**Original Article**

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Experimental Neurobiology

# Absence of Glia Maturation Factor Protects from Axonal Injury and Motor Behavioral Impairments after Traumatic Brain Injury

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Traumatic brain injury (TBI) causes disability and death, accelerating the progression towards Alzheimer's disease and Parkinson's disease (PD). TBI causes serious motor and cognitive impairments, as seen in PD that arise during the period of the initial insult. However, this has been understudied relative to TBI induced neuroinflammation, motor and cognitive decline that progress towards PD. Neuronal ubiquitin-C-terminal hydrolase-L1 (UCHL1) is a thiol protease that breaks down ubiquitinated proteins and its level represents the severity of TBI. Previously, we demonstrated the molecular action of glia maturation factor (GMF); a proinflammatory protein in mediating neuroinflammation and neuronal loss. Here, we show that the weight drop method induced TBI neuropathology using behavioral tests, western blotting, and immunofluorescence techniques on sections from wild type (WT) and GMF-deficient (GMF-KO) mice. Results reveal a significant improvement in substantia nigral tyrosine hydroxylase and dopamine transporter expression with motor behavioral performance in GMF-KO mice following TBI. In addition, a significant reduction in neuroinflammation was manifested, as shown by activation of nuclear factor-kB, reduced levels of inducible nitric oxide synthase, and cyclooxygenase-2 expressions. Likewise, neurotrophins including brain-derived neurotrophic factor and glial-derived neurotrophic factor were significantly improved in GMF-KO mice than WT 72 h post-TBI. Consistently, we found that TBI enhances GFAP and UCHL-1 expression and reduces the number of dopaminergic TH-positive neurons in WT compared to GMF-KO mice 72 h post-TBI. Interestingly, we observed a reduction of TH-positive tanycytes in the median eminence of WT than GMF-KO mice. Overall, we found that absence of GMF significantly reversed these neuropathological events and improved behavioral outcome. This study provides evidence that PD-associated pathology progression can be initiated upon induction of TBI.

**Key words:** Traumatic brain injury, Neuroinflammation, Glia maturation factor, Parkinson's disease, Motor behavior

## INTRODUCTION

Traumatic brain injury (TBI) is a leading cause of disability, death, and approximately 50 million people experience TBI [1]. More than 1.7 million people have TBI in the United States of America [2-4], and the economic burden from TBI has been estimated to be more than $400 billion annually [5-7]. Worldwide, the occurrence of TBI is increasing, particularly in developing

countries [8]. TBI affects veterans, professional athletes, and boxers that cause decelerating muscular movements (athetosis) occasionally combined with difficulty in speech, tremors of the hands, gait disturbance, drowsiness and mental illness [9]. TBI encompasses a series of events that cause both physical and cognitive impairments that interfere with locomotor ability in normal life [4, 10-12]. TBI involves a mechanical injury that is the primary event followed by secondary damages that cause pathological changes at the site of injury, the penumbra or contusion region. The secondary damages including the inflammatory response, apoptosis, and breach of the blood-brain barrier (BBB), formation of edema, oxidative stress, and excitotoxic damage that leads to neuronal death [13-15]. Ubiquitin carboxy terminal hydrolase-1 (UCHL-1) is a multifactorial protein selectively expressed in neurons and GFAP is a glial filament protein specifically expressed by astrocytes. Both these proteins represent the severity of astrocyte mediated neuronal damages upon TBI [16, 17]. Earlier studies have shown that TBI in WT mice activates the NF-kB pathway, but we would like to emphasize that the molecular responses to TBI mediated by GMF such as changes in NF-kB and neurotrophic factors have not been described before and are reported here for the first time. Recently, Mettang et al, reported that NF-kB activation aggravates and activates apoptosis mediated neuronal cell death that activates proinflammatory gene expression, severely causes behavioral impairments and increases mortality rate [18, 19]. Previous studies have shown that double peaks of increased NF-kB activity found in the subarachnoid hemorrhage [20], enhances astrocyte swelling mediated brain edema volume in TBI [21]. In addition, NF-kB activation selectively participates in neurotrophic factors expression such as neuronal growth factor (NGF), brain-derived neurotrophic factor (BDNF) and excitatory neurotransmitters [22, 23]. Enhanced expression of neurotrophic factors activates NF-kB [24-26]. However, the pathophysiology of TBI is complex, and the mechanism of neurodegeneration in TBI remains poorly understood. The consequences of TBI cause secondary complications that are closely linked with the advancement to neurodegenerative diseases including Parkinson's disease (PD), dementia pugilistica (DP) and Alzheimer's disease (AD) among other pathologies [27-29]. PD is an age-associated neurodegenerative disorder, pathologically characterized by a loss of dopaminergic (DAnergic) neurons and subsequent decline of dopamine concentration in the nigrostriatal region of midbrain [30].

TBI causes focal brain tissue damage, diffused axonal injury and suppresses the electrical and chemical transmission between the neuronal cells in the brain [31]. These neuro-anatomical alterations have been examined through epigenetic and behavioral studies [32, 33]. There are two major pathological markers

identified and recognized that contribute to the development of PD or PD like pathology such as DAnergic system degeneration and α-synuclein inclusions in the SN region of the brain [34, 35]. However, increasing evidence suggests that dysfunctions of the DAnergic system may be a significant contributing mechanism for behavioral and cognitive deficits after TBI [36, 37]. Alterations in the DAnergic signaling pathway may be a potential mechanism for the persistent cognitive dysfunction seen after TBI [31]. Primarily, TBI causes cognitive impairments due to damage and loss of hippocampal [38, 39], and frontal cortex [40] neurons that affect neural transmission between these regions to striatum [41]. Termination or fluctuations of dopamine neural transmission from these regions lead to impairments in attention, executive function, learning, memory, and motor decline [42-45]. Even though restoration and recovery are possible, certain factors affect the recovery rate such as different personal and social influences [12].

Experimental studies have demonstrated that small protein molecules play a crucial role in several intracellular functional deteriorations, which are closely involved in a number of pathophysiological changes. Currently, a number of studies have investigated the novel therapeutic targets of small proteins with essential biological properties to halt the secondary consequences associated with TBI. One of these small proteins is the glia maturation factor (GMF; 17-kDa) a neuroinflammatory protein, which was first isolated, purified, and characterized in our laboratory [46-48]. GMF is abundantly present in central nervous system and principally expressed by the neuronal populations such as neurons and astroglia [47, 49, 50]. The expression of GMF was increased with intracellular stress and rapidly phosphorylated by protein kinases at various phosphorylation sites [51-53]. The present study was undertaken to better understand the mechanisms of action of GMF on the pathological events that are associated with TBI leading to the development of movement disorder like PD, and understanding how GMF mediates neuroinflammation and influences behavioral deficits in mice.

## MATERIALS AND METHODS

### Animals

Wild type (WT) C57BL/6 mice (25~30 gm), aged between 10~12 weeks, were maintained and housed at The University of Missouri, Columbia laboratory animal care facility, and they were used in accordance with the guidelines approved by the IACUC and National Institutes of Health. The C57BL/6 global glia maturation factor knockout (GMF-KO) mice were generated in our laboratory [50, 54, 55], and colonies of these mice were permanently sustained in our laboratory for our studies. Mice were housed four per cage and

maintained under a 12:12 h light/dark cycle at 21±1℃ and 50±5% humidity. Standard laboratory diet and drinking water were available *ad libitum*.

### Closed head injury induced by a weight-drop method

TBI experiment was performed using the closed-head weight drop model as reported previously [56, 57] with a few modifications. In brief, mice were deeply anesthetized with 2% Isoflurane and O$_2$, allowed to respire spontaneously without tracheal intubation. The WT and GMF-KO mice were subjected to TBI or to sham-treatment, where mice underwent identical surgical procedures. The severity of brain injury was a determinant of weight and the height from which it is dropped, that can range from mild to severe. In the present study, mice were subjected to mildTBI (mTBI) by placement on a platform covered by foam. The head was not fixed to enable a predominantly diffuse injury induced by a shearing force, to mimic real-life accidents. The head injury was induced by using the concussive head trauma device, as described previously [58, 59] as shown in schematic diagram (Fig. 1A). The device is a hollow metal tube that was placed vertically above the head of mice. An iron metal weight (3 mm diameter, 35 gm) that drops freely from a height of 50 cm was used to induce high acceleration and upon impact cause a shearing in the target area of injury as needed which was identified as the right frontal anterior region of the brain area (1.5 mm lateral to the midline in front of the coronal plane). For the sham-operated control mice, outer skin was opened and the skull was exposed under anesthesia, then the skin incision was closed with silk sutures without brain injury. Body temperature was maintained at 37℃ throughout all procedures using a small animal temperature control chamber and immediately returned to the home cage after complete recovery from anesthesia. This model simulates traumatic brain injuries seen in road accidents or falls, as it imparts a diffuse injury.

