

Role of glycogen synthase kinase-3 β in early depressive behavior induced by mild traumatic brain injury

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Traumatic brain injury (TBI) is a triggering event for a set of pathophysiological changes and concomitant depressive behavior. Glycogen synthase kinase-3 (GSK-3) is a potent *in vivo* regulator of cell apoptosis and, in addition, is implicated in depressive behavior. In this study, we investigated the role of GSK-3 in the physiological model of mild TBI (mTBI) at both the cellular and behavior levels. mTBI resulted in increased phosphorylation of inhibitory site serine⁹ of GSK-3 β , which coincided with increased serine⁴⁷³ phosphorylation of its upstream kinase PKB and accumulation of its downstream target β -catenin in the hippocampus. mTBI induced a depressive behavior which was evident as early as 24 h post-injury. Pretreatment with GSK-3 inhibitors, lithium, or L803-mts prevented mTBI-induced depression. We suggest that mTBI elicits a pro-survival cascade of PKB/GSK-3 β / β -catenin as part of a rehabilitation program. Furthermore, the use of selective GSK-3 inhibitors may have therapeutic benefits in treatment conditions associated with brain injury.

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Introduction

Traumatic brain injury is a common neurological condition associated with neurological and psychological dysfunctions. The pathophysiology of TBI is characterized by cellular consequences leading to formation of reactive oxygen species, inflammation, and tissue damage (Shohami et al., 1999; Leker et al., 2002; Cernak, 2005). TBI elicits early intracellular pathways that involve apoptotic or pro-survival signaling. For example, TBI activates apoptogenic proteins, such as caspases *p53*, or the stress-activate kinase c-Jun NH₂-terminal kinase (JNK) (Rink et al., 1995; Kaya et al., 1999; Larner et al., 2005; Kuan et al., 2003). On the other hand, recent studies reported activation of protein kinase B (PKB) in response to

various insults, including brain injury, and production of neurotrophic factors, such as BDNF, in response to TBI, or Bcl2 (Mocchetti and Wrathall, 1995; Noshita et al., 2002; Chen et al., 2005; Neary et al., 2005; Zhang et al., 2006; Clark et al., 1997). TBI is also associated with cognitive and mental disabilities, in which depression is a common symptom (Robinson, 1992; Levin, 1998; Milman et al., 2005). In this context, it is noteworthy that recent studies correlated depressive behavior with neuron loss and neuron damage, particularly in the hippocampus (Pierce et al., 1998; Starkstein and Lischinsky, 2002; MacQueen et al., 2003; Sheline et al., 2003; Leuner et al., 2004; Rola et al., 2006). Thus, it is tempting to speculate that apoptosis and depressive behavior induced by TBI may share common mechanisms.

Glycogen synthase kinase (GSK)-3 is a serine threonine kinase that was originally identified as a key regulator of glucose metabolism (Woodgett and Cohen, 1984). In recent years, it became apparent that GSK-3 may play a critical role in the pathophysiology of neurodegenerative and affective disorders (Phiel et al., 2003; Chen et al., 2004; Cole et al., 2004; Hye et al., 2005). The GSK-3 β isoform has been linked directly to cell mortality and has been shown to provoke pro-apoptotic signals in various neuron cell systems (Pap and Cooper, 1998). On the other hand, use of selective GSK-3 inhibitors produced neuroprotective effects in a variety of pro-apoptotic insults (Cross et al., 2001; Li et al., 2002; Hongisto et al., 2003). Recent work suggested a central role for GSK-3 in bipolar disorder, because mood stabilizers, such as lithium and valproic acid, were found to inhibit GSK-3 (Klein and Melton, 1996; Chen et al., 1999). In addition, treatment with selective GSK-3 inhibitors produced anti-depressive activity in preclinical animal models (Gould et al., 2004; Kaidanovich-Beilin et al., 2004; O'Brien et al., 2004), and suggested that GSK-3 may have a broader role in the behavior domain.

Given that TBI is associated with both depression and cell death, we sought to evaluate the role of GSK-3 in a TBI model. Our studies suggest that GSK-3 is inhibited by TBI, and this likely supports an anti-depressive behavior imposed by the injury.

