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BEHAVIORAL REACTIONS AND STRUCTURAL ALTERATIONS OF HIPPOCAMPAL TISSUE AFTER REPETITIVE MILD TRAUMATIC BRAIN INJURY IN MICE

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SUMMARY. Mild traumatic brain injury (TBI), often identified with concussion, is not considered to be life threatening in close perspective. However, the information about its delayed neurodegenerative effects is becoming more common. Especially this relates to repeated cases of concussion in military service members and athletes. The aim of our study was to show behavioral and histological changes in a mouse model of repetitive mild traumatic brain injury (rmTBI). No macroscopic brain abnormalities such as skull fractures or intracranial bleeding were found in mice after rmTBI. To detect behavioral alterations, we carried out the "Open field" test on day 5, 10 and 30 since the first impact of five. The behavioral data revealed decrease of motor activity and increase of anxiety level when compared to controls. Immunohistochemical data provided strong evidence of astro- and microgliosis that persisted for weeks after rmTBI. Our results indicate that new mouse model of human rmTBI can be useful in development of neuroprotective approaches for treatment of rmTBI consequences.

Keywords: hippocampus, immunohistochemistry, mouse model, "Open field" behavioral test, repetitive mild traumatic brain injury.

Introduction

Symptoms of mild traumatic brain injury (mTBI) are more difficult to detect than those observed in severe traumatic brain injury due to moderate range of noticeable abnormalities like skull fractures and intracranial bleeding. According to Centers for Disease Control and Prevention (CDC), common concept of TBI indicates brain damage due to bump, blow or jolt or a penetrating injury that disrupts normal function of the brain. However, TBI represents not a single event, but a whole cascade starting with initial damage (primary injury) and continuing with secondary processes (secondary injury) that can last from minutes to months and years (Xiong *et al*., 2013). Latter is mainly associated with cytotoxic and inflammatory processes accompanied by

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metabolic alterations of brain due to vascular failure (Werner and Engelhard, 2007; Beauchamp *et al*., 2008; Granacher, 2008). It should be noted that mild TBI is a type of closed trauma, and its outcome is primarily determined by secondary processes, while severe forms are much more dependent on initial mechanical injury (Betrus and Kreipke, 2012).

Acute signs of a concussion can include loss of consciousness, amnesia, irritability, slowed reaction times, sleep disturbances and emotional lability (McCrory *et al*., 2009). The critical fact is that these deficits are observed in the absence of structural brain damage when using such standard diagnostic methods as magnetic resonance imaging (MRI), computer tomography (CT) and electroencephalography (EEG) (Belanger *et al*., 2005; Davis *et al*., 2009). Nevertheless, there's still a danger of long-term consequences. Specifically, the results of the survey showed that retired professional football players who experienced three or more concussions were more likely to report about memory impairment and mild cognitive impairment (Guskiewicz *et al*., 2005). Moreover, the analysis of post-mortem data indicated that it was among retired football players who had played for five or more years there were elevated rates of death due to Alzheimer's disease (AD) or amyotrophic lateral sclerosis (ALS). However, the authors assumed that actual causes of death also might have included chronic traumatic encephalopathy − CTE (Lehman *et al*., 2012; McKee *et al*., 2014).

Taking into account all depicted above, modeling of mTBI and capturing its consequences at short and long time intervals after immediate impact appear to be extremely important. There are several kinds of models reproducing single and repeated mTBI. Mostly they represent variations of schemes that existed before and were used for mimicking more severe forms of trauma. On a similar principle, the model of repetitive mTBI (rmTBI) designed by Michael J. Kane et al. (Kane *et al*., 2012) and used in our research represents a modification of Marmarou's weight-drop model (Marmarou *et al*., 1994). The main point of its key features was to make experimental conditions of receiving a concussion closer to reality by lowering rates of mortality and cases of skull fractures and hemorrhages in animals. Since hippocampus is integral for multiple aspects of behavior and shows high sensitivity to any pathological changes of brain (Levita *et al*., 2014; Kim *et al*., 2014), we focused our research on this particular structure.

Materials and methods

All stages of the experiment were conducted in accordance to European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986).

