Moderate traumatic brain injury is linked to acute behaviour deficits and long term mitochondrial alterations.

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Abstract

Traumatic Brain Injury (TBI) remains one of the leading causes of death and disability, worldwide. Mild TBI may lead to neuropsychiatric sequelae, including memory loss and motor impairment. Mitochondrial dysfunction and oxidative stress have a contributory role in several neurological disorders; however, their association with mitophagy in mild TBI is unclear. TBI was induced in female Sprague Dawley (SD) rats using a New York University Impactor (10g, impactor head 2.5mm diameter, weight drop 50mm). The novel object recognition and error ladder tests were performed at 24h and for 6 weeks post-injury, and the brains were examined histologically to confirm the extent of injury at both times. Mitochondria Manganese Superoxide Dismutase (MnSOD) and the Oxidative Phosphorylation (OXPHOS) complexes I-V (CI-CV), as well as mitophagy markers, dynamin related protein 1 (DRP-1), LC3A/B and PTEN-induced putative kinase 1 (PINK-1), were measured in the penumbra by western blot. At 24 hours sham rats spent similar times exploring the two identical objects, but spent longer with the novel object when it was introduced; while TBI rats devoted similar time with the new object as the old one. TBI rats also made more errors while walking on the ladder, compared with the sham rats. MnSOD, OXPHOS CI, CIII and CV protein levels were significantly lower in the TBI group at 24 hours. DRP-1, LC3A/B I and II, and PINK-1 were increased at 6 weeks suggesting abnormal mitophagy. Moderate TBI caused immediate cognitive and mild motor functional deficits in the rats that did not persist. Reduced antioxidative capacity and possibly compromised mitochondrial function may affect the long-term functional recovery.

Key words: Traumatic brain injury, mitochondria, mitophagy, oxidative stress, Immunohistochemistry, behavioural studies.

1. Introduction

Traumatic brain injury (TBI) is an increasingly common event. The WHO predicted that TBI will become the major cause of death and disability in the world by 2020 (1). There is no specific therapeutic treatment for TBI, particularly mild injury where there is no evidence of excessive swelling or bleeding. TBI induces rapid neuropathological effects that may cause cumulative neural damage and degeneration. Even for mild TBI, the majority of cases result in acute neurologic deficits, including motor and cognitive impairments, which may resolve over time or may continue to significantly influence quality of life (2).

The brain relies heavily on glucose as its energy source, while mitochondria are involved in the final phase of producing ATP from glucose through oxidative phosphorylation (OXPHOS), which takes place within the electron transfer chain (3). Thus, mitochondrial density is often indicative of the cell's requirements for ATP production and neurons tend to have high mitochondrial densities. The respiratory chain is also the main source of reactive oxygen species (ROS) (4). When intracellular antioxidants are unable to counteract the overproduction of ROS, irreversible oxidative modification occurs in all cellular components, thus affecting cell structure, function, and viability (4). Increased oxidative stress has been found during acute brain injury (5), which can directly damage the cellular ‘power-house’ mitochondria as the major source of ROS, leading to fuel deficiency and apoptosis during ischemia (6-12). As such, mitochondrial dysfunction is believed to play a major role in neural damage during TBI (5, 7, 11, 13) and the early trigger of neural degeneration following TBI (9, 14). As such, mitochondria are vital for the function and survival of neurons.

Autophagy, as ‘self-eating’, can degrade proteins and even entire organelles; whereas mitophagy is responsible for removing damaged mitochondria; breaking them down to basic constituents that are recycled to generate new healthy mitochondria through the process of fission and fussion, respectively (15, 16). Mitophagy dysfunction has been found to be closely linked to neurodegenerative disease (17). Increased mitophagy has been shown in brains with ischemic and

reperfusion injury, suggesting a role in protecting the neural injury site (18). However, during hemicerebellectomy induced axonal injury, fission is increased however fussion is inhibited in remote axotomized neurons, suggesting increased mitochondrial damage but reduced regeneration capacity (19). It is unclear how mitophagy is changed in the penumbra area in response to a moderate TBI induced by direct force to the cortex. This type of injury is less likely to be actively treated in clinic, and yet it is related to significant neural apoptosis and abnormal neural functional outcome. Therefore, this study aimed to investigate the acute and long term changes in mitochondrial integrity as well as behaviour changes following a moderate TBI.