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Results

Phosphorylation of GSK-3 β at serine 9 inhibits the kinase activity (Sutherland et al., 1993). Thus, we first examined how serine phosphorylation of GSK-3 β is affected by the insult. Mice were subjected to mTBI, and hippocampi tissue extracts prepared from sham and injured animals were subjected to immunoblot analyses. Results indicated that mTBI led to a significant increase in serine 9 phosphorylation of GSK-3 β at 3, 6, and 24 h post-injury (Figs. 1A and B, and about 1.8-fold over control at 24 h time point). As PI3kinase/PKB is a key signaling pathway responsible for the phosphorylation of GSK-3 β (Cross et al., 1995), we examined the phosphorylation levels of PKB in the same hippocampi extracts. Indeed, mTBI increased PKB serine 473 phosphorylation at all time points (Figs. 1A and B, and 1.7 fold of control at 24 h post-injury). As this phosphorylation is necessary for PKB activation (Alessi et al., 1996; Bellacosa et al., 1998), it is concluded that mTBI led to PKB activation that, in turn, phosphorylated GSK-3 β . Further evidence for the role of PI3 kinase/PKB pathway GSK-3 β phosphorylation in the hippocampus is demonstrated in Fig. 2C also.

GSK-3 β phosphorylates β -catenin at three residues: Ser33, Ser37, and Thr41. This, in turn, destabilizes the protein and enhances its proteosomal degradation (Yost et al., 1996; Ikeda et al., 1998; Liu et al., 2002). Thus, the accumulation of β -catenin is often considered a “good” marker for *in vivo* inhibition of GSK-3 β . β -catenin levels were determined in the hippocampus tissue extracts prepared from control and injured animals by Western blot analysis. Results indicated that β -catenin levels were increased

significantly in response to mTBI (Fig. 2A). Moreover, the use of a specific β -catenin antibody (ABC- β -catenin), which recognizes the dephosphorylated β -catenin only, verified that the accumulation of β -catenin was associated with specific dephosphorylation at GSK-3-sites (Fig. 2A). Indirect immunofluorescence analyses of the hippocampus sections further showed a significant increase in β -catenin in the ipsilateral-injured hippocampus. β -catenin accumulation was evident in the dentate gyrus (DG) and CA3 regions particularly, as compared with non-injured hippocampus (Fig. 2B).

To further evaluate the role of PI3kinase/PKB signaling in the phosphorylation of GSK-3 β and, subsequently, the regulation of β -catenin, a selective PI3 kinase inhibitor, LY294002, was injected intracerebroventricularly (i.c.v.), in animals. The impact on serine phosphorylation of GSK-3 β or expression levels of β -catenin was determined. As shown in Fig. 2C, treatment with LY294002 significantly reduced serine phosphorylation of GSK-3 β . In addition, LY294002 reduced β -catenin levels (Fig. 2C). These results supported a role for the PI3K/PKB pathway in regulating GSK-3 β phosphorylation and, subsequently, β -catenin in the hippocampus.

Inhibition of GSK-3 was shown previously to produce anti-depressive-like effect (Kaidanovich-Beilin et al., 2004; Gould et al., 2004; O'Brien et al., 2004). We next examined whether GSK-3 may affect depressive behavior associated with mTBI. The studies used the well-established preclinical model for depressive-like activity, the forced swimming test (FST) (Porsolt et al., 1977). FSTs were performed 24 h post-injury, and the results indicated that mTBI increased immobility time duration of the injured