Animals

Subjects were 56 8 to 12 weeks old males of wild-type mouse (24 – 40 g). Animals were housed in cages on a 12-hour light–dark cycle, with free access to food

and water. In the experiment, animals were divided into following groups: 1) not anesthetized, not injured; 2) anesthetized, not injured; 3) anesthetized, injured. Before injury mice were lightly anesthetized with diethyl ether. The positive effect of anesthesia was determined by absence of reaction to toe pinch.

Model of repetitive mild traumatic brain injury

We induced rmTBI by using a model proposed by Michael J. Kane et al. (Kane *et al*., 2012). Its design represents a modification of classical Marmarou's weight-drop model (Marmarou *et al*., 1994) which reproduced single diffuse TBI from mild to severe. Both in original scheme and in modified, the skull of a mouse is exposed to an impact with a weight guided by a vertical tube. The tube is pre-fixed to a stationary object like wall or rack. In both schemes, the severity of injury depends on the height from which the load falls and the weight of the latter. Respectively, in our study the 95 g weight fell from the 1 m height. Lightly anesthetized animal were not fixed in the apparatus, and after the weight fell, they fell too (through a piece of aluminium foil pre-attached on top of the H-shaped box), landing on a damper support. We avoided rebounding of the weight and, consequently, additional blows to the skull by using a nylon fishing line that stopped the load from falling after the impact due to its length. According to Kane's model, neither scalp incision nor attachment of helmet to the mouse skull were done. Mice were subjected to 5 head impacts (1/day for 5 successive days).

Recovery of righting reflex

After each of five impacts mice (n=29) were immediately placed on their backs on a flat surface, and time required for taking a normal position was fixed. Thus, we evaluated time required for neurological restoration. Measures of injured mice then were compared to controls (anesthetized, not injured, n=20).

Behavioral assessment

To investigate the behavioral effects of rmTBI, mice were tested in the Open field test before (control, n=16) and after the series of impacts (n=27) on day 5 (n=10), day 10 (n=9) and day 30 (n=8) since the first injury. In addition, we divided control animals into two groups, one of which was anesthetized without delivering the impact (n=8) in order to compare it with the group of animals tested shortly after the last injury (day 5). Presumably, animals from the group of day 5 could still be under the influence of anesthesia. Another control group (n=8) wasn't exposed to ether inhalations.

Animals were tested for changes in motor activity, rest duration, grooming activity and anxiety level. Each mouse was kept on a platform for 30 minutes. The area of the "field" was 60 cm x 60 cm and consisted of 16 equal squares. Motor activity was defined by counting lines (sides of the squares) crossed within respective period of time, rest duration was defined by fixing the time spent in corner zones of the platform, grooming activity was defined by number of acts of grooming and anxiety level was defined by number of acts of defecation.

Edema

Both controls (n=8) and injured mice (n=8) were sacrificed to detect possible signs of edema formation. The presence of edema was evaluated by comparing water content between controls and mouse brains after 4 hours since the last impact of five. We used the method which was previously described (Kawai et al., 2001), but slightly modified. Mice were decapitated, and brains were rapidly removed from the skull. Whole fresh brain was weighed on aluminum foil, dried for 90 hours at 73°C in an oven and reweighed. Percentage water content of each brain was calculated according to the formula: (wet weight – dry weight)/(wet weight) × 100.

Immunohistochemical staining and image processing

Immunohistochemical analysis was carried out as previously described (Pivneva *et al*., 2005). Controls (n=6) and mice of day 5 (n=5), 10 (n=5) and 30 (n=8) were anesthetized by injecting an overdose of calipsol and transcardially perfused with 0.1 M phosphate buffer (PB) followed by 4% paraformaldehyde in 0,1 M PB. After perfusion, extracted brains were immersed in the same fixative and postfixed overnight at 4^{o}C. Frontal brain sections (50 μm) were prepared using a Leica VT1000 A vibratome (Wetzlar, Germany). The sections were left overnight at 4^{o}C for incubation with primary antibodies diluted in the solution (0.1 M PB, 1% bovine serum albumin, 0,3% Triton X-100). To visualize astrocytes and microglial cells, we used rabbit polyclonal antibodies anti-GFAP (1:1500, Dako, Denmark) and rabbit polyclonal antibodies anti-Iba-1 (1:1000, Wako, Japan). On the next day, brain sections were treated with secondary anti-rabbit Alexa Fluor 594-conjugated antibodies (1:1000, Invitrogen, USA). Stained sections then were mounted to glass slides using fluorescent mounting medium Immu-Mount (Thermo Scientific, Waltham, USA) and examined with a FluoView FV1000 confocal microscope (Olympus Inc., Japan).