2 Results

2.1 Behaviour test

The novel object recognition (NOR) test showed that at 24h post injury sham rats spent most of the total time on the novel block during the test phase as expected ($P=0.05$, $t=2.08$ $df=20$ $F=1.11$, Fig 1A). From week one post injury (W1) the sham rats spent slightly less time on the novel block suggesting a lack of ongoing interest. On day one post injury (D1) and also at W1 the TBI rats spent less time of the novel object, indicating a cognitive deficit. The error ladder test showed that at 24h post injury the TBI rats made more stepping errors with their left forepaw than the sham rats ($P<0.01$, $t=1.59$ $df=13$ $F=25.9$, Fig 1B). After D1, both groups made a similar percentage of stepping errors during the test (Fig 1B).

2.2 Histology and IHC

The cortex, hippocampus, and thalamus were intact in the sham rats (Fig 2A). Examination of the brain slices of the right (contused) hemisphere of the TBI rats at 24 hours showed extensive haemorrhage. It extended from the cortex (S1Tr and S1BF, Bregma -3.6mm) to the hippocampus (CA2 and CA3, Bregma -3.6mm) and the thalamus (LDVL, DLG, VLG, VPM, VPL, Rt, Bregma -

3.6mm) (Fig 2B). The brains in the TBI rats at 6 weeks showed a distinct area with yellow colouration indicating an older injury in the area of the contusion (Fig 2C).

H&E staining showed that there was no brain injury in the sham rat brains (Fig 2D). The structural integrity of the cortex, the hippocampus, and the thalamus below the impact site was damaged in the TBI rats at 24 hrs (Fig 2E) and at 6 weeks (Fig 2F).

Astrogliosis, reflected by glial fibrillary acidic protein (GFAP) staining (Fig 3), was increased at 24 hours in the cortex ($P<0.05$, $t=2.46$ $df=9$ $F=1.5$), hippocampus ($P<0.05$, $t=2.73$ $df=9$ $F=1.5$) and thalamus ($P<0.05$, $t=1.23$ $df=9$ $F=10.5$) of the TBI rats compared to the sham rats. At 6 weeks there were increases in GFAP staining intensity in all three TBI brain areas albeit without statistical significance.

At 24 hours there were no macrophages ($ED1^+$cells) visible in the brain in either the sham or TBI rat brains. At 6 weeks there were no macrophage cells ($ED1^+$cells) present in the sham rats but macrophages were present in the cortex, hippocampus, and thalamus areas of the TBI rats.

2.3 Mitochondrial markers

At day 1 post surgery, right brain protein Manganese Superoxide Dismutase (MnSOD, $P<0.05$, $t=1.45$ $df=8$) and OXPHOS Complex (C)I ($P<0.05$, $t=3.64$ $df=10$ $F=9.5$), CIII ($P<0.05$, $t=2.77$ $df=9$ $F=15.3$) and CV ($P<0.05$, $t=2.39$ $df=10$ $F=15.8$) levels were significantly reduced in the TBI rats compared to the shams (Fig 4A). However, translocase of outer membrane (TOM)20, which imports proteins into mitochondria, was not changed in the TBI rats (Fig 4B). Among all the mitophagy markers, only LC3A/B-I protein was significantly reduced in the mitochondria ($P<0.05$, $t=2.90$ $df=6$ $F=2.1$), while dynamin-related protein 1 (DRP-1), PTEN-induced putative kinase (PINK)1, optic atrophy (OPA)1 and LC3A/B-II proteins were not significantly different from the sham rats (Fig 4B).

By week 6, brain MnSOD, TOM20 and OXPHOS CI-CIV were similar between the groups albeit non-significant reduction in CV which was still nearly 30% lower in the TBI rats (Fig 4C).

Mitochondrial DRP-1 ($P<0.05$, $t=3.56$ $df=4$ $F=2.0$), LC3A/B-I ($P=0.06$) and LC3A/B-II ($P<0.05$, $t=3.78$ $df=4$ $F=2.2$) levels were nearly doubled in the TBI rat brains (Fig 4D), while PINK1 ($P<0.05$, $t=2.99$ $df=4$ $F=1.2$) was significantly reduced in the TBI rat brains, Fig 4D).

