simancas et al., 2014), and a wide variety of pathophysiological and neurochemical events (widespread inflammatory reactions and oxidative stress, demyelination, axonal injury, and alterations of several neurotransmitter systems, impaired neuroendocrine function, etc) (Biegon et al., 2004; Budde et al., 2011; Zhang, Han, Zhang, Sun, & Ling, 2014), may also contribute to ORM deficits after CCI in rodents.

The animals were 7 weeks old at the time of initial injury. In spite that not a clear definition of adolescence in rats is available, for male rats this age seems to correspond to late adolescence (McCormick & Mathews, 2010; Schneider, 2013). During adolescence multiple neurodevelopmental phenomena, such as substantial synaptic pruning in several brain areas, changes in the activity of multiple neurotransmitter systems, etc, take place (Schneider, 2013). Some of these neurodevelopmental processes have been linked to the specific neuroendocrine status associated to this period of life, such as the substantial increase of gonadal steroid

hormones and growth hormone (GH), as well as to differential reactivity of the hypothalamic pituitary adrenal (HPA) axis and higher stress vulnerability compared to other ages (Masel & Urban, 2014; McCormick & Mathews, 2010; Schneider, 2013). Besides a role in development, gonadal hormones, GH, and hormones of the HPA axis also participate in a variety of emotional and cognitive functions (Masel & Urban, 2014; McCormick & Mathews, 2010; Sisk & Zehr, 2005). In turn, altered neuroendocrine function is common after TBI in humans, including children and adolescents (Masel & Urban, 2014). Animal research has reported reduced levels of GH and testosterone after repeat (but not single) mild closed head injury in adolescent rats (Greco, Hovda, & Prins, 2014, 2013). In adult rats, disrupted hormonal stress responses after mild LFP (Griesbach, Hovda, Tio, & Taylor, 2011), and long-term alterations of HPA axis, gonadal hormones and GH after CCI (Kasturi & Stein, 2009; Taylor et al., 2008, 2010; Zhang et al., 2014) have been reported. Since no endocrine measurements have been done in the present work, an influence of altered hormonal status on the behaviors tested cannot be either confirmed or disregarded. For example, it is known that stress-related increase of corticosterone has disruptive effects on ORM in male rats, while both estrogens and testosterone exert positive modulatory effects on this task (Luine, 2014). In turn, GH participates in a wide variety of cognitive and non-cognitive functions, and during adolescence this hormone and its downstream mediator insulin like growth factor 1 regulate the expression of a wide variety of genes related to brain function (Yan et al., 2011). Zhang and colleagues (Zhang et al., 2014) found that CCI injured adult male rats had lower levels of overall objet exploration in an ORM task, similar to what has been found in the present work. Interestingly, object exploration was increased by GH replacement therapy, but only in GH deficient rats (which constituted 54.28% of all injured animals). No ORM deficits were found by Zhang and colleagues, probably

because memory was only tested after a relatively short delay (1 h). Thus, GH deficiency may contribute to altered levels of object exploration during ORM training, but not be its sole cause. Whether GH deficiency might contribute to the detrimental effects of TBI on ORM at longer testing intervals remains to be tested. Anyhow, the possibility that CCI-related endocrine alterations in rodents may lead to different emotional and cognitive outcomes during adolescence than at other periods of life awaits further investigation.