Statistical Analysis

Statistical analysis was performed in StatSoft Statistica 6.0. The two-tailed Student's t–test was used to assess the significance of differences between samples ($p < 0.05$ was considered to indicate statistical significance). All data are shown as mean ± SEM.

Results and discussion

No cases of skull fractures, intracranial bleeding or seizures were registered. Preliminary data also showed no signs of general edema (Fig. 1).

To confirm the alterations of neurological state immediately after trauma, we compared time intervals necessary for righting reflex restoration in anesthetized controls and in injured mice after each impact (Fig. 2). Thus, we observed that receiving of an injury leaded to delay in full recovery of consciousness.

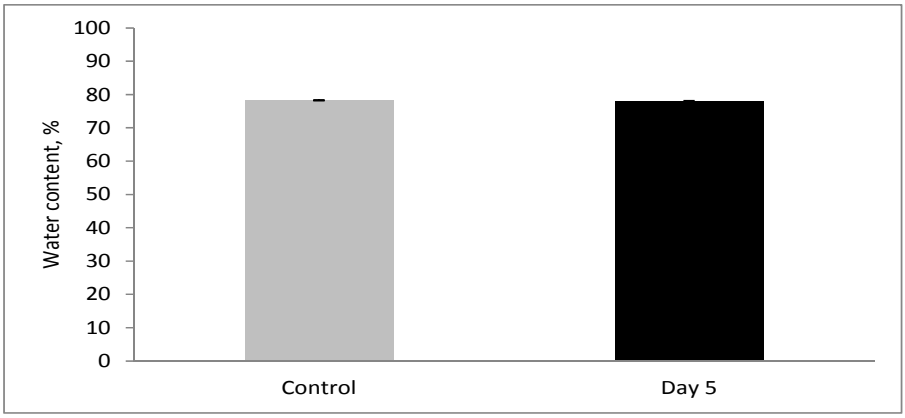

Figure 1. Edema measuring on day 5 since the first impact. Ocurrence of edema was defined by comparison of water content in control (n=8) and damaged (n=8) mouse brains.

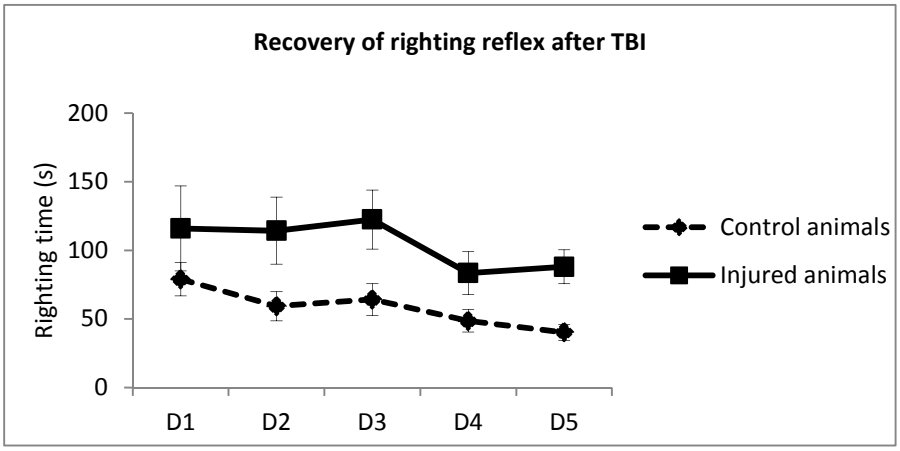

Figure 2. Recovery of righting reflex after each inhalational anesthezia (control animals, n=20) or after each impact (injured animals, n=28) on five successive days.
Results are the mean ± SEM.

Behavioral data indicated neurological deficits as well (Fig. 3). We observed decreasing of motor activity on day 5 and its gradual restoration by day 30 (Fig. 3 A). However, the level of control wasn't reached. Statistically significant difference was shown for groups of non-anesthetized control animals and injured animals tested at day 5 ($p < 0.01$). Since there was a decrease in motor activity of animals exposed to trauma, the time spent in corner zones increased (Fig. 3 B). Statistically significant difference was shown for groups of non-anesthetized control animals and injured animals tested at day 5 ($p<0.01$).