3 Discussion

In this study, moderate TBI in the rats caused noticeable motor and cognitive deficit within 24 hours of the contusion. In the acute phase, markers related to mitochondrial energy metabolism were significantly reduced and astrogliosis was increased. However at 6 weeks post-injury, high levels of inflammatory cell infiltration and mitophagy dysfunction occur, albeit with recovered motor-cognitive functions.

In this study, a discrete contusion was observed at 24 hours, while at 6 weeks, cystic cavities were observed in the tissue. Immediately after the injury, the repair mechanism is turned on, reflected by an increase in reactive astrocytes in the injured area of the brain. Therefore, the increase in astrocytes at 24h reflects a quick adaptation for damage control. Astrocytes are important supporting cells, which form the homeostatic regulator of the neurons, by delivering important nutrients into neurons, including glucose and lactate to support normal neural function (20). During neural trauma induced destruction of neurons trigger nearby astrocytes to be activated to form scar tissues that limit the damage (21, 22). Continuous astrogliosis is also involved in the progress of certain neurodegenerative diseases, such as Alzheimer's disease due to increased inflammation, production of intermediate filament proteins, and unknown mechanisms (22, 23). This perhaps forms the link between mild TBI and increased risk of neurodegenerative disease in such patients (24).

The neurobiological consequences of traumatic brain injury vary from patient to patient, determined by the genetic susceptibility and brain resilience of the individuals (25). In this study, there is a significant variation in the behaviour data, which is expected for such studies. Abnormal NOR and error ladder results are indication of short memory defect and motor skill dysfunction,

respectively. Both are commonly observed in patients suffering from concussion, the mildest form of brain injury without any observable tissue damage. Although extensive tissue loss was evident at 6 weeks, behavioural function had spontaneously recovered, highlighting the strong plasticity for the intact brain area to compensate. However, it also needs to be noted that only two simple behavioural tests were chosen in this study and followed up for only six weeks, which does not reflect the whole spectrum of neural functional outcome after milder forms of TBI. More tests are needed in future studies, including those testing sensory function and anxiety, and more long term follow up, such as 6 months.

When TBI occurs, there are dramatic changes in energy metabolism and demand in the surrounding brain tissues due to the halted blood supply and reactive inflammatory responses. With a traumatic impact to the brain, an inflammatory response is initiated by the activation of resident glial cells, microglia, and astrocytes, and the migration of pro-inflammatory cells to the injury site (27). This migration is only evident at 6 weeks, which plays an important role in scavenging the damaged cells. However, inflammatory cells contain NADPH oxidase, an enzyme attributed to reducing molecular oxygen to form ROS (28, 29). Additionally, TBI causes the up-regulation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase resulting in excessive production of ROS subsequently promoting oxidative stress (29). Thus oxidative stress is integral to the general inflammatory response (30). Such increased levels of ROS can directly impair mitochondrial function to regulate growth, tissue maintenance, cellular metabolism and viability (4, 31, 32). In addition, mitochondrion itself is a major site of ROS production during OXPHOS to generate ATP (33). During an inflammatory response, there is a high consumption of oxygen and release of the superoxide free radicals (O^{-}_{2}) by the mitochondria (34), which can, in turn, impair mitochondrial function (35). Without a properly functioning mitochondria, cell death is likely to ensue, this is particularly true for neuronal cells of the brain given their high energy demands (36). Thus mitochondrial dysfunction has been identified as a significant influence in the cell death in numerous neurological disorders (37-40). Indeed, in this study antioxidant MnSOD was

significantly reduced at 24h suggesting increased need for scavenging free radicals which is commonly linked to oxidative stress. TOM20 transfers proteins into the mitochondria for energy metabolism (26), which was not changed at any time point suggesting substrate supply mechanism is not impaired; whereas reduced OXPHOS C1,III and IV in the acute stage might reduce energy synthesis capacity, which has been suggested to adversely affect neural repair and functional recovery in the literature (41). As such, in the long term neural tissue loss is inevitable as observed at 6 weeks here, although simple cognitive and motor functions seem to be compensated well at this time point. However, low brain mitochondrial OXPHOS CV seems to persist in the TBI rats long after the injury was recovered suggesting a potential long term effect on neurological repair and functional recovery following TBI, which is still yet to be confirmed in future studies.