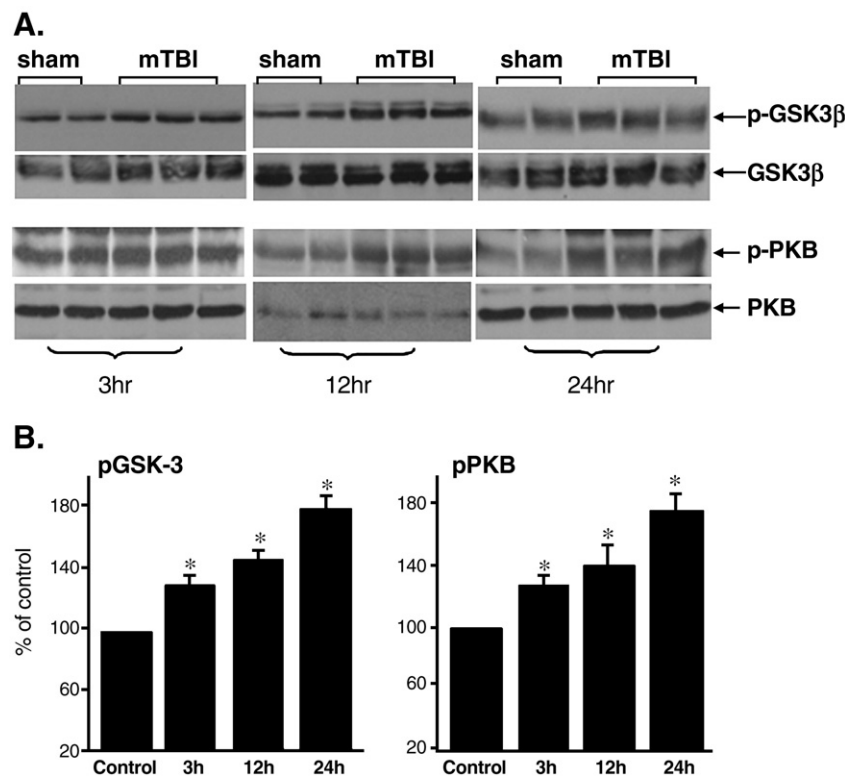


Fig. 1. GSK-3 β and PKB phosphorylation is enhanced after mTBI. (A) Hippocampus protein extracts prepared from Sham or mTBI-animals at indicated time points were subjected to gel electrophoresis followed by immunoblot analysis using antibodies against pGSK- β (Ser9), GSK-3 β , pPKB (Ser473), and PKB as indicated. (B) Phosphorylation of GSK-3 β or PKB was evaluated by densitometry analyses. The non-injured values of (for each time point) were set a 100%, and the percentage of respective treatment was calculated accordingly. Results are mean \pm SEM of 6–8 animals. * p < 0.05 mTBI vs. sham.