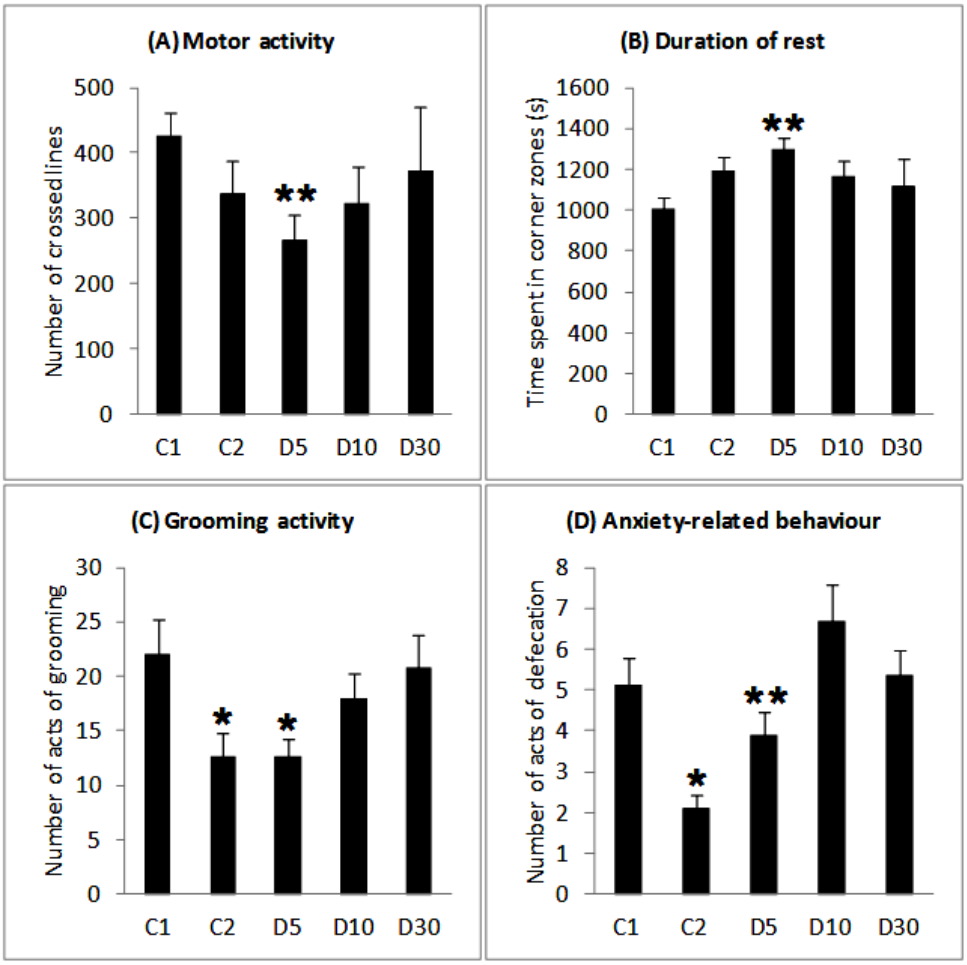

Figure 3. Results of behavioral test "Open field" in control (C1 – non-anesthetized, n=8, C2 – anesthetized, n=8), on day 5 (D5 – anesthetized, n=10), day 10 (D10, n=9) and day 30 (D30, n=8) since the first impact. * and ** - $p<0.05$ and $p<0.01$ in comparison with non-anesthetized control (t-test). Day 5 was compared to anesthetized control. Graph (D) shows statistically significant difference between anesthetized control and day 5. Rest of indicators represents the result of comparison with non-anesthetized control. Results are the mean ± SEM.

The observations of grooming activity showed that the number of grooming acts decreased when compared to non-anesthetized control, but remained the same when compared to the group of anesthetized control animals (Fig. 3 C). By day 10 it increased when compared to anesthetized control and day 5. By day 30 it became comparable to non-anesthetized control. Statistically significant difference was shown

between both control groups ($p<0.05$) and between non-anesthetized control and day 5 ($p<0.05$). The changes of the number of acts of defecation (Fig. 3 D) were analogous to those of grooming activity. Statistically significant difference was shown between both control groups ($p<0.01$) and between anesthetized control and day 5 ($p<0.05$).