### Behavioral assessments

At the end of the experiment, all the behavioral performances were assessed between 09:00 AM - 5:00 PM at the regulated room temperature in a quiet room as detailed before [57, 60]. The laboratory-trained staff conducted all the behavioral performances and data analysis of the TBI and sham-operated control. We followed the procedure for the blinded behavioral assessments of both WT and GMF-KO mice after TBI induction as we have previously reported [60, 61].

### Tail suspension test

The tail suspension test (TST) was used to assess the depressive-motor behavior in mice, as previously reported [62, 63]. In brief, experimental mice were individually suspended at the height of 50 cm from foam mat by using a piece of adhesive tape, approximately 2 cm from the tail tip of the mice. The immobility time was recorded during 3 minutes period.

### Hang test

Neuromuscular impairment and motor coordination in mice were measured by hang test performance as described earlier [64, 65]. In brief, experimental mice were placed 50 cm from foam mat on a horizontal grid and the grid was gently inverted so that the mice were allowed to hang upside down. The grid was placed 50
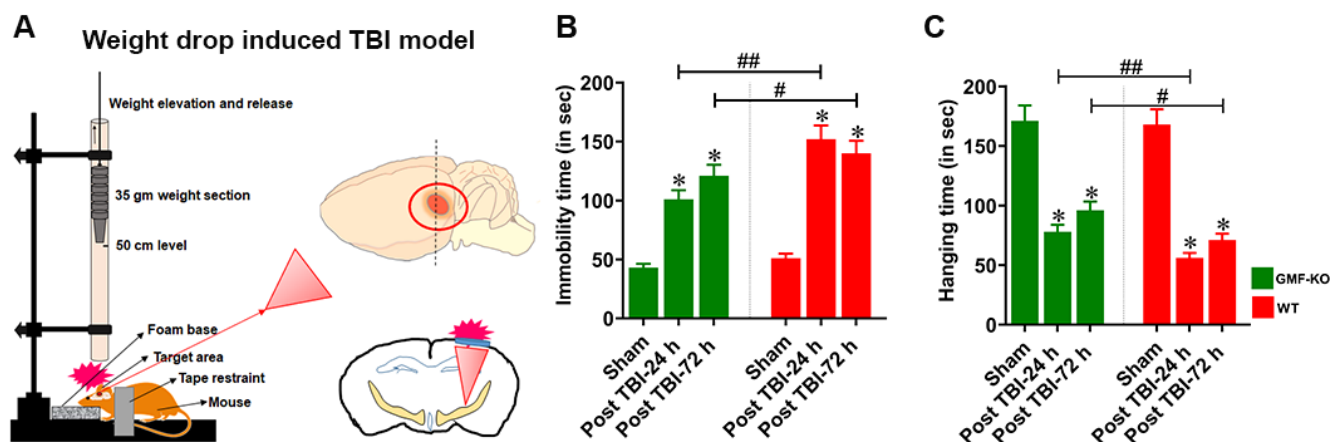


**Fig. 1.** Absence of GMF improves behavioral performance after weight drop induced TBI in mice. Induction of TBI by weight drop method in the cortical region (A) that significantly causes motor behavioral impairments in tail suspension test (B) and hang test (C) from WT and GMF-KO mice. The absence of GMF significantly reverses these behavioral abnormalities compared with WT TBI subjected mice. Values are presented as mean±SEM (n=6). *p<0.05 control vs TBI subjected mice; #p<0.05 and ##p<0.01 GMF-KO TBI subjected mice vs WT TBI subjected mice.

cm above the foam mat to prevent the injury to the mouse when it falls. The total staying time until the mice lost their grasp was measured as hanging time.

### Nissl staining and measurement of contusion volume

Nissl staining method was opted to measure the contusion volumes of TBI or sham-operated control mice, as reported previously [66, 67]. Nissl staining was performed at different times such as post-TBI-24 h and post-TBI-72 h after experimental TBI. The brains of experimental mice were prefixed with a 4% paraformaldehyde solution. To freeze and cut the brain sections we used an Optimal-cutting temperature (OCT) embedding medium. Coronal sections (25~30 µm-thick) were made in a cryostat (Leica Biosystems, Germany), and the sections were deeply washed with 1X PBS (ThermoScientific, Waltham, MA). The brain regions containing target lesions were collected every 200 mM. After sectioning, brain slices were deeply washed with PBS for 5 min, and stained with working buffer solution, which contains 0.1 M acetic acid and 0.1 M sodium acetate (94:6) and Cresyl Violet acetate at a ratio of 5:1. Then the sections were dehydrated with 100% ethyl alcohol and mounted in gelatin-coated glass slides. After Nissl staining, the sections were digitized using a Nikon microscope and quantified by using ImageJ software (NIH, Bethesda, MD). Contusion volume was assessed based on the previous reports [68]. The contusion volume of the areas of the hemispheres were quantified by an investigator blinded to the TBI and non-TBI subjected animals (n=6 per group).

### Western blot

The levels of protein expression was quantified by the Western blot method as we have described previously [69-71]. The extracted brain protein samples were equally loaded (ranging from ~20 to 35 µg) in each well, electrophoresed followed by transfer to polyvinylidene fluoride (PVDF) membrane. Immediately the membranes were blocked for 1 h at 4℃, then incubated overnight at 4℃ with following primary antibodies diluted in blocking solution: Tyrosine hydroxylase (TH) (ThermoScientific, Cat No. P21962; 1:800), dopamine transporters (DAT) (ThermoScientific, Cat No. PA1-4656; 1:800), glial fibrillary acidic protein (GFAP; Cell Signaling Technology (CST), Beverly, MA, USA; Cat No. 12389S, 1:1,000), C-terminal hydrolase-L1 (UCH-L1; CST, Cat No. 13179S, 1: 800), total and p-NFkB (CST, Cat No. 3033S, 1:800), BDNF (CST, Cat No. 3897S, 1:800), GDNF (CST, Cat No. 3223S, 1:800), iNOS (CST, Cat No. 13120S, 1:800), COX-2 (CST, Cat No. 12282S, 1:1,000) and β-actin (Sigma-Aldrich, Cat No. A2228, 1:1,000) at 4℃ overnight with gentle shaking. Subsequently, the membranes were washed and incubated with corresponding secondary antibodies (HRP-conjugated anti-rabbit or anti-mouse IgG). The band signals were visualized by adding ECL reagent and densitometry analyses were performed.

### Immunofluorescence analysis

Immunofluorescence double staining and positively stained cells were counted in the SN and STR region as described previously [60, 61, 72]. Midbrain tissues were collected from mice subjected to TBI or sham-operated control mice for immunofluorescence and performed as previously reported [73, 74]. As we mentioned previously, brain sections were washed and blocked with blocking buffer (containing 1% BSA in TBST) for 30 mins at 4℃. Following this the, sections were probed with the following primary antibodies: MAP2 (CST, Cat No. 8707S, 1:500); UCHL1 (1:500), NFL (CST, Cat No. 2837S, 1:500) and TH antibody (1:500) at 4℃. These sections were then incubated with their matching Alexa flour conjugated secondary antibodies (Alexa Fluor 488; Cat No. A-11001, Alexa Fluor 568; Cat No. A-11004, ThermoScientific). Finally, stained sections were transferred and mounted on a gelatin-coated slide with coverslip glued with mounting medium containing DAPI (VECTASHIELD antifade, Vector Laboratories). Then the slides were examined and images were acquired under a Nikon fluorescent microscope and number of nigral TH positive dopaminergic neurons were determined as described previously [75-77]. To determine the TH-positive area and the axonal length of the dopaminergic neurons in the SN from immunostained sections, we used NIH ImageJ software. Results are expressed in arbitrary unit (AU) on analysis obtained from five different random fields and compared to controls.

For the confocal imaging, the immunofluorescent stained microscopic slides were examined and the images were acquired under a Leica TCP SP8 rapid laser scanning confocal microscope (Leica, Biosystems, Germany) as we demonstrated previously [69-71].

### Statistical analysis

All the results obtained from the experiments were analyzed by GraphPad InStat 3 software. All the data are expressed as mean±SEM. To determine statistically significant differences we used a one-way analysis of variance (ANOVA) followed by Tukey-Kramer post hoc test. In addition, an unpaired $t$-test was performed when comparing only two groups. $p<0.05$ was considered statistically significant in all the experiments in this study.