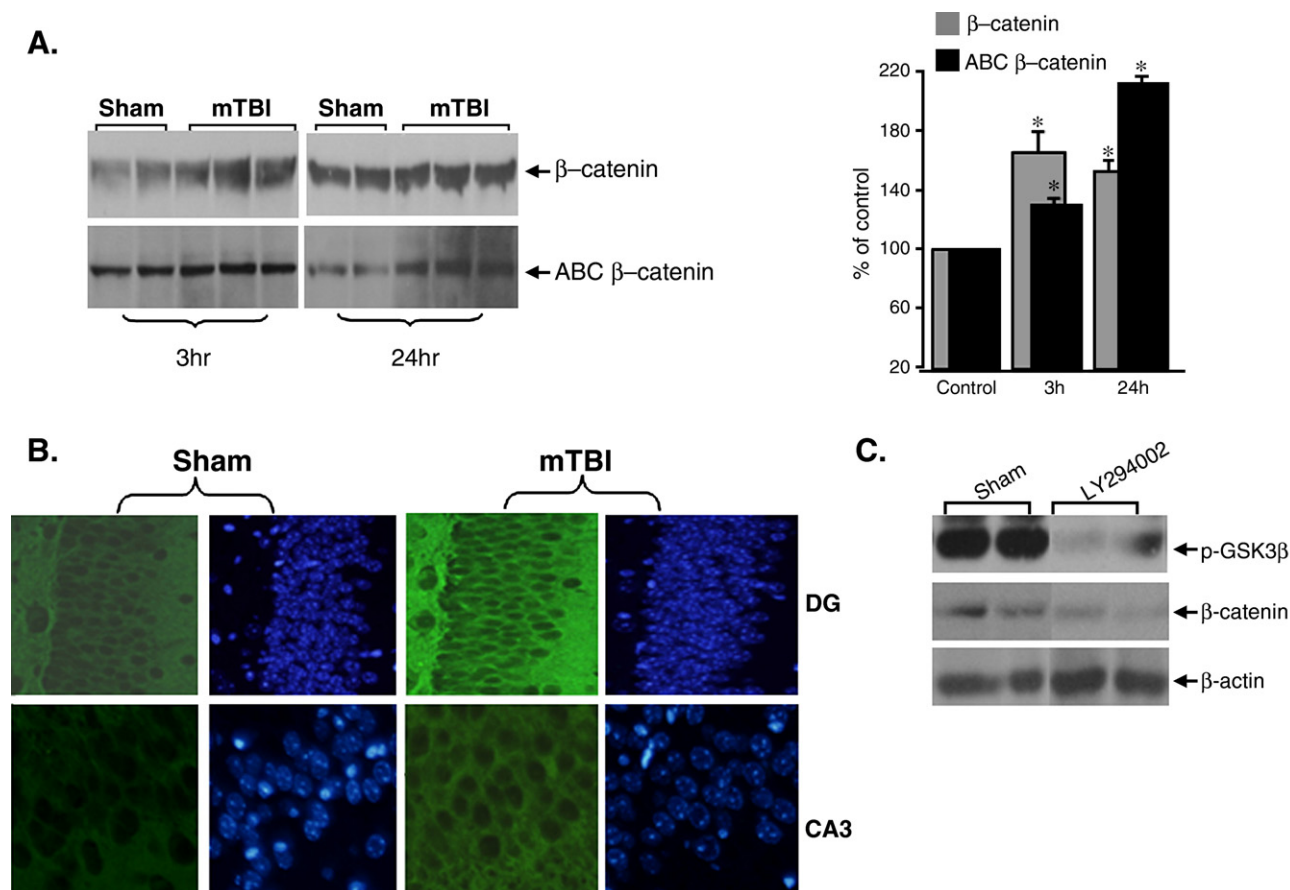


Fig. 2. β -catenin is upregulated by mTBI. (A) Hippocampus protein extracts prepared from Sham or mTBI-animals at indicated time points were subjected to gel electrophoresis, followed by immunoblot analysis using antibodies against β -catenin or active β -catenin (ABC), as indicated (left panel). Total β -catenin (gray bars) or active β -catenin (black bars) were evaluated by densitometry analyses. The non-injured values (for each time point) were set 100%, and the percentage of respective treatment was calculated accordingly. Results are mean \pm SEM of 6–8 animals (right panel). * $p < 0.05$ mTBI vs. sham. (B) Immunofluorescence analysis of β -catenin in the hippocampus. Paraffin-embedded tissue sections were prepared from sham or injured animals 24 h post-injury. Sections were stained with anti- β -catenin antibody, as described in Experimental methods, and visualized by fluorescence microscopy. A representative photomicrograph of ipsilateral dentate gyrus granule cells or CA3 is shown. (C) Animals were i.c.v. injected with PI3 kinase inhibitor LY294002. Hippocampus tissue extracts were prepared 3 h post injection and were subjected to gel electrophoresis, followed by immunoblot analysis with antibodies against pGSK-3 β (Ser9) β -catenin or β -actin (protein load verification). GSK-3 phosphorylation and β -catenin levels are reduced by the PI3kinase inhibitor.

animals (Fig. 3A). Notably, since we found that this type of mTBI did not produce motor deficits (see Experimental methods), it is apparent that the increased immobility time reflected depressive-like behavior. Next, we tested whether inhibition of GSK-3 may affect this response, using two selective GSK-3 inhibitors: L803-mts, recently developed in our laboratory (Plotkin et al., 2003), or lithium, a known GSK-3 inhibitor (Klein and Melton, 1996). GSK-3 inhibitors were injected i.c.v. 30 min before mTBI, and FSTs were performed 24 h post-injury. As shown in Fig. 3B, either L803-mts or lithium reduced the immobility time of the injured animals, as compared with the mTBI-vehicle-treated animals. In fact, the immobility of the inhibitors-treated animals was comparable with that of the non-injured animals (as seen in Fig. 3A), indicating that treatment with GSK-3 inhibitors had prevented mTBI-induced depression. To further examine whether the effect is associated with changes in β -catenin, hippocampus extracts of both control and treated animals were subjected to immunoblot analysis with β -catenin antibody. Indeed, treatment with L803-mts further increased β -catenin levels in the injured hippocampus (Fig. 3C).