Quantitive immunohistochemical analysis revealed that astro- and microglisosis in CA1-zone of hippocampus persisted for weeks (Fig. 4).

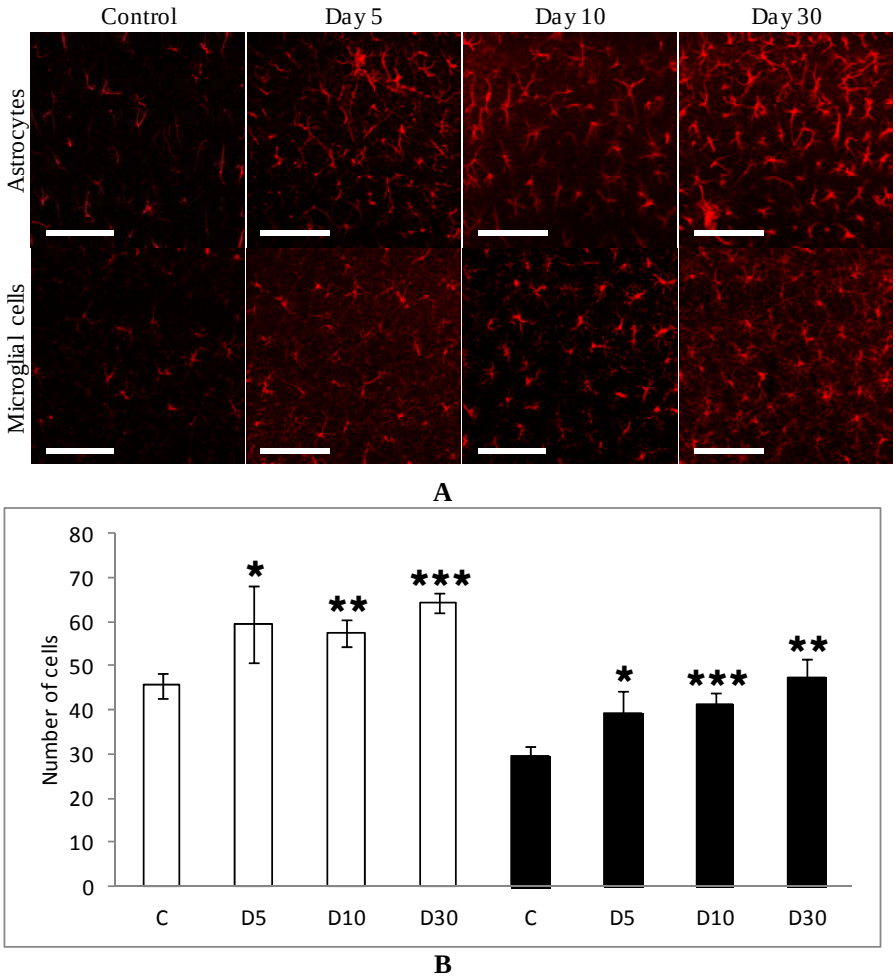

Figure 4. Changes in number of astrocytes and microglial cells of hippocampal CA1-zone after delivering brain injury in control, on day 5, day 10 and day 30 since the first impact. A – microphotographs of hippocampal CA1-zone. Bar=100 μm. The number of cells were counted in 0.1 mm^2. B – Comparative cell counts (astrocytes – white, microglial cells - black) in control and after delivering brain injury. *, ** and *** – $p<0.05$, $p<0.01$ and $p<0.001$ in comparison with control (t-test). Results are the mean ± SEM.

Specifically, the amount of astrocytes on day 5 since the first impact (Fig. 4 B) was significantly higher than in controls. Remaining roughly the same on day 10, the number of astrocytes increased by day 30. Statistically significant difference was shown between controls and each of injured groups: day 5 ($p<0.05$), day 10 ($p<0.01$) and day 30 ($p<0.001$). The tendency was similar for dynamics in microglia activation. In numbers of microglial cells, statistically significant difference was shown for all the groups related to rmTBI: day 5 ($p<0.05$), day 10 ($p<0.001$) and day 30 ($p<0.01$). Noteworthy, the activation of astro- and microglia was combined with restoration of behavioral performance.