Several studies have analyzed the temporal evolution of memory deficits after TBI in juvenile rodents by testing memory functions at different post injury times. Using CCI in rats lesioned at postnatal day 17, no spatial memory deficits were found when animals were tested 30 and 60 days post injury (Ajao et al., 2012), but a longer follow-up study found spatial memory deficits 3 and 5 months post injury that seemed to have resolved by 6 months. Using models of diffuse TBI, and in rats lesioned at postnatal day 17, persistent memory deficits along three months post injury were reported (Adelson, Dixon, & Kochanek, 2000). In another work, diffuse TBI in rats of the same age was reported to induce deficits in acquisition of a spatial task when the animals were tested a few days post injury as well as 3 weeks later, but spatial memory was only affected at the latter time period (Huh et al., 2008). Thus, although lingering memory deficits are generally reported after TBI in immature rodents, there are also some instances of late-onset disturbances, and/or attenuation of the severity of deficits after a long period of time. With regard to rodents lesioned during adolescence, the majority of studies have tested memory function at a single post injury time (For example, Appelberg, Hovda, & Prins, 2009; Jacotte-Simancas et al., 2014; Mannix et al., 2014; Mehan & Strauss, 2012; Prins, Hales, Reger, Giza, & Hovda, 2010). A recent study examined the long-term evolution of step down avoidance memory in rats

submitted to CCI at 4 weeks of age, which would correspond to late childhood/beginning of adolescence. Memory deficits were found to persist from the first post injury time tested (7 days) to the last testing time, at 12 weeks (Park et al., 2014). The results of the present work indicate that CCI also causes persistent memory deficits in late adolescent rats, since impairment of 24-h ORM was present three weeks after injury and remained unchanged well into adulthood, six weeks after injury.

TBI animals had similar locomotion amounts (distances moved) than sham rats in the ORM cage during acquisition and retention trials. In contrast, they exhibited lower object exploration times in the retention tests, but not in the neophobia and acquisition sessions. These data might reflect a somehow reduced exploratory drive after CCI in adult and immature rats, in concordance with other reports (Ajao et al., 2012; Wagner, Postal, Darrah, Chen, & Khan, 2007; Zhang et al., 2014). Since ORM is based on exploratory activity, reduced object exploration during retention might have mediated the ORM deficits. This seems unlikely, though, because TBI rats spent a similar proportion of time exploring the novel object than sham animals in the first retention test, in spite of lower overall exploration times. Furthermore, the specific ORM measure used is known to minimize any possible influences of overall object exploration on memory (Akkerman et al., 2012). The ORM deficits cannot be attributed, either, to a side bias (which was not detected in any group) or to any putative influence of the object (familiar or novel) that was visited in the first place in the retention tests on percent time exploring the novel object.

In contrast to the detrimental effects on 24 h ORM, TBI only had minor effects on emotional reactivity. Thus, the EPM measures more directly related to anxiety, such as open arm entries and time ratio, were not affected by TBI. The only significant difference between TBI and sham groups in the EPM was the finding that TBI animals

spent less time in the central platform than sham rats, an effect opposite to a report indicating that male (but not female) preadolescent rats submitted to mild TBI/concussion by means of a modified weight drop injury spent more time in the central platform of an EPM than control rats when tested shortly after injury (Mychasiuk, Farran, & Esser, 2014). The meaning of time in the central platform is not clear, but it has been suggested that this measure may be related to risk assessment and decision making (about whether or not to enter the unsafe areas) (Casarrubea et al., 2013; Cruz, Frei, & Graeff, 1994). Thus, focal TBI with contusion might be associated to a lower risk assessment capacity in face of new and potentially threatening environments, without any significant alteration of anxiety-like behaviors. A comparison of anxiety-related behaviors after TBI in animal literature has led to rather inconsistent results, as indicated in the introduction section (Malkesman et al., 2013). With regard to immature rodents, Kamper and colleagues (Kamper et al., 2013), using rats submitted to CCI at postnatal day 17, failed to detect any change in anxiety-like behavior in the zero maze at any of the post injury testing times (3, 5, and 6 months); however, with the same model increased anxiety was found 60 days post injury, but not earlier (Ajao et al., 2012). This indicates that the effects of TBI on anxiety may vary depending on the time elapsed since injury. In concordance with this, using a model of concussion Mychasiuk and colleagues found that rats injured at 30 days of age did not differ from shams in time in open arms of an EPM when testing took place one day after injury (Mychasiuk et al., 2014). In contrast, increased anxiety-like behaviors were found when testing took place 33 days after injury regardless of whether the animals had received a single concussion or two concussive injuries separated by one month. There were, however, some differences between male and female rats (Mychasiuk, Hehar, van Waes, & Esser, 2015). Overall, these results suggest that anxiety-like behaviors may

vary depending on post-surgery delay, as well as on other variables, such as age and sex of the animals, kind of animal model of TBI, amount of prior handling, etc.