The fact that mTBI induced a depressive-like behavior already at 24 h post-injury, which was also associated with inhibition of GSK-3 β , raised the question of whether this effect is modified by cell apoptosis. We conducted histological analyses to evaluate the damage of mTBI in the hippocampus. Hippocampus sections stained with H and E detected small, dense, hyperchromatic cells in the inner blade of the dentate gyrus in the injured ipsilateral hippocampus (Fig. 4A), which indicated that mTBI led to cell damage in the dentate gyrus. However, TUNEL assays did not detect nucleus DNA fragmentation in either the injured dentate gyrus or other respective regions in the hippocampus (Fig. 4B). Thus, mTBI led to some damage, but did not induce apoptosis within 24 h after the injury.

Discussion

Numerous studies have implicated GSK-3 β as a potent *in vivo* regulator of cell apoptosis and have demonstrated its involvement in bipolar disorders and depressive behavior (see introduction). This prompted us to investigate how GSK-3 β is manipulated in

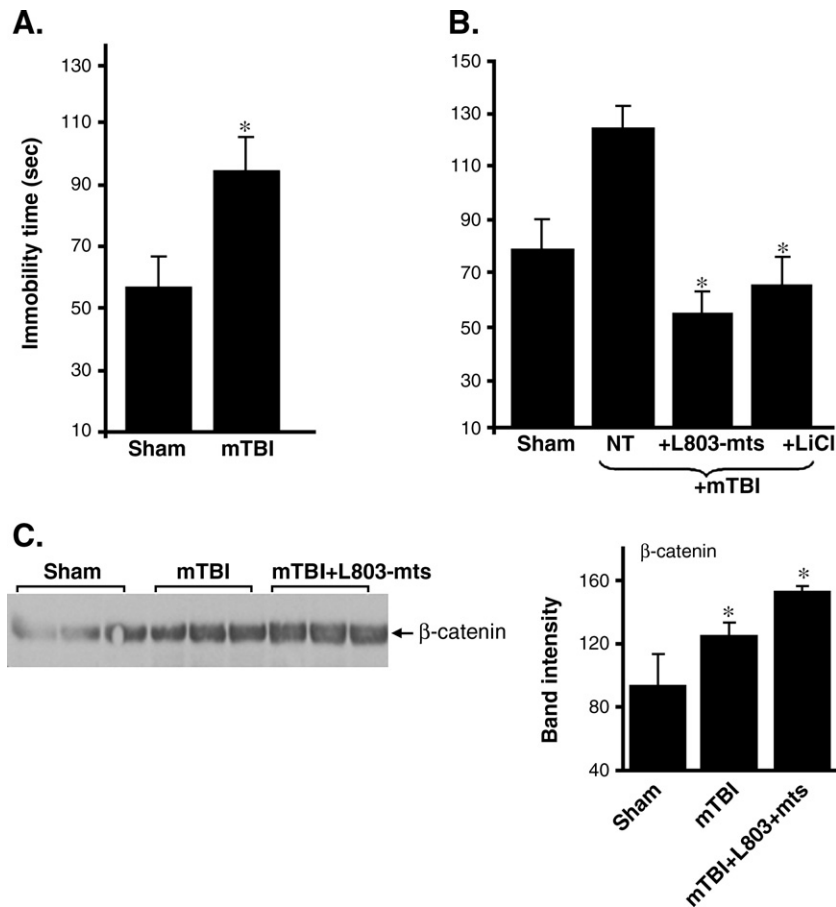


Fig. 3. GSK-3 inhibitors prevent mTBI-induced depression. (A) Animals were subjected to FST 24 h after mTBI. Bar graphs represent mean of immobility \pm SEM of sham and injured animals. $n=12-15$, $*p<0.05$ (mTBI vs. sham). (B) Animals were subjected to mTBI after administration of L803-mts, lithium, or respective vehicle, as described in Experimental methods. Animals that were injected with vehicle served as control (NT). Non-injured animals were also included in the experiment (Sham). Animals were subjected to FST 24 h after mTBI, and immobility time was determined. Bar graphs represent mean of immobility \pm SEM. $n=7-9$, $*p<0.05$ (treated vs. vehicle-treated (NT)). (C) Hippocampus extracts of Sham-, mTBI-, and L803-mts-treated mice were subjected to gel electrophoresis followed by immunoblot analysis with antibodies against β -catenin. Right panel shows densitometry analysis of β -catenin in the indicated treatments. Results are mean \pm SEM of 7–9 animals. $*p<0.05$ treated vs. sham.