Conclusions

In a novel model of rmTBI, no cases of skull fractures, intracranial bleeding or seizures were registered. "Open field" behavioral testing revealed two tendencies related to decrease of motor activity by day 5 and its gradual recovery by day 30, as well as increase of anxiety level by day 5 and day 10 and its return to control measures. Astro- and microgliosis took place on day 5, day 10 and day 30, which indicates the presence of secondary injury even after a long time interval. This allows us to use the model in further research of rmTBI features and selection of potential treatments.

REFERENCES

Beauchamp, K., Mutlak, H., Smith, W.R., Shohami, E., Stahel, P.F. (2008) Pharmacology of traumatic brain injury: where is the "golden bullet"?, *Mol. Med.*, **14**, 731–740

Belanger, H.G., Curtiss, G., Demery, J.A., Lebowitz, B.K., Vanderploeg, R.D. (2005) Factors moderating neuropsychological outcomes following mild traumatic brain injury: A meta-analysis, *Journal of the International Neuropsychological Society*, **11**, 215–227

Betrus, C., Kreipke, C.W. (2013) Historical Perspectives in Understanding Traumatic Brain Injury and in Situating Disruption in CBF in the Pathotrajectory of Head Trauma, In: *Cerebral Blood Flow, Metabolism, and Head Trauma*, Kreipke, C.W., Rafols, J.A. (ed), Springer Science+Business Media, New York, pp 1-29

Davis, G.A., Iverson, G.L., Guskiewicz K.M. et al. (2009) Contributions of neuroimaging, balance testing, electrophysiology and blood markers to the assessment of sport-related concussion, *Br. J. Sports Med.*, **43**, 36–45

Granacher, R.Jr. (2008) Traumatic Brain Injury: Methods for Clinical and Forensic Neuropsychiatric Assessment, 2nd Edition, CRC Press, Boca Raton, pp 586

Kane, M.J., Angoa-Pérez, M., Briggs, D.I., Viano, D.C., Kreipke, C.W., Kuhn, D.M. (2012) A mouse model of human repetitive mild traumatic brain injury, *J. Neurosci Methods*, **203(1)**, 41-49

Kawai, N., Kawanishi, M., Okauchi, M., Nagao, S. (2001) Effects of hypothermia on thrombin-induced brain edema formation, *Brain Res*., **895**, 50–58

Kim, J., Parker, D., Whyte, J., Hart, T., Pluta, J., Ingalhalikar, M., Coslett, H.B., Verma, R. (2014) Disrupted structural connectome is associated with both psychometric and real-world neuropsychological impairment in diffuse traumatic brain injury, *J. Int. Neuropsychol. Soc*., **20**, 887-896

Kunz, A., Dirnagl, U., Mergenthaler, P. (2010) Acute pathophysiological processes after ischaemic and traumatic brain injury, *Best Pract. Res. Clin. Anaesthesiol.*, **24(4)**, 495–509

Levita, L., Bois, C., Healey, A., Smyllie, E., Papakonstantinou, E., Hartley, T., Lever, C. (2014) The Behavioural Inhibition System, anxiety and hippocampal volume in a non-clinical population, *Biol. Mood Anxiety Disord*., **4(4)**

Marmarou, A., Foda, M.A., van den Brink, W., Campbell, J., Kita, H., Demetriadou, K. (1994) A new model of diffuse brain injury in rats. Part I: Pathophysiology and biomechanics, *J. Neurosurg.*, **80**, 291–300

McCrory, P., Meeuwisse, W., Johnston K. et al. (2009) Consensus statement on concussion in sport - the 3rd International Conference on concussion in sport, held in Zurich, November, *J. Clin. Neurosci.*, **16(6)**, 755–763

McKee, A.C., Daneshvar, D.H., Alvarez, V.E., Stein, T.D. (2014) The neuropathology of sport, *Acta Neuropathol.*, **127(1)**, 29–51

Pivneva, T.A., Tsupikov, O.M, Pilipenko, M.N., Vasilenko, D.A., Skibo, G.G. (2005) Structural modifications of astrocytes in the hippocampus after experimental cerebral ischemia in gerbils, *Neurophysiology*, **37**, 359-364

Werner, C., Engelhard, K. (2007) Pathophysiology of traumatic brain injury, *Br. J. Anaesth.*, **99(1)**, 4–9

Xiong ,Y., Mahmood, A., Chopp, M. (2013) Animal models of traumatic brain injury, *Nat. Rev. Neurosci. Nature,* **14(2)**, 128–142