## RESULTS

### GMF reduces behavioral performance in TBI mice

Neuromotor behavioral impairment is a common sequela in

weight drop induced TBI (Fig. 1A), as we determined by motor behavioral impairments in mice through assessing immobility time in tail suspension test (Fig. 1B) and hanging time in hang test (Fig. 1C). Results show a significant reduction of motor behavioral performance found in TBI subjected WT mice. Notably, we found that the absence of GMF in mice (GMF-KO mice) subjected to TBI showed a significant decrease in behavioral impairments at 24 h and 72 h post-TBI compared to WT mice. No significant differences were found between the sham-operated controls of WT and GMF-KO mice. These results indicate that GMF may be involved in the neuromotor behavioral performances in TBI induced mice through the neuroinflammatory sequence in the affected region of the brain.
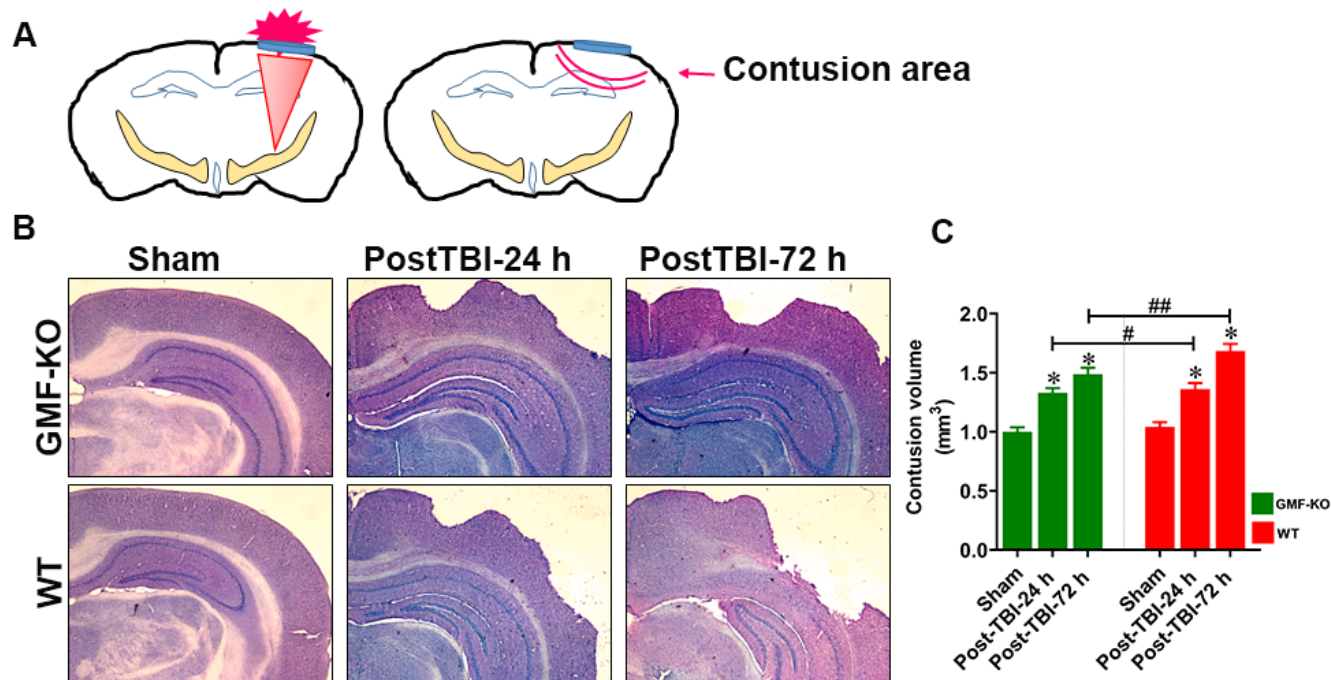
### GMF augments TBI induced contusion volume and amplifies brain tissue damage

The Nissl stained brain sections revealed that there was a significant cortical cell loss, which was examined by lack of cresyl violet staining and the representative images show contusion volumes following unilateral TBI in right hemisphere (Fig. 2A). Brain edema and contusion volume were assessed on day 3 after TBI, since weight drop induced more brain cortical tissue damage during this time (Fig. 2B). Quantitative bar graph shows that there

were no significant differences in the contusion volume between the WT and GMF-KO mice after 24 h post-TBI. Meanwhile, the contusion volume significantly increased in WT mice compared to GMF-KO mice after 72 h post-TBI (Fig. 2C). No obvious brain contusion volume was observed in the sham-operated control group. However, GMF-KO mice showed less contusion volume compared with WT mic following 24 h and 72 h TBI.

### GMF attenuates TBI induced expression of dopaminergic markers in SN of midbrain

Next, we examined the consequence of GMF on the expression of dopaminergic markers in mice brain after 72 h post-TBI. We examined TH (essential rate-limiting enzyme of catecholamine dopamine biosynthesis) and dopaminergic transporter DAT (a membrane-spanning protein that propels the catecholamine exchange between the nerve synaptic clefts) as shown in Fig. 3A. Representative immunofluorescence images show the TH-positive dopaminergic neurons in the SN at different magnifications (Fig. 3D; 10X and 63X). Bar graphs show that the expressions of TH (Fig. 3B), DAT (Fig. 3C) densitometry, TH-positive area (Fig. 3E) and the number of TH-positive dopaminergic neurons (Fig. 3F) were significantly reduced in both the WT mice and GMF-KO mice when compared with control mice. However, we noticed



**Fig. 2.** Absence of GMF reduces TBI induced contusion volume and amplifies brain tissue damage. TBI caused an expanding contusion volume in WT and GMF-KO mice to brain cortical lesions. The illustration of the cortical tissue lesion after weight drop induced TBI (A). Nissl staining showed the temporal pattern of anatomical structure change (B). Bar graphs show the quantification of cortical contusion volume or cavity (C; n=6). Values are presented as mean±SEM (n=6). *$p<0.05$ control vs TBI subjected mice; #$p<0.05$ and ##$p<0.01$ GMF-KO TBI subjected mice vs WT TBI subjected mice.
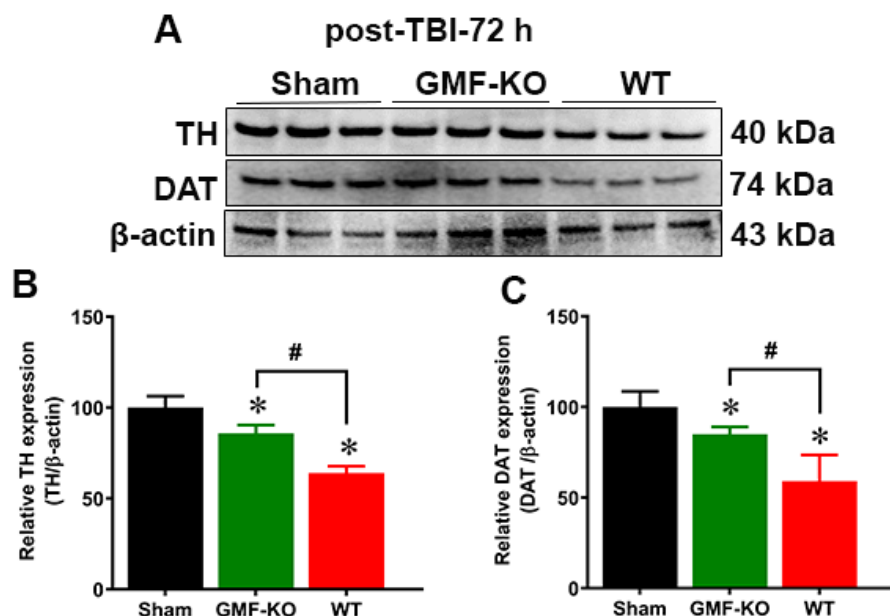
**Fig. 3.** Removal of GMF improves TBI induced dopaminergic markers expression in SN of the midbrain. Induction of TBI by weight drop method in the cortical region causes significant reduction in dopaminergic markers such as TH and DAT expression as determined by western blot (A) and immunofluorescence (D; red fluorescence) in the SN region of the midbrain of WT and GMF-KO mice. Representative images show the TH-positive dopaminergic neurons in the SN at different magnifications (10X and 63X). Bar graphs show the quantitation of the western blot band intensity with TH (B), DAT (C) expression, TH-positive area (E; as arbitrary units) and the number of TH-positive dopaminergic neurons (F) as compared to the controls. Values are presented as mean±SEM (n=6). *$p<0.05$ control vs TBI subjected mice; #$p<0.05$ GMF-KO TBI subjected mice vs WT TBI subjected mice.

that GMF-KO mice showed a significantly enhanced expression of these proteins when compared with WT TBI subjected mice. No significant reductions were found between the sham-operated controls. Together, these results suggest that GMF aggravates TBI induced dopaminergic neuronal loss as represented by markers TH and DAT expression in SN of the midbrain.