the physiological model of brain injury, mTBI, and whether this may affect depression, a mood disorder often associated with TBI symptoms (Robinson, 1992; Levin, 1998; Milman et al., 2005). Unexpectedly, mTBI led to inactivation of GSK-3 β through its increased phosphorylation at serine 9. mTBI increased the phosphorylation of PKB, a known upstream kinase to GSK-3 (Cross et al., 1995), at serine 473 residue, which is required for PKB activation (Alessi et al., 1996). Thus, mTBI induced the inhibition of GSK-3 β via activation (at least in part) of PKB. Inhibition of GSK-3 β has been implicated in neuroprotective activity (Cross et al., 2001; Li et al., 2002; Hongisto et al., 2003). Likewise, activation of PKB has been linked with anti-apoptotic activity and neuroprotection in various insults, including excitotoxicity, neuronal axotomy, hypoxia, and oxidative stress (Friguls et al., 2001; Yano et al., 2001; Chong et al., 2005). These anti-apoptotic effects were explained by the ability of PKB to phosphorylate and inhibit pro-apoptotic proteins, such as caspase-9, Bad, JNK, Forkhead transcription factors, and GSK-3 β (Noshita et al., 2002; Neary et al., 2005; Kilic et al., 2006; Zhang et al., 2006). Thus, mTBI-induced PKB/GSK-3 β could support a pro-survival pathway. Accumula-

tion of β -catenin observed in the injured hippocampus provided indication for the *in vivo* inhibition of GSK-3 β by mTBI. Moreover, this observation further coincided with the view that mTBI elicits a pro-survival signaling, because β -catenin itself is a “strong” survival factor. β -catenin is a key mediator of the canonical *Wnt* signaling pathway and mediates *Wnt*-neuroprotective or anti-inflammatory effects in neurons (Zhang et al., 1998; Chen et al., 2001). Additionally, β -catenin has been implicated as a modulator in brain development, dendritic morphogenesis, and synaptic plasticity (Chenn and Walsh, 2002; Yu and Malenka, 2003; Bamji et al., 2006). Altogether, it appears that the PKB/GSK-3 β / β catenin pathway elicited by mTBI established an anti-apoptotic signaling that likely supported neuroprotection and repair mechanisms. The negative TUNEL results obtained in the injured hippocampus (Fig. 4B) indeed suggest that the apoptotic signaling that could be activated by TBI (Rink et al., 1995; Clark et al., 1997; Kaya et al., 1999; Larner et al., 2005) was eliminated or attenuated, at least within the time frame of 24 h.

At the behavior level, we observed a depressive-like activity 24 h after injury. This depressive effect was diminished in