Anxiety-like behaviors, while not being influenced by TBI, were affected by post-surgery delay. Thus, in both TBI and sham conditions, animals tested six weeks after surgery (when they were 13 weeks old) showed a higher number of entries into the open arms and higher entries ratio than animals tested three weeks after surgery (at an age of 10 weeks old). This time-dependent effect on anxiety-like behaviors may be indicative of a slight reduction of anxiety with age, a finding which would be concordant with the progressive reduction of anxiety-like behaviors reported from adolescence to young adulthood, and from the latter to middle adulthood, by Lynn & Brown (2010). Additionally, or alternatively, the differences between the two time points might be due to the different lengths of the interval in which rats were left essentially undisturbed, from surgery to testing, rather than age. Surgery-testing interval also had an effect on locomotion during the neophobia test, where sham-3W animals moved more than sham-6W rats, while this effect was not seen in TBI rats. These data are concordant with a report of higher locomotion at postnatal day 72 than at postnatal day 117 in rats introduced for the first time in a cage containing novel objects (Saul et al., 2012), a condition with some similarities to the neophobia test. These results might, therefore, reflect the existence of possible age-related differences in locomotion under certain circumstances in sham-operated rats, which would be blocked by TBI.

No significant differences were found in the gross volumetric histological measures of brain damage between the two TBI groups (and, thus, between the two post injury times examined). Therefore, similar histological outcomes paralleled similar behavioral deficits in both TBI groups. The possibility, however, that differences in other measures related to brain damage may exist cannot be disregarded. Also

differences among groups might have arisen at longer follow-up periods, as it has been described after several TBI models in adult and juvenile rodents (Kamper et al., 2013; Osier et al., 2014).

In summary, experimental TBI by means of CCI during late adolescence (7 weeks old) induced ORM deficits when the animals were challenged with a 24-h (but not with a 3-h) retention delay. These deficits were evidenced at the two post injury times examined, three and six weeks, when the animal ages were 10 and 13 weeks old, respectively, indicating persistence of memory disturbances well into adulthood. TBI also had subtle effects on behaviors related to exploratory drive and risk assessment, but did not have a major impact on the main anxiety-like behaviors. Longer follow-up studies should be carried out after late adolescent CCI injury, as well as after other TBI models, to examine whether this behavioral profile is modified at older ages and whether temporal evolution of memory deficits and emotional reactivity differs depending on the kind of lesion inflicted and its severity.