### GMF amplifies TBI induced UCHL1 and glial activation in SN of midbrain

To determine the expression of UCHL1 and GFAP, which are promising biomarkers for TBI, we performed western blot on protein homogenates from the SN of midbrain after 72 h post-TBI (Fig. 4A). UCHL1 is a thiol protease that helps to recognize and hydrolyze a peptide bond at the C-terminal glycine of ubiquitinated proteins in the brain. GFAP is a filament protein of astroglial skeleton and represents glial injury in the brain. However, the direct relationship between these markers have not been studied in TBI [78]. Results show that UCHL1 expression (Fig. 4B) significantly increased, along with the GFAP expression (Fig. 4C) in the SN of midbrain after TBI when compared with sham mice. In addition, we found that TBI subjected GMF-KO mice showed significantly lower expressions of UCHL1 and GFAP when compared with WT TBI subjected mice. There are no significant

differences in UCHL1 and GFAP expressions observed between the sham-operated controls from WT and GMF-KO mice. Taken together, our results indicate that GMF is involved in the TBI induced UCHL1 expression that may mediate glial injury in SN of midbrain 72 h post-TBI.

### GMF induces NF-kB activation and neuroinflammatory proteins expression in SN of midbrain of TBI subjected mice

To study the role of GMF in the activation of nuclear factor-κB (NF-kB) and neuroinflammatory markers expression, we performed western blot on protein lysates from the SN of the midbrain of 72 h post-TBI mice (Fig. 5A and 5C). NF-κB is a key transcriptional regulator of inflammatory genes. iNOS is pathologically upregulated in the brain after neuronal damage or injury, including TBI. COX-2 is an inducible isoform upregulated by reactive oxygen species (ROS), inflammatory cytokines and mitogens. Results shows a significant enhancement in the phosphorylated-NF-κB (p-NF-kB; Fig. 5B), iNOS (Fig. 5D) and COX2 (Fig. 5E) expression in WT and GMF-KO TBI subjected mice groups as compared with control mice. Furthermore, we found that GMF-KO mice subjected to TBI showed a significant reduction in p-NF-kB, iNOS and COX2 expressions as comparison with WT TBI subjected mice. No significant alterations were found in p-NF-kB
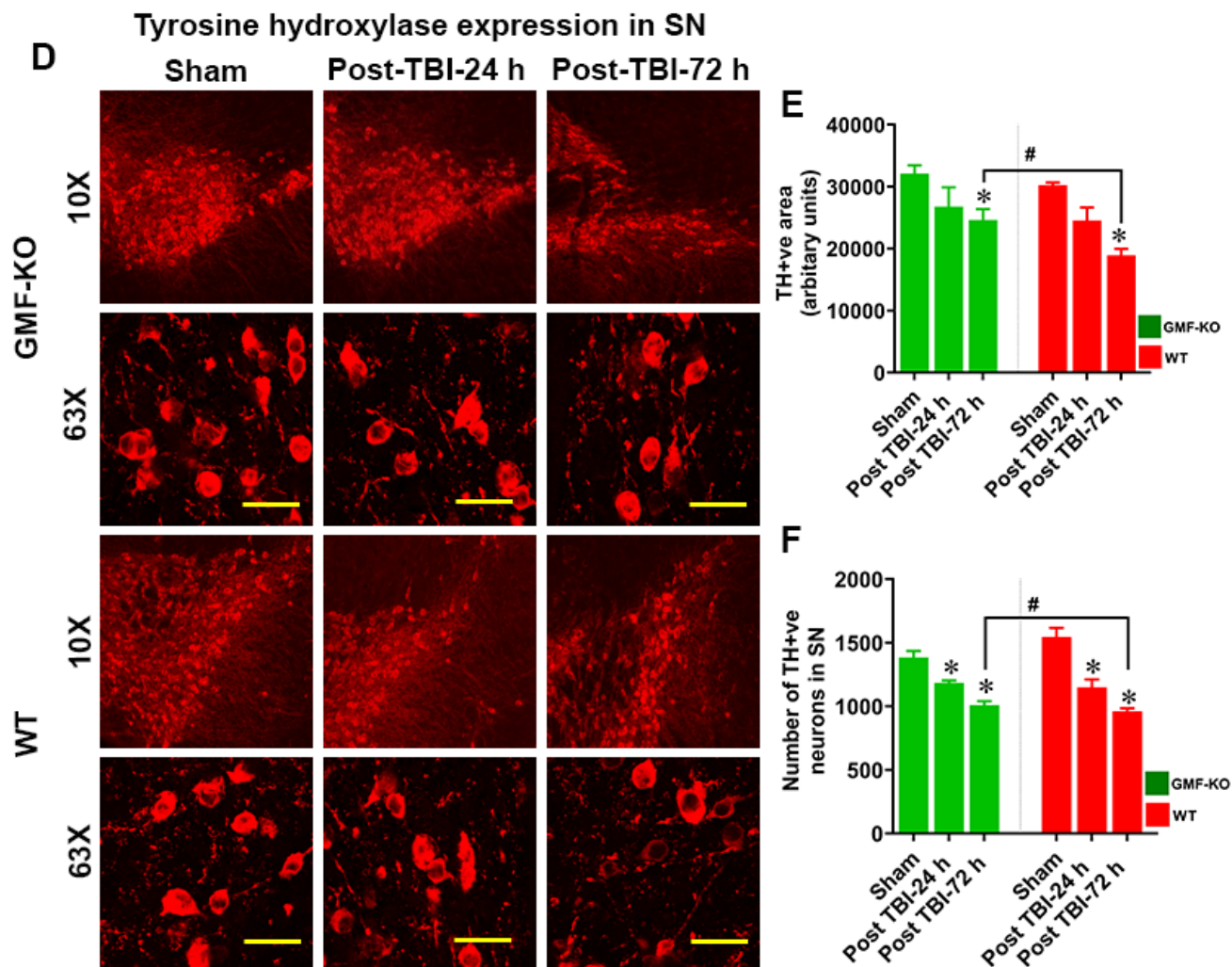
**Fig. 3.** Continued.

iNOS and COX2 expressions between the sham-operated control mice. These results indicate that GMF is associated with the aggravation of TBI induced neuroinflammation in SN of midbrain 72 h post-TBI.

### GMF attenuates TBI induced expression of neurotrophic factors in SN of midbrain

We examined the involvement of GMF in the expression of neurotrophic factors such as BDNF and GDNF in mice brain after 72 h TBI. We analyzed BDNF and GDNF (key neurotrophic proteins, involved with the survival and function of selected populations of brain dopaminergic, serotoninergic, and GABAergic neurons) expression, as shown in Fig. 6A. Bar graphs show that BDNF and GDNF expressions were significantly reduced in both the WT mice and GMF-KO mice when compared with sham mice. However, GMF-KO mice showed significantly increased expressions in

BDNF (Fig. 6B) and GDNF (Fig. 6C) expressions when compared with WT TBI subjected mice. No significant differences between the expressions of these markers were observed in the sham-operated controls from WT and GMF-KO mice. These results indicate that GMF may be involved in the neuronal survival and population after TBI by controlling BDNF and GDNF expression in SN of the midbrain.