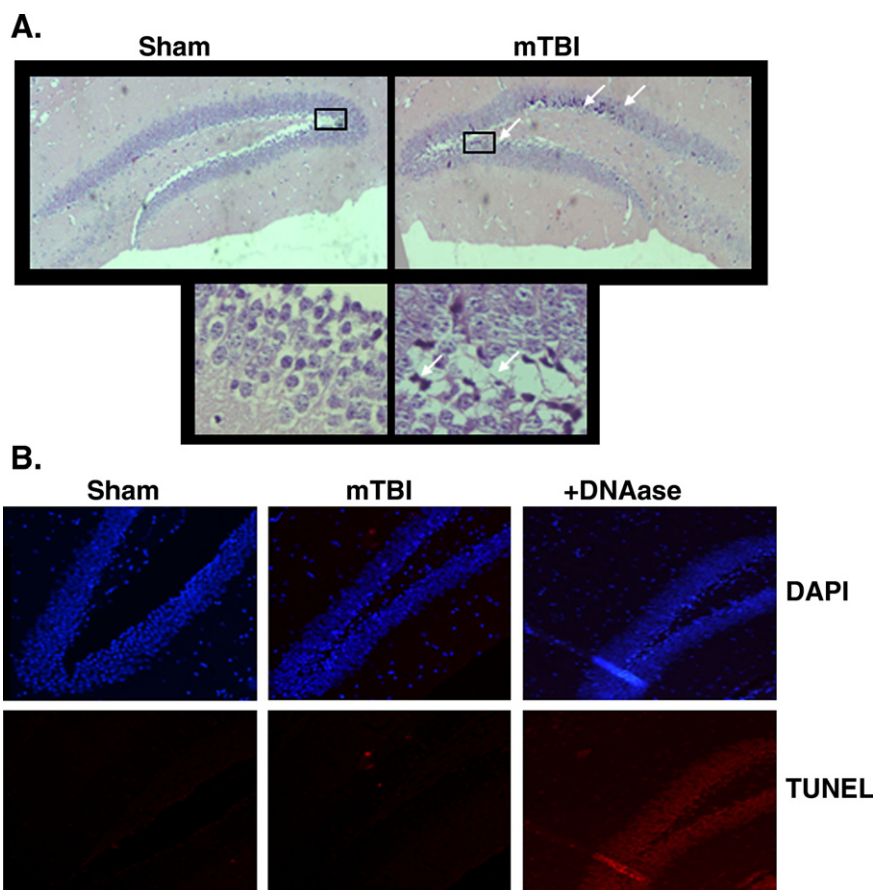


Fig. 4. Histological and TUNEL analyses of the injured hippocampus. (A) Injury was evaluated by H&E staining in sham- or mTBI-coronal brain tissue sections to determine damaged cells in the hippocampus. White arrows point toward hyperchromatic shrunken cells in the dentate gyrus of the injured hippocampus that were not observed in its respective sham control (original magnification $\times 10$ or $\times 20$). (B) Hippocampus tissue sections prepared from sham and injured (mTBI) animals were subjected to TUNEL assays (red), and counterstained with DAPI for nuclei detection (blue) as described in Experimental methods. Sections treated with DNAase served as a positive control and show apoptotic nuclei (+DNAase). No apoptotic signal is detected in the injured hippocampus.

animals that were first treated with the GSK-3 selective inhibitors, Lithium or L803-mts. Hence, inhibition of GSK-3 is capable of opposing the depressive events activated by mTBI. We believe that the physiological inhibition of GSK-3 β and, subsequently, the accumulation of β -catenin by mTBI, observed in this study, were beneficial in reducing depression, however, not sufficiently. On the other hand, the use of the GSK-3 inhibitors, which provided sustained inhibition of the enzyme, resulted in a more robust effect that prevented the depressive behavior. Certainly, additional intracellular signaling pathways are activated by mTBI and may affect behavior. For example, TBI elicits the activation of members of the mitogen activated protein kinase family (MAPK's) including ERK1/2 and JNK (Otani et al., 2002; Kuan et al., 2003). Recent studies also reported activation of the mammalian target of rapamycin, mTOR, and calcium/calmodulin-dependent protein kinases by TBI (Chen and Atkins, *in press*; Atkins and Chen, 2006). However, whether these protein kinases are actually involved in the mechanisms underlying the depressive behavior remains to be elucidated. The use of selective inhibitors may be a useful way to address this question.

In summary, our study suggests that inhibition of GSK-3 β by mTBI is part of a rehabilitation scheme elicited in response to the insult, and that the use of selective GSK-3 inhibitors may have

therapeutic benefits in treatment conditions associated with brain injury.

Experimental methods

Animals and trauma model

The study was performed according to the guidelines of the Institutional Animal Care Committee of Tel Aviv University. C57Bl/6J mice were housed in individual cages, with free access to water in a temperature-controlled facility with a 12 h light/dark cycle. Animals aged 12–13 weeks were used, and each experimental group consisted of 6–10 mice chosen randomly. Experimental mTBI was performed, using a weight-drop device described previously (Yatsiv et al., 2002). In brief, animals were anesthetized with ketamine/xylazine (100 mg/kg; 20 mg/kg). After a midline longitudinal incision, the skull was exposed, and a 75-g weight was dropped on a cone placed 1 mm lateral to the midline from a height of 12 cm, resulting in a focal injury to the left hemisphere. Hippocampi were removed, at indicated time points and frozen in liquid nitrogen. Mice that were anesthetized only and their skull subsequently exposed served as sham controls. Sham controls were sacrificed at the same time points as the mTBI mice to verify that results did not reflect anesthesia effects (Li et al., 2005). One hour after induction of mTBI, the functional status of the mice, which reflects the severity of injury, was evaluated using the Neurological Severity Score (NSS) described by Beni-Adani et al. (2001). NSS is a scale of 10 tasks, and one point is awarded for failure to perform a task. Thus,