Under environmental stress, mitochondrial integrity and self-repair largely depend on mitophagy. In previous studies, the autophagy markers for whole cells were analysed following ischemic brain injury (42, 43), which does not reflect mitochondrial specific autophagy. In this study, we measured the autophagy markers in the mitochondrial fraction only. Mitophagy is so far the only identified mechanism by which the defective and malfunctioning mitochondria are entirely degraded and recycled (15), as opposed to the elimination of the whole cell by autophagy. Several factors are involved in the breaking down of damaged mitochondria and reassembling the new healthy mitochondria, namely fission and fusion respectively. Mitophagy also shares similar functional molecules with autophagy. Here we found that in the acute phase, mitophagy fission and fusion markers seem to be unchanged by moderate TBI despite increased oxidative stress and reduced mitochondrial OXPHOS complex levels, suggesting impaired mitophagy response to preserve intact mitochondrial fragment (44). Rather, damaged mitochondria were scavenged as whole by autophagosomes, reflected by overconsumed microtubule-associated protein LC3A/B-1 in the acute phase. To date, only LC3A/B is known to exist on autophagosomes, and therefore, it is widely used marker for autophagosomes. During TBI, the damaged neurons have high demand for energy supply (45). Therefore, the most economical way is to preserve functional intact fragment of

injured mitochondria to reassemble into new healthy mitochondria, namely mitophagy. Indeed, here mitophagy activity seems to be increased in the long term reflected by high protein levels of mitophagy machinery in injured brain.

During cellular insult, mitophagy machinery element DRP-1 can stabilize p53 on the mitochondria to trigger necrosis *in vitro* and *in vivo* (46). DRP-1 facilitates the fission process which splits the damaged mitochondria into a damaged and a healthy component. However, in the neurons with ischemic hypoxic injury, inhibition of DRP-1 can protect from neural loss (48); Following fission, PINK1 is recruited and attached to the damaged mitochondrial component (50, 51), which is further engulfed by autophagic vacuoles composed by LC3A/B for degradation (52). Thus LC3A/B forms a reliable marker of mitophagy actions. In this study, increased LCA/B-I and II at 6 weeks is in line with increased DRP-1 to increase the clearance of damaged mitochondria, which also overconsumed PINK1. Furthermore, increased DRP-1 and its related fission can increase ROS production to further damage both mitochondria and neurons (49). Hence at 6 weeks, increased DRP-1 levels well correlates with reduced OXPHOS CV and tissue loss on the injured side. On the other hand, the intact components of several damaged mitochondria are reassembled by OPA1-1 to form new healthy mitochondria via the process of fusion. Therefore the upregulation of the mitochondrial OPA1-1 has been shown to restore mitochondrial morphology and protect neurons against excitotoxic injury (54). In this study, albeit increased markers involved in fission process, the fusion marker OPA1-1 was not increased, suggesting less functional mitochondria were regenerated afterwards. Such imbalanced fission and fusion action may directly contribute to reduced mitochondrial OXPHOS CV level that is involved in ATP synthesis. This may in turn lead to fuel shortage and continuously degeneration in the longer term after moderate TBI, which requires further investigation.

In addition to the rescue mechanism by mitophagy, it has been suggested that estrogen processes protective effect against injuries. However, this seems to be prominent when high pharmacologic doses were injected in animals with very low endogenous estrogen level, such as

male rats and female rats with ovariectomy (55-58). As such, in female rats with natural endogenous estrogen production as we have used in this study, there was very little variation in the injury, oxidative stress and mitophagy markers within the injury group and within the control group.

Although neural functions, such as motor and short term memory, can be compensated in the short term, in the long term, the development of certain neurogenerative disease can be inevitable. Future studies can focus on interventions to improve mitophagy machinery, in order to improve neural cell survival post moderate TBI.