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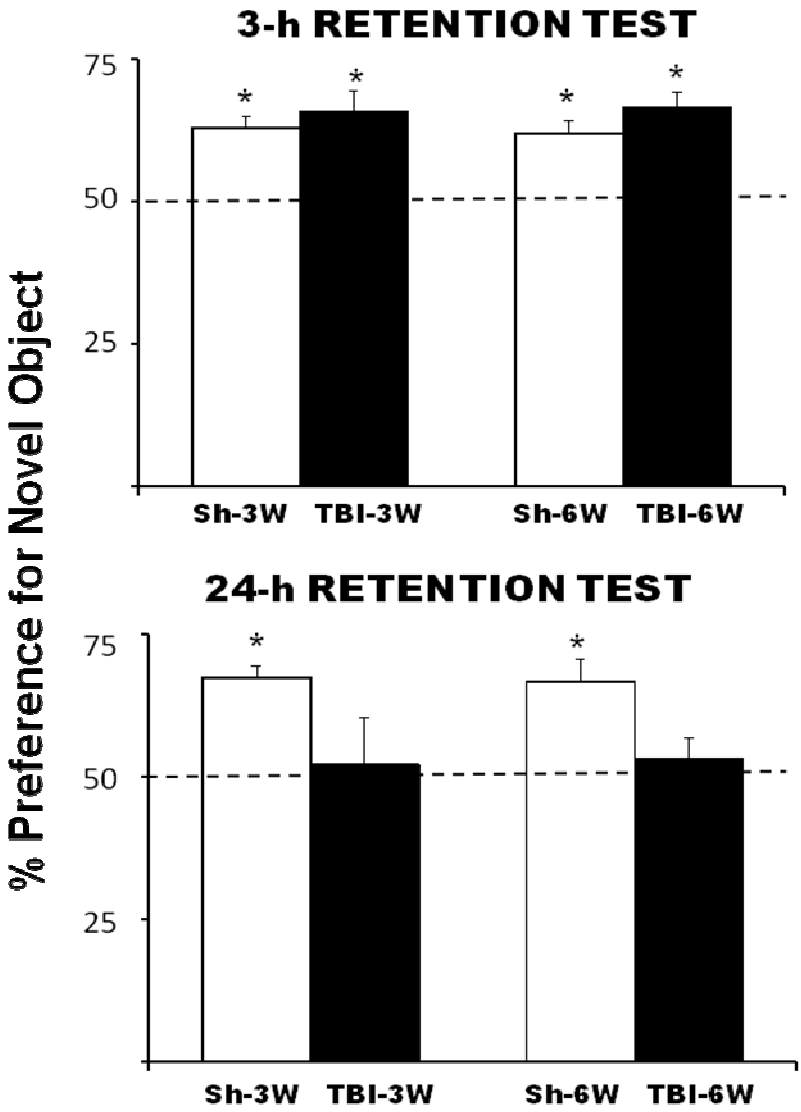

Figure 1. Performance in the object recognition memory tests: Mean (+SEM) percent time exploring the novel object in the 3-h and 24-h retention tests for each experimental group. Significant effect of the main factor lesion (P=.004) was found in the 24-h retention test, indicating that TBI disrupted memory in this test regardless of whether training started three or six weeks after injury.

* : Significantly different from chance level (50%) ($P \leq .001$). Chance level is depicted by dashed lines.