### Involvement of GMF in TBI induced UCHL1 expression in the neurons and its fiber

We performed double immunofluorescence staining to show that UCHL1 expression was increased in the neuronal cell body (MAP2; Fig. 7A) as well as in the neurofilament region (NFL, Fig. 7D) of TBI subjected mice after 24 h and 72 h. Representative immunofluorescence images show that MAP2 and NF-L (red fluorescence) expression colocalized with UCHL1 expression (green
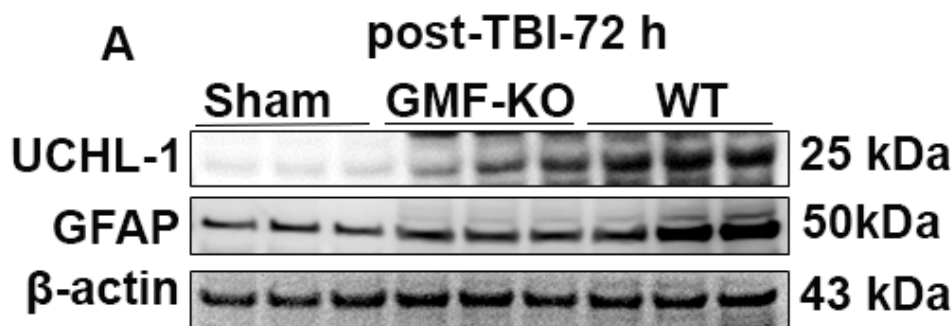
**Fig. 4.** Effect of GMF on TBI induced ubiquitin-protein hydrolase enzyme and astrocyte activation in SN of the midbrain. Weight drop TBI in the cortical region significantly increases the TBI key markers such as UCHL-1 and GFAP expression in SN region of midbrain of WT and GMF-KO mice as determined by western blot (A). Bar graphs show the quantitation of the western blots with UCHL-1 (B) and GFAP (C) expression as compared to the controls. Values are presented as mean±SEM (n=6). *p<0.05 control vs TBI subjected mice; #p<0.05 GMF-KO TBI subjected mice vs WT TBI subjected mice.
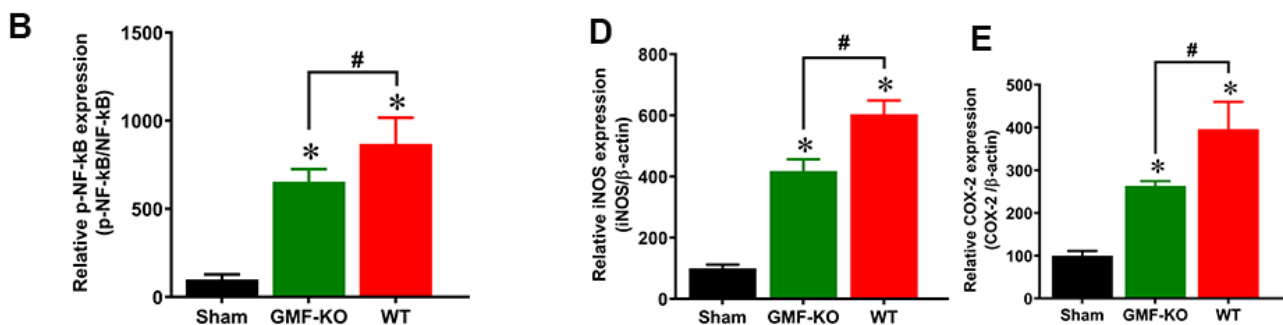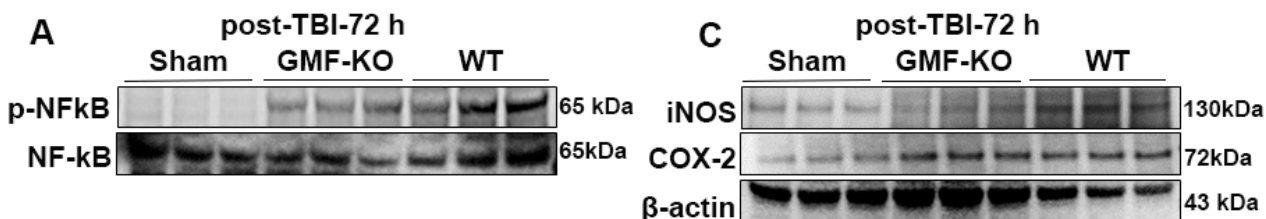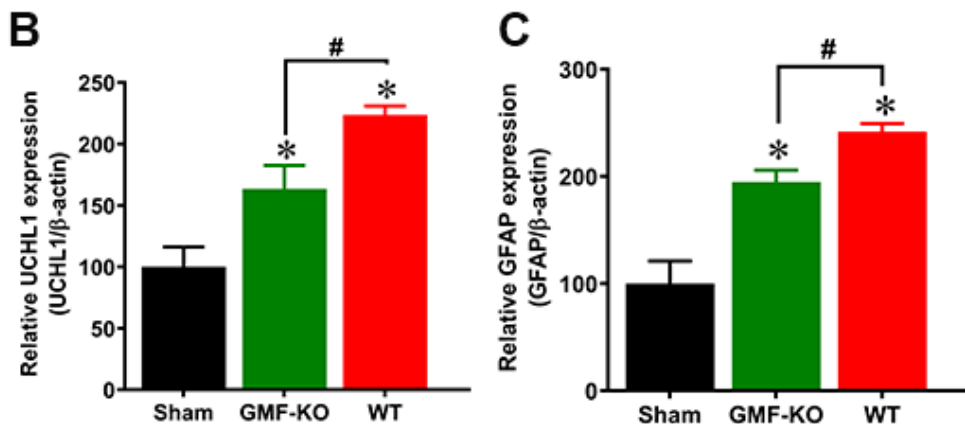


**Fig. 5.** Deficiency of GMF reduces NF-kB activation and neuroinflammatory proteins expression in SN of midbrain from mice subjected to TBI. Weight drop TBI in the cortical region significantly increases the activation of NF-kB, neuroinflammatory proteins such as iNOS and COX2 expression in SN region of the midbrain of WT and GMF-KO mice as determined by western blot (A and C). Bar graph shows the quantitation of blot intensity (B, D and E) expression as compared to the controls. Values are presented as mean±SEM (n=6). *p<0.05 control vs TBI subjected mice; #p<0.05 GMF-KO TBI subjected mice vs WT TBI subjected mice.

fluorescence) in the neuronal body (as stained with MAP2, red fluorescence, Fig. 7A) as well as in neurofilament region (as stained with NFL, red fluorescence, Fig. 7D). Bar graphs show the quanti-

tation of the total average intensity of MAP2 (Fig. 7B and C), NF-L (Fig. 7E) were significantly reduced meanwhile UCHL-1 (Fig. 7F) expression significantly increased as compared to the sham oper-
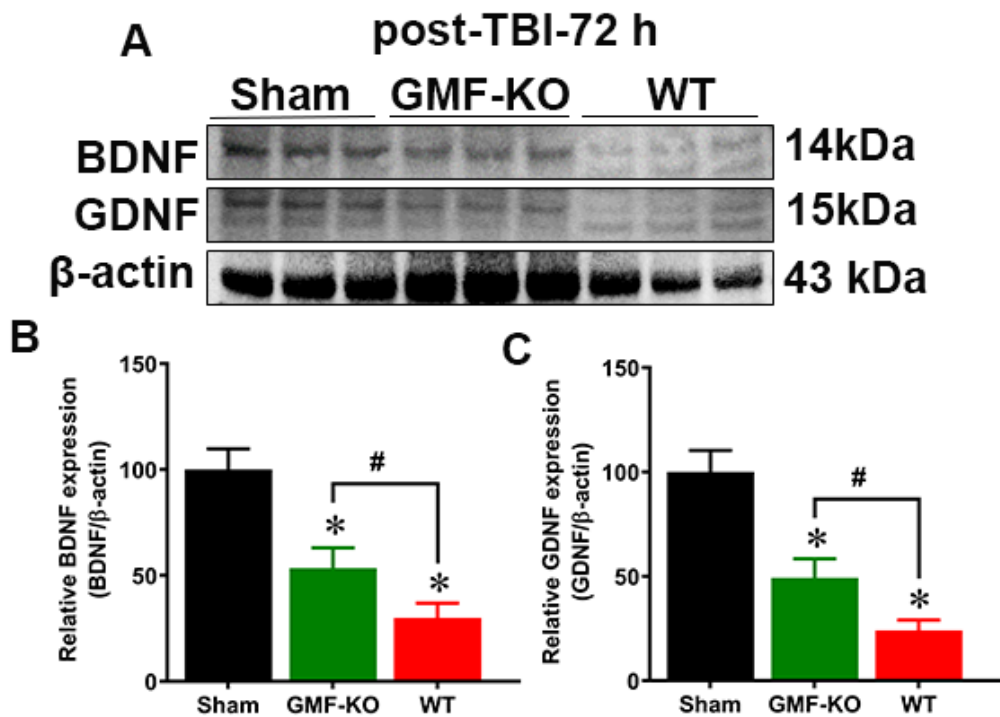
**Fig. 6.** Absence of GMF improves TBI induced neurotrophic factors expression in SN of the midbrain. Induction of TBI by weight drop method in the cortical region causes significant reduction in neurotrophic factors such as BDNF and GDNF expressions in the SN region of the midbrain of WT and GMF-KO mice as determined by western blot (A). Bar graphs show the quantitation of the western blot intensity with BDNF (B) and GDNF (C) as compared to the controls. Values are presented as mean±SEM (n=6). *p<0.05 control vs. TBI subjected mice; #p<0.05 GMF-KO TBI subjected mice vs WT TBI subjected mice.
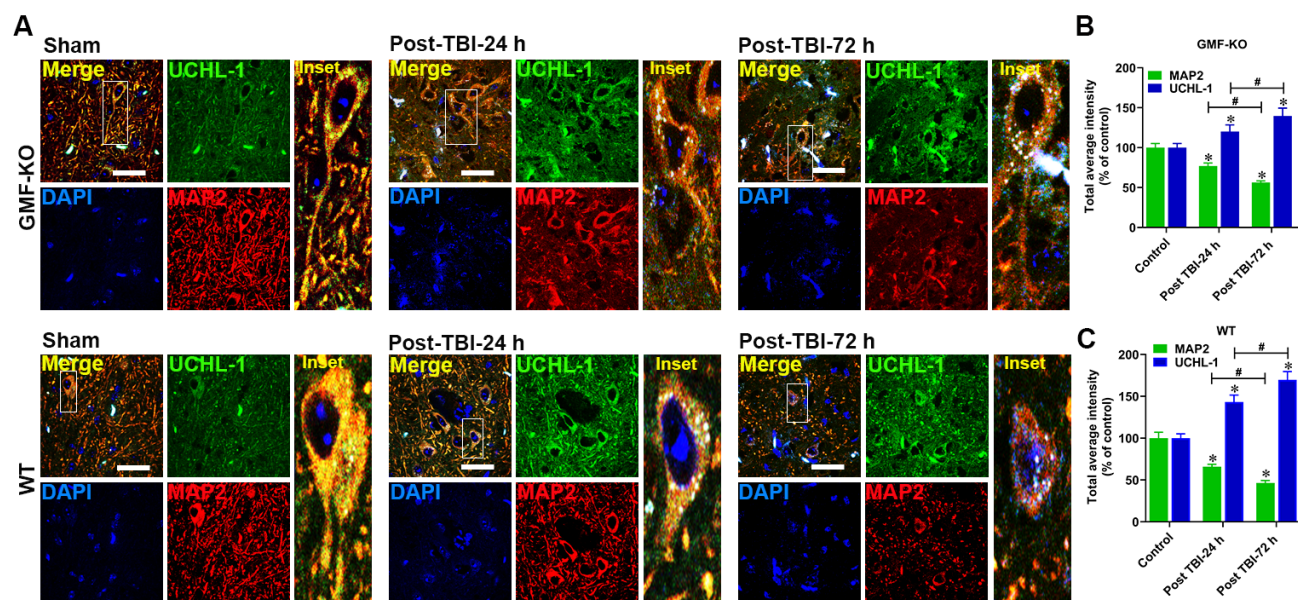