4. Methods

4.1 Modelling moderate TBI

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Health and Medical Research Council. The protocol was approved by the Animal Care and Ethics Committee at University of Technology Sydney (ACEC#2014-208). All surgery was performed under iosflurane anesthesia, and all efforts were made to minimize suffering. 30 adult female Sprague Dawley rats (~250g) were used. 20 rats were submitted to a moderate cortical contusion and 10 underwent sham surgery only. Sham rats did not undergo the weight drop impact but all other procedures were identical. The rats were allowed to recover and half of each group were kept for 24 hrs while the other half were kept for 6 weeks. Analgesics (Buprenorphine hydrochloride-Temgesic 0.03mg/kg s.c.), antibiotics (Cefazolin sodium 33mg/kg s.c.) and Hartmans replacement solution (Compound sodium lactate 15ml/kg s.c.) were given prior to the surgery. Animals were anesthetised with isoflurane (4% induction, 2% maintenance) with 1% oxygen then a craniectomy was made on the right side of the skull 2.5mm posterior and 3.0 mm lateral to bregma using a dental drill. A 10g weight, 2.5mm diameter impactor head, was dropped from 5cm onto the surface of the brain (S1Tr and S1BF areas) using a New York University Impactor (59, 60) modified for brain injury. This produces a moderate brain injury, with a small focal lesion and good recovery in the animals. The contusion was confirmed visually in all

TBI rats. Bone wax was placed over the skull hole and the skin sutured closed. Animals received subcutaneous antibiotics, analgesics and fluids as above every 12 hours for 3 days post-surgery.

Rats were culled after anaesthetic overdose with Lethobarb (pentobarbitone, 1ml/100g, i.p). The first cut of the brain was made at the lesion site and a 2mm slide was made. The brain was dissected into half at the mid-line and the right hemisphere was halved again horizontally. The inner piece containing injury was kept for western blotting.

4.2 Behavioural test

Animals were assessed for cognition (novel object recognition (NOR) test) (61) and forelimb function (error ladder walking test) (62) at 24h, 1 week, 3 weeks and 6 weeks after the surgery. All rats were habituated to the testing apparatus for 1 week prior to the surgery. During the NOR test, the rat was exposed to 2 sets of 5 minutes exposure session, one with two identical blocks (familiarisation phase), and one with one old and one novel block (test phase). The time when the rat sniffed or touched each block was recorded. All data for the NOR tests is presented as the percentage of time spent on the novel blocks in the test stage. For the error ladder walking test, the rat walked on a horizontal ladder with uneven runs for 2 minutes. The number of front left paw stepping errors was divided by the total numbers of steps and multiplied by 100 to give a percentage left paw stepping error score. Analysis was undertaken from digital video recordings by two researchers blind to the groups.

4.3 Western blotting

The protein levels of antioxidant MnSOD, protein transporter TOM20, and mitochondrial energy synthesis units OXPHOS complexes I-V, autophagy marker microtubule-associated protein 1 light chain (LC)3A and LC3B complexes proteins were measured in brain lysis; while DRP-1, PINK1, and OPA-1 were measured in the mitochondria. The brain was extracted for whole protein and mitochondrial protein as previously described (63, 64). Protein samples of 40µg were separated

on NuPage® Novex® 4-12% Bis-Tris gels (Life Technologies, CA, USA) and then transferred to PVDF membranes (Rockford, IL, USA), which were then blocked with non-fat milk powder and incubated with the primary antibodies (MnSOD (1:1000) & TOM20 (1:2000, Santa Cruz Biotechnology, Texas, USA) and Mitoprofile Total® OXPHOS complex Rodent WB antibody (1:2500, Abcam, Cambridge, UK), LC3A/B (1:2000, Cell Signalling Technology, MA, USA), DRP-1 (1:2000, Novus Biologicals, CO, USA), PINK-1 (1:000, Novus Biologicals, CO, USA), OPA1-1 (1:2000, Novus Biologicals, CO, USA)) for overnight and then goat anti-rabbit or rabbit anti-mouse IgG horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). Protein expression was detected by SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher, MA, USA) by exposure of the membrane in FujiFlim (Fujiflim, Tokyo, Japan). Protein band density was determined with Image J software (NIH, MD, USA).