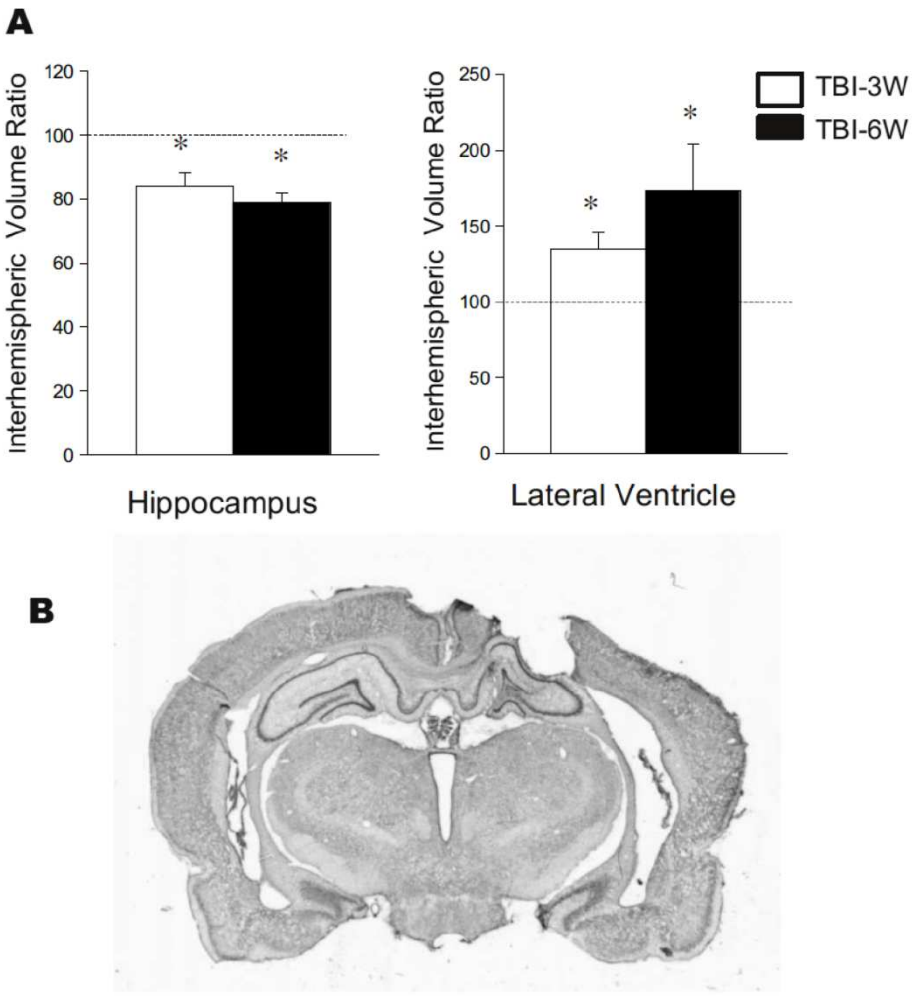

Figure 2. **A.** Mean interhemispheric ratio scores (+ SEM) for the volume of the hippocampal formation and the lateral ventricle in TBI-3W and TBI-6W groups. **B.** Microphotograph of a cresyl violet-stained section of the brain in a representative animal in the TBI conditions. * : Significantly different from 100, which is the reference (contralateral) value.

EPM measure	TBI-3W	Sham-3W	TBI-6W	Sham-6W	Statistical Effects
Open arm entries	2.1 (.8)	2.3 (1.7)	4.1 (4.0)	4.6 (4.4)	6W groups >3W groups (*P*=.026)
Open arm entries ratio	14.2 (6.8)	14.7 (9.4)	19.8 (16.2)	29.8 (26.0)	6W groups >3W groups (*P*=.037)
Time in open arms (s)	20.2 (18.4)	19.8 (22.3)	46.2 (55.2)	34.7 (55.8)	NS
Time ratio	10.1 (8.1)	10.7 (12.4)	22.4 (27.3)	17.8 (26.0)	NS
Closed arm entries	13.1 (3.3)	12.9 (2.6)	12.6 (3.8)	10.5 (5.6)	NS
Time in closed arms (s)	179.4 (37.1)	173.6 (33.3)	164.6 (63.3)	148.4 (60.9)	NS
Time in central platform (s)	91.3 (26.4)	106.5 (18.1)	89.14 (24.2)	116.92 (42.3)	TBI<Sham (*P*=.011)
Defecations	0	0.3 (0.5)	0.7 (1.2)	1 (1.6)	NS
Micturitions	0.3 (.5)	0.7 (.9)	0.6 (.6)	0.8 (.5)	NS
Open arm ends	0.3 (.7)	0.5 (.8)	1.6 (2.9)	0.5 (.7)	NS
Head dip	3.2 (2.6)	2.3 (3.7)	3.4 (3.7)	2.1 (1.9)	NS
Rearing	8.3 (3.0)	9.4 (4.1)	8.7 (4.0)	9.3 (4.3)	NS
Grooming	1.8 (1.3)	1.8 (1.5)	1.0 (.9)	1.5 (1.5)	NS

Table 1. Mean values (standard deviation) of the measures taken in the EPM for each experimental group. Statistical effects are indicated in the last column.

6W groups >3W groups: Indicates a significant effect of the main factor surgery-testing interval. Specifically, the mean pooled values of TBI-6W and Sh-6W groups were higher than the mean pooled values of TBI-6W and Sh-6W.

TBI<Sham: Indicates a significant effect of the main factor lesion, with the mean pooled values of the two TBI groups being lower than those of the two sham groups.