NSS of 9–10 at 1 h predicts mortality within 24 h, a moderate trauma, is defined by NSS levels of 4–8 and mild injured mice have NSS of <4. No motor deficits were recorded by NSS confirming that the trauma was indeed mild.

Hippocampus extracts and Western blots

Hippocampus tissue (ipsilateral left side) was removed from brain and immediately homogenized with ice-cold buffer H (150 mM β -glycerophosphate pH 7.3, 50% glycerol, 5 mM EGTA, 5 mM EDTA, 50 mM NaF, 25 mM NaPPi, 25 μ g/ml leupeptin, 25 μ g/ml aprotinin, 500 nM and 1% NP40). The extracts were centrifuged for 20 min at 15,000 \times g, and supernatants were collected. Equal amounts of proteins (30 μ g), as determined by Bradford analysis (Bradford, 1976), were boiled with Laemmli sample buffer and subjected to gel electrophoresis (10% polyacrylamide gel), transferred to nitrocellulose membranes, and immunoblotted with selected antibodies for GSK-3 β , β -catenin (Transduction laboratories, USA), phospho PKB (Ser473), phospho GSK-3 β (Ser9) (Cell Signaling Technology Beverly, MA, USA), and active (ABC) β -catenin antibody (Upstate, Lake Placid NY, USA).

Forced swim test (FST)

The FST procedure that was used is similar to that initially described by Porsolt (Porsolt et al., 1977). Animals were placed in a large cylinder (30 cm \times 45 cm) filled with water at a temperature of 25 $^{\circ}$ C, for a 6-min period. The duration of immobility was monitored during the last 4 min of the 6-min test. Immobility period is defined as the time spent by the animal floating in the water without struggling and making only those movements necessary to keep its head above the water. Immediately afterwards, the trial mice were placed under a heating lamp to dry. All testing took place between 11:00 and 15:00. For treatment with GSK-3 inhibitors or LY294002, mice were anesthetized as described and unilaterally intracerebroventricularly injected (i.c.v.) with L803-mts (1 μ l of 25 mM in DMSO), 1 μ l vehicle DMSO, lithium (1 μ l of 50 mM), or 1 μ l saline. Our previous work confirmed that saline-DMSO and scramble-control peptide injections resulted in comparable immobility values (Kaidanovich-Beilin et al., 2004). Animals were subjected to mTBI 30 min after i.c.v. injection.

Histology and immunofluorescence

Brain tissues obtained from control and injured animals (24 h post mTBI) were fixed with a 4% paraformaldehyde solution. Coronal sections of paraffin-embedded tissues (10 μ m) were stained with hematoxylin/eosin (H&E) to detect damaged cells. For immunofluorescence, paraffin-embedded tissue sections were blocked with 1% gelatin and permeabilized with PBS containing 0.25% Triton X-100 for 10 min. Tissue sections were incubated with monoclonal antibody to β -catenin at 4 $^{\circ}$ C for 15 h. Slides were incubated with Cys2 goat anti-mouse antibody and counterstained with DAPI. For TUNEL assay (terminal transferase-mediated dUTP nick-end labeling), a commercial apoptosis detection kit (TMR red, Roach Ltd., using the manufacturer's instructions) to detect DNA fragmentation was employed in the paraffin-embedded tissue sections. Sections incubated with DNase served as a positive control.

Statistics

Graphics and statistical analyses were performed by Origin Professional 6.0 software. The significance of differences between experimental conditions was determined using the two-tailed Student's *t*-test. Data were deemed significant when $p < 0.05$. Results are expressed as group mean with standard error of the mean (SEM).

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