4.4 Histology and immunohistochemistry (IHC)

Frozen brain tissues were sectioned (15µm) in the coronal plane and stained with hematoxylin and eosin (H&E) prior to immunohistochemistry on adjacent sections. Immunohistochemistry was carried out using the following primary antibodies: rabbit anti-GFAP (1:1000, Dako, Denmark) to label astrocytes and mouse anti-CD68 (ED1, 1:2000, AbD Serotec, Germany) to label macrophages. Prior to the primary antibody incubation, slides were incubated in 5% normal goat serum in phosphate buffered saline with Triton X-100 at pH 7.4 (PBST) for 30 minutes. Primary antibodies were diluted with 5% normal goat serum in phosphate buffer (PBG) and incubated overnight at 4 ºC. Slides were washed in PBST and incubated in goat anti-rabbit Alexa Fluor 488 (1/200, Invitrogen, USA) or goat anti-mouse Alexa Fluor 568 (1/200, Invitrogen, USA) in 5% PBG for 2 hours at room temperature. Slides were washed with PBST and counterstained by Hoechst (1:5000, Invitrogen, USA) for 10 minutes. All slides were cover slipped in fluorescent mounting medium (Dako, Denmark).

Imaging was carried out using an Olympus BH-2 light microscope with an Olympus BX-51 light microscope with an Olympus U-RFL-T fluorescence burner. Image analysis was carried out on samples taken from the cortex, hippocampus and thalamus of the left and right hemispheres, using ImageJ software (National Institutes of Health, version 2X). Analysis of astrocytes was undertaken by measuring the staining intensity (mean grey scale value) of GFAP in one low power field of view for each brain region near the center of the lesion after normalising against the black background for each section using the internal features of the software. ED1 positive cells were counted in each brain region at the same level as for GFAP in six high power fields taken from the edge of the lesion for TBI, the hippocampus and the thalamus directly beneath the lesion or at the equivalent location for shams.

4.5 Statistical method

The results are expressed as mean ± SE. The difference between the Sham and TBI groups was analysed by unpaired Student's *t* test and multiple comparisons were not undertaken (Statistica 9, Statsoft, USA). $P<0.05$ was considered significant.

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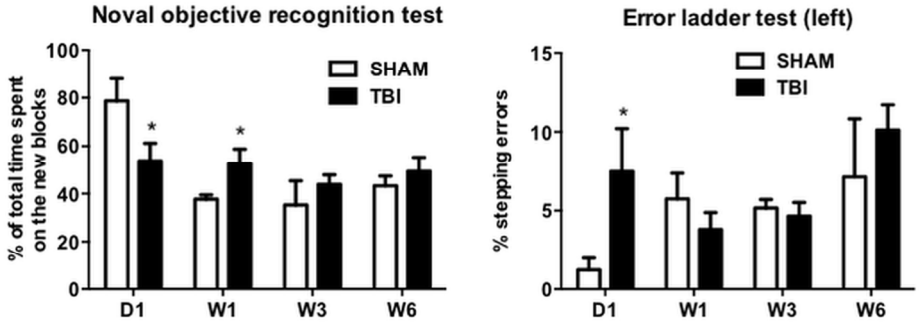
Figure legends

Fig 1. Behaviour test result. Percentage of time spent on (A) the new block during novel objective recognition test and (B) the percentage of errors made with the left forepaw during the error ladder test at 24h, 1 week, 3 weeks and 6 weeks post-injury. Results are expressed as mean ± SE. * $p < 0.05$, compared with the corresponding sham group.

Fig 2. Coronal brain slices at the level of the lesion for (A) sham and (B) injury at 24h showing obvious bleeding and (C) injury at 6 weeks post injury showing discoloured tissue on the contused (right) side of the brain. Bar = 5mm. Coronal brain sections stained with Haematoxylin and Eosin for (D) sham and (E) injury at 24h showing haemorrhage and tissue disruption and (F) injury at 6 weeks post injury showing a distinct cavity. Bar = 1mm.

Fig 3. Glial Fibrillary Acidic Protein (GFAP) staining. GFAP immunohistochemistry in the right (A) cortex, (B) hippocampus and (C) thalamus of the sham and injured brain at 24 hours and 6 weeks post injury. Graphs show the staining intensity as the mean greyscale value (GSV). * $p < 0.05$ compared with the corresponding sham group. Bar = 1mm

Fig 4. Protein levels of mitochondrial related markers. Protein levels of brain MnSOD, TOM20, and OXPHOS complex, as well as mitochondrial mitophagy markers DRP-1, PINK1, OPA1-1, LC3A, and LC3B at (A, B) 24h and (C, D) 6 weeks post-injury. Results are expressed as mean ± SE.* $P<0.05$, compared with the sham.