**Fig. 7.** The involvement of GMF in TBI induced UCHL-1 expression in the neurons and its fiber. Induction of TBI in the cortical region enhances UCHL-1 protein expression in neurons of the midbrain as determined by immunofluorescence. Representative images show a qualitative enhancement of UCHL-1 expression in neurons (A) and its fiber (D) after weight drop induced TBI in mice brain at 24 h and 72 h time point. Bar graphs show the quantitation of the total average intensity of MAP2 (B), UCHL1 (C), NF-L (E) colocalized with UCHL-1 (F) as compared to the sham operated controls. Values are presented as mean±SEM (n=6) and expressed as percentage of control. *p<0.05 control vs. TBI subjected mice; #p<0.05 GMF-KO TBI subjected mice vs WT TBI subjected mice. Scale bar 25 μm.

ated controls. However, this increase in UCHL1 expression was significantly lower in the GMF-KO TBI- subjected mice compared with WT TBI- subjected mice. No significant differences were found between the sham-operated controls from WT and GMF-KO mice. These immunofluorescence results imply that GMF a neuroinflammatory protein may be involved in the UCHL1 ex-
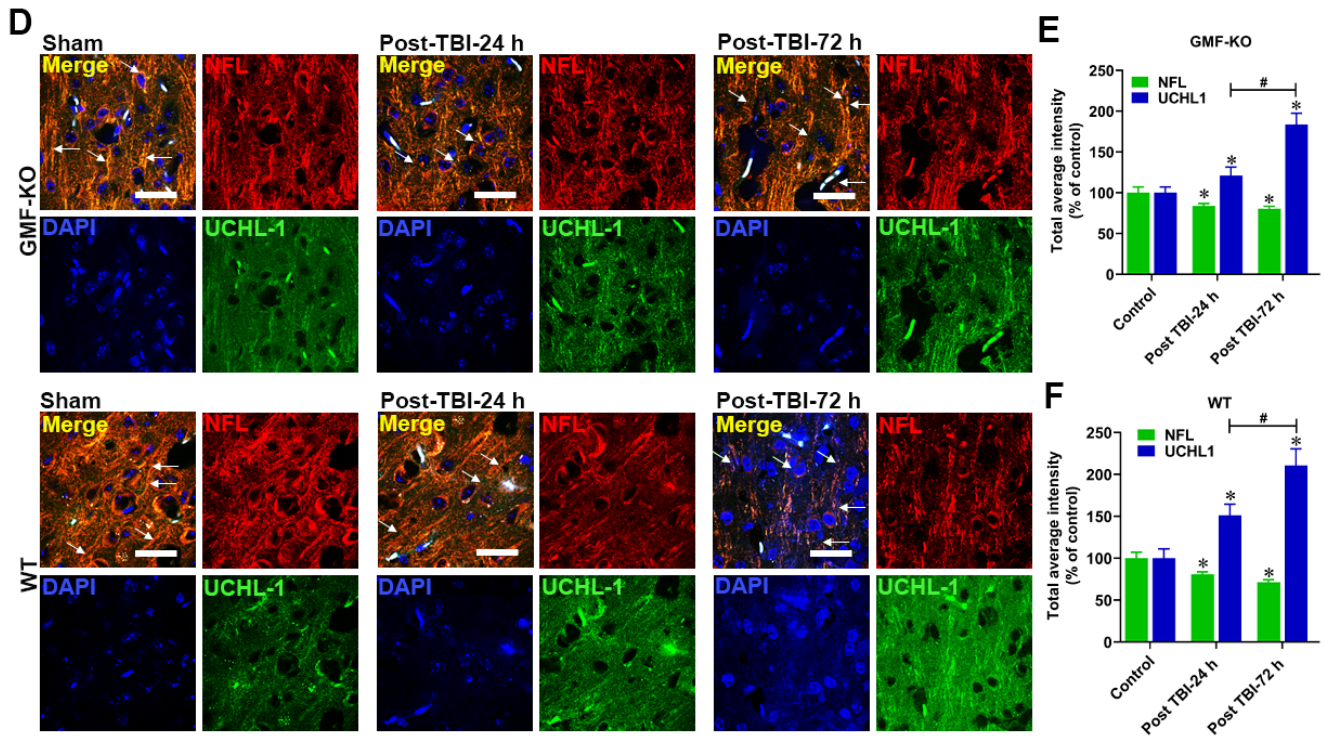
**Fig. 7.** Continued.

pression in neurons during the TBI induced neuroinflammation in the brain.

### GMF influences the TBI induced axonal injury in dopaminergic neurons of the midbrain

To determine the involvement of axonal injury of dopaminergic neurons after traumatic brain injury we performed immunofluorescence and axonal measurement as shown in Fig. 8. Results show that weight induced TBI significantly increases the axonal injury when compared with control mice (red fluorescence; Fig. 8A, B and C). However, we noticed that GMF-KO TBI- subjected mice show significant preservation in the axonal injury of TH positive neurons as compared with WT TBI subjected mice. No significant differences were found between the sham-operated controls from WT and GMF-KO mice.

### GMF affects TBI induced UCHL1 expression and TH positive dopaminergic neurons of the median eminence

We further performed double immunofluorescence staining to show the TH positive cells in the median eminence after WT mice and GMF-KO mice were subjected to TBI (Fig. 9). Representative images show that TH positive (red fluorescence) and UCHL1 expressions (green fluorescence) in WT and GMF-KO mice subjected to TBI (Fig. 9A). Bar graphs show the number of TH-Positive

tanycytes were significantly higher in the median eminence after TBI of GMF-KO TBI subjected mice as compared with WT TBI subjected mice (Fig. 9B). However, we found that GMF-KO TBI-subjected mice showed a significantly lowered reduction in TH positive and UCHL1 expressions as compared with WT TBI-subjected mice. No significant differences were found between the sham-operated controls from WT and GMF-KO mice. These immunofluorescence results specify that GMF, a neuroinflammatory protein may be involved in the capillary plexus in the median eminence of TBI subjected mice brain.