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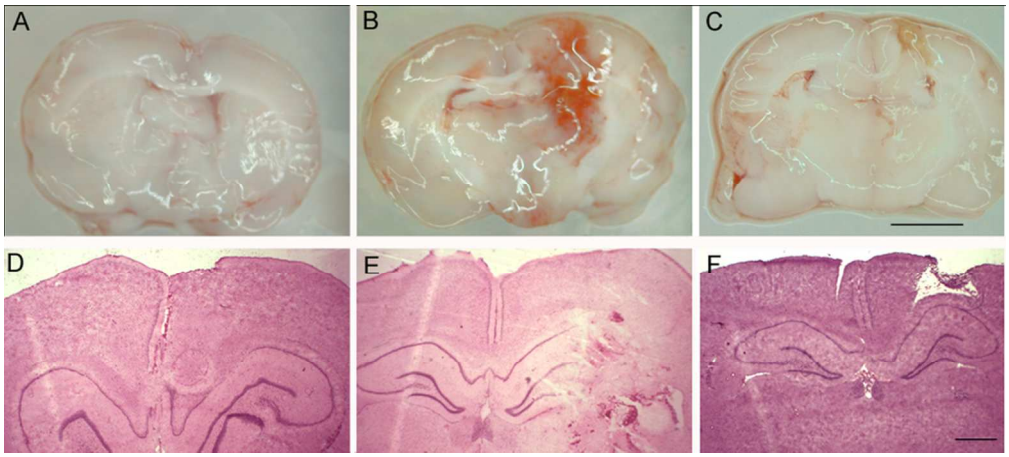

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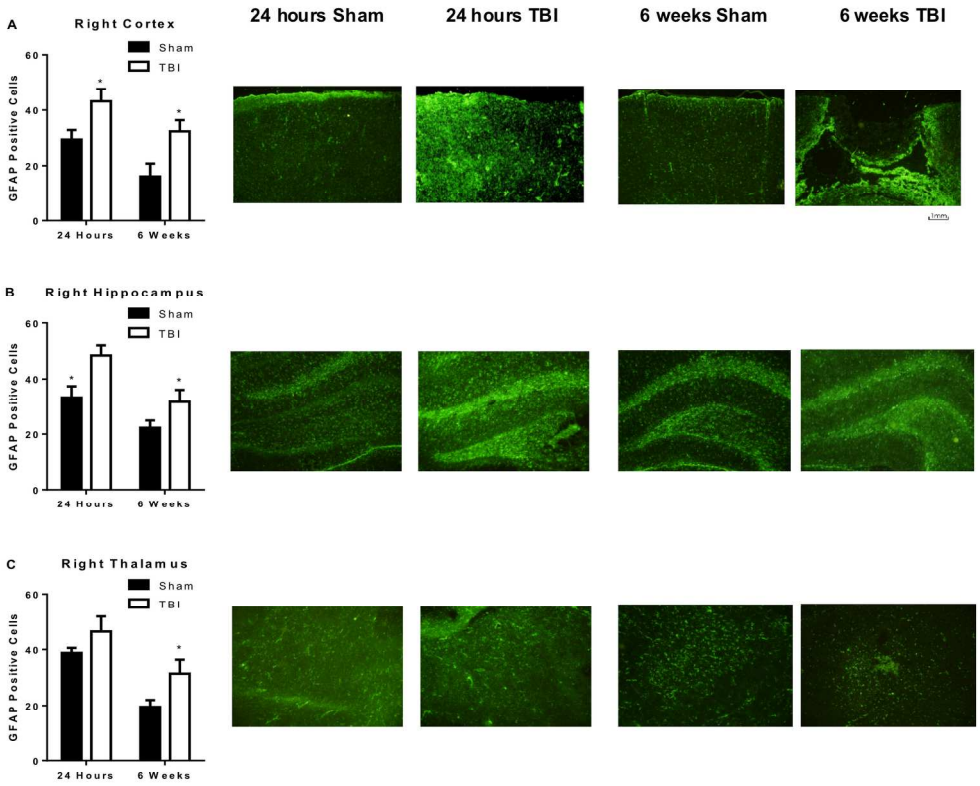

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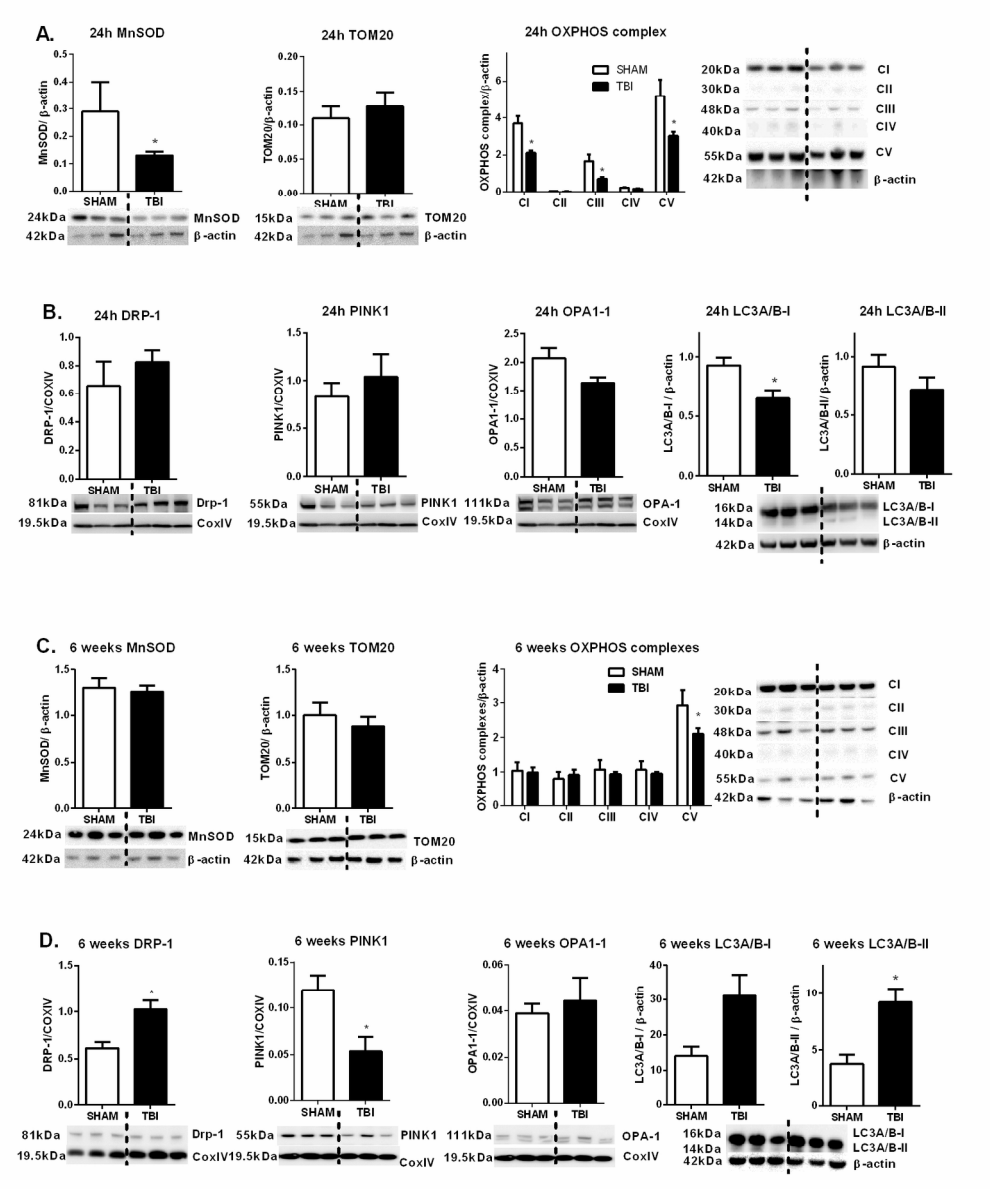

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1490x1775mm (72 x 72 DPI)