## DISCUSSION

In the present study, we demonstrate the sequence of events along with a strong association of GMF within the TBI pathology to expand secondary consequences that lead to dopaminergic neuronal loss in the SN of the midbrain, which further leads to motor deficits in mice. The salient finding of this study includes a significant reduction in the expression of dopaminergic markers such as TH and DAT after TBI. In addition, the weight drop model induced TBI causes UCHL1 dysfunction and activates NF-kB that drives iNOS and COX-2 to further reductions in GDNF and BDNF expressions that ultimately result in decline of motor behavior in mice. Furthermore, TBI affects TH and UCHL1 ex-
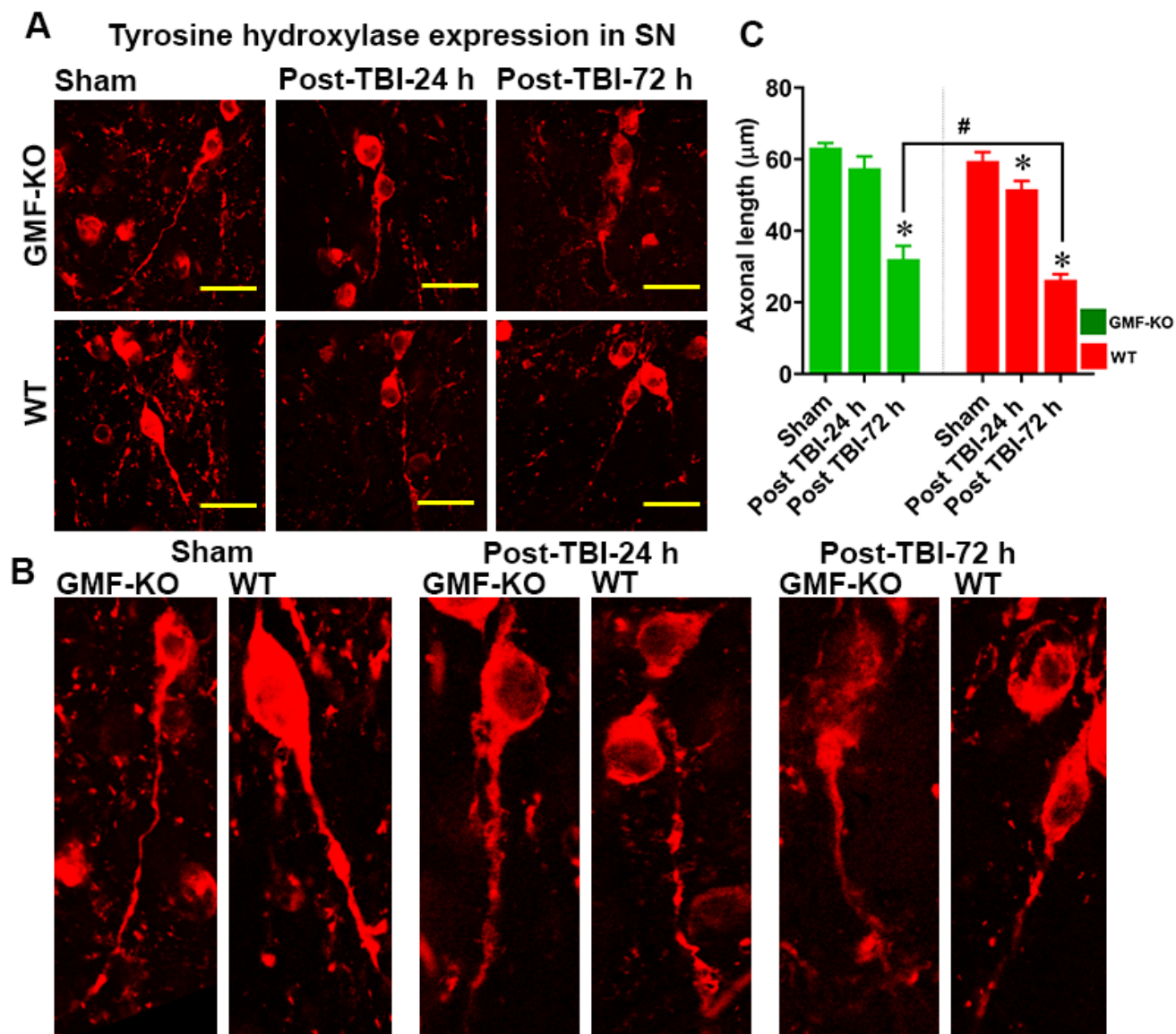
**Fig. 8.** GMF is involved in TBI induced axonal injury in dopaminergic neurons of the midbrain. TBI-induced cortical brain tissue damage causes significant axonal injury of the TH-positive dopaminergic neurons in the SN region of the midbrain of WT and GMF-KO mice as determined by immunofluorescence (A; Insets B). Bar graph shows the measured axonal length of TH-positive dopaminergic neurons in the field (C), as compared to the controls. Values are presented as mean±SEM (n=6). *p<0.05 control vs TBI subjected mice; #p<0.05 GMF-KO TBI subjected mice vs WT TBI subjected mice.

pression in median eminence that may causally be linked to neuroendocrine dysfunction in TBI lesioned mice brain.

Globally, TBI is a form of acquired brain injury that causes death and disability, with variable behavioral impairments in survivors. Earlier reports showed that generally, TBI has been examined and viewed as producing a static neurological insult [79]. However, accumulating evidence suggests that TBI can trigger severe secondary insults, including progressive neurodegeneration that induces dementia, cognitive impairments such as loss of memory,

processing speed problems, and executive dysfunctions [79, 80] as shown in PD [81, 82]. Experimental models of TBI that validate and demonstrate the principal pathological processes associated to PD have been a significant advancement in this field [29, 83]. Furthermore, some TBI survivors experienced cognitive impairments after injury, due to dementia [84]. Previously, it was reported that neuromotor impairments is a common phenomenon in TBI [85]. However, a recent study demonstrates that individuals sustaining a single mTBI suffered long-term cognitive behavioral disability
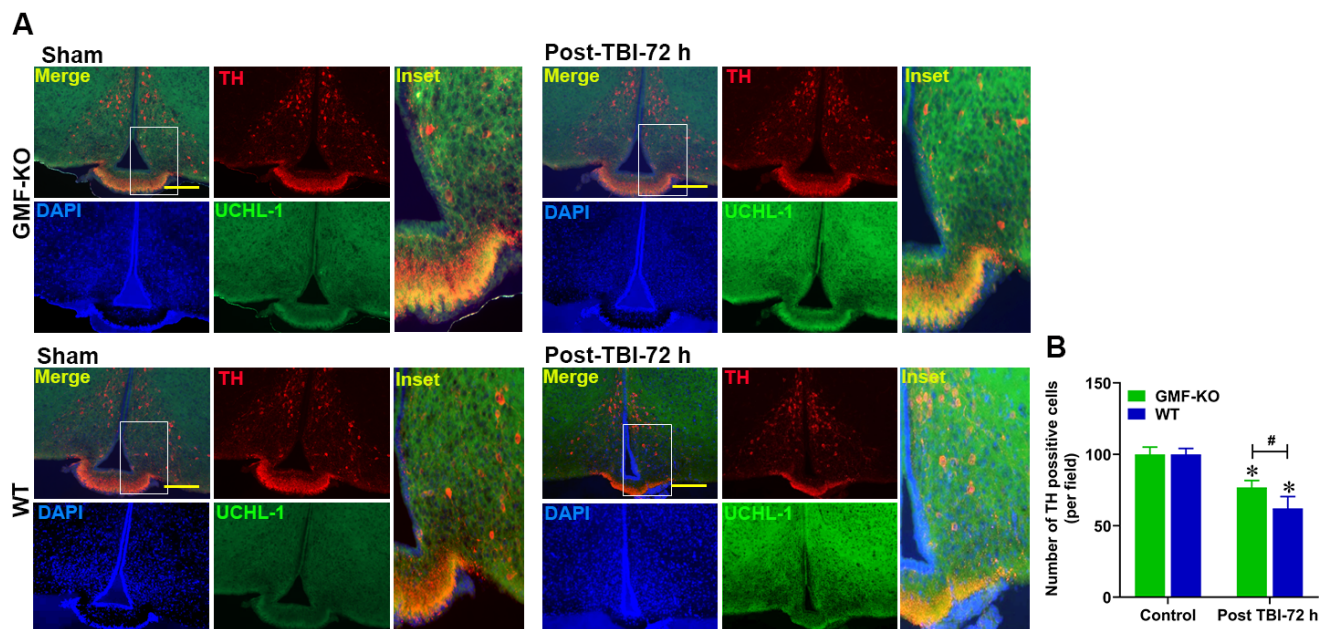
**Fig. 9.** GMF influences the TBI induced UCHL1 and TH positive dopaminergic neurons expressions in the median eminence. Induction of TBI in the cortical region enhances UCHL-1 protein expression and reduces TH-positive tanycytes expression in the median eminence as determined by immunofluorescence. Representative images show a qualitative enhancement of UCHL-1 expression and reduced TH-positive tanycytes in the median eminence after weight drop induced TBI in mice brain at 72 h time point (A). Bar graphs show the number of TH-Positive tanycytes in the median eminence after TBI of WT and GMF-KO mice (B). Scale bar 50 μm. *p<0.05 control vs TBI subjected mice; #p<0.05 GMF-KO TBI subjected mice vs WT TBI subjected mice.

[86] indicating that >50% of the population with mTBI progress to secondary insults that is associated with neurodegeneration and motor complications. Previous reports showed that 94% of people recover after 3 months post-TBI; however, the recovery of independent walking after TBI takes on an average 1.5 months [87]. In a group of severe TBI subjects, about 60% to 65% people were incapable of performing locomotor activities such as hopping, jumping, and running [88]. In mouse models, TBI mice exhibit decreased motor and cognitive behavior outcomes compared with control mice [89-93]. Previously it was reported that the functional recovery rate depends upon the type (mild, moderate and severe), mechanism of injury and the specific region of injury in the brain [94]. However, measures of fine motor control fail to identify deficits with mild TBI, even when participants are tested within the first 10 days postinjury. Furthermore, longer term follow-up of these findings has not been reported, though in adults, motor findings have been identified years after mild TBI [95]. However, substantial spontaneous recovery occurs in the weeks to months following TBI. In addition, the functional and cognitive outcomes during early intensive neurorehabilitation compared to the potential recovery patterns of patients presenting with cognitive motor dissociation (CMD) shows more disorders of consciousness (DOC) and non-DOC, but not the functional outcome [96].

However. understanding how the remaining motor and sensory cordination can support the recovery of such functions has been a primary goal of recent neuroscientific research. Currently we are focusing on the chronic (upto 30 days post-TBI) model of TBI to elucidate the functional recovery of WT and GMF-KO mice that will answer the recovery phase and chronic pathological changes that are associated with and without GMF in mice.

Our present findings show that the absence of GMF significantly prevents TH and DAT expression leading to improvement in motor behavioral performance in mice [60, 61, 97]. The present study shows that balance, coordination and neuro muscular strength are significantly reduced and the increased immobility time may be due to reduced expression of TH and DAT in WT mice subjected to weight drop induced TBI. However, GMF-KO mice subjected to TBI show a significant improvement of these behavioral performances compared with WT mice.

Both UCHL1 and GFAP showed promise as biomarkers after brain injury, especially in TBI. GFAP is an intermediate filament skeletal protein of astrocytes [98] and its levels could be indicative of glial cell injury [99]. Neuronal UCHL1 is involved in either adding or removing ubiquitin from the intracellular proteins and increase in its levels signifies neuronal injury [100-102]. Increased UCH-L1 concentrations and GFAP expression in TBI have been

linked to injury severity and worse outcome after TBI. Increased GFAP expression has been correlated with axonal injury, elevated intracranial pressure, and mortality [78, 103-107] and has outperformed S-100β in detecting intracranial injuries on patients with extracranial injuries [104, 108]. Our earlier findings indicated that the absence of GMF in mice (GMF-KO mice) significantly reduced GFAP expression when compared with WT mice [60, 61, 97]. In the present study, we show that UCHL1 and GFAP expressions were significantly increased in WT and GMF-KO mice subjected to TBI when compared with controls. However, we found that GMF-KO mice showed less expression of these proteins compared with WT TBI mice.

NF-κB is a crucial transcriptional regulator of inflammation that is involved in the cellular processes such as apoptosis, neuronal cell survival and damage. The activation and translocation of NF-κB may persist in TBI and it has been shown that NF-κB plays a key role in the inflammatory processes in neurotrauma [19]. Our data also shows an increased expression of NF-κB after 72 h TBI. Furthermore, midbrain NF-κB activation was accompanied with transcriptional increases in inflammatory markers COX-2 and iNOS. COX-2 production in turn leads to high levels of neurotoxic NO and superoxide radicals. COX-2, a major source to generate prostaglandins, is represented as a potential risk factor for degeneration of dopaminergic neurons. Previous reports showed that increased COX-2 expression was localized to dopaminergic neurons of the SN in the post-mortem brains of PD patients [109]. Here, we hypothesize that activation and translocation of NF-κB in the midbrain could play a crucial role in determining dopaminergic neuronal fate through COX-2 and iNOS. This activation may contribute to the neuroinflammatory processes in the nigrostriatal pathway, which influences the neurodegeneration.

GDNF is an essential factor, that influences the survival of dopaminergic neurons in the SN of midbrain by increasing synaptic excitability [110] and decreasing neuronal loss [111]. BDNF, NT-3, and GDNF increase dopamine uptake by binding to high affinity receptors to stimulate survival and morphological differentiation of nigral dopaminergic neurons [112-115]. Impellizzeri et al. [116] reported a significant reduction of neurotrophic factors expression such as BDNF, GDNF, and NT-3 in the midbrain region after TBI insult. This is the first report where we are showing the involvement of GMF in the association of TBI related pathological progression towards PD- like pathology. We found that reduced expression of TH, DAT, BDNF and GDNF is associated with increased expression of UCHL1, GFAP, p-NF-kB, iNOS and COX2 expresion. In the present study, we found that weight drop induced TBI significantly decreased the expression of BDNF and GDNF compared to control mice. In addition, the absence of GMF in

mice subjected to TBI showed a significant improvement in the expression of these trophic factors , indicating that GMF may be involved in the inhibition of neurotrophic factors expression in the midbrain region. Since, median eminence contains a number of neurondocrine nerve endings [117]; accordingly, endocrine dysfunctions are known consequences of TBI in patients, and endocrine axes remain impaired when assayed up to 5 years after TBI [118, 119]. Previously, it has been reported that dysfunction of neuroendocrine system enhances the outcome of secondary complications after TBI that causes severe neuropsychiatric symptoms and negatively regulates the quality of behavioral outcomes even after recovery from TBI [117, 120]. However, accumulating evidence suggest that dysfunctions of tanycytes in median eminence plays an important role in the etiology of motor dysfunction, impairments in concentration, depression, anxiety, fatigue, and loss of facial expression due to the astroglial activation upon TBI [117, 121]. In this study, we found that WT TBI subjected mice show significantly less number of TH-positive tanycytes in the median eminence when compared with GMF-KO mice. Reduced number of TH-positive tanycytes may reduce the axonal transportation between the median eminence and nigrostriatal pathway leading to motor behavioral impairments in the TBI subjected mice as we have shown in this study.

In conclusion, our present findings render insights into the possible pathophysiological regulatory mechanism of GMF action in the weight drop induced TBI that induces motor deficits in mice. We demonstrate that GMF may serve as the pathological factor amd a link between TBI and the development of PD-like pathology. Our data indicate that TBI induces altered expression of dopaminergic markers such as TH and DAT in neurons, and it may be upregulated by GMF in the SN, providing neurodegenerative pathology in TBI. In addition, upregulated expression of UCHL1 and GFAP indicate the severity of TBI that leads to activation of sequential neuroinflammatory responses via NF-kB mediated iNOS and COX-2 expression. Furthermore, reduction in expression of neurotrophic factors such as BDNF and GDNF leads to neurofilament loss along with UCHL1 expression. Ultimately these changes in the midbrain lead to motor behavioral impairments. The global knockdown of GMF significantly reduced these pathological changes in mice after 72 h TBI. These findings warrant further therapeutic relevance of the aberrant expression of GMF as a potential predictive biomarker for the progressive secondary insults post TBI that is mediated by neuroinflammation and may enhance potential neurodegeneration (with PD-like pathology) in TBI. Furthermore, GMF may serve as a key regulatory factor between the TBI and associated PD symptoms which opens up new therapeutic avenues.

## ACKNOWLEDGEMENTS

## ETHICS STATEMENT

The animal study was reviewed and approved by The University of Missouri animal care facility, and they were used in accordance with the guidelines approved by the IACUC and National Institutes of Health, Unites States of America.

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