

Stimulation of dopamine D2 receptor via quinpirole protects against brain damage after brain injury in mice

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Research

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Abstract

Background Brain injury is a major risk factor for the development of chronic neurodegenerative diseases. The disease still lacks a potential candidate to treat brain injury associated neurodegeneration. In the present study we aim to investigate the expression of Dopamine D2 receptor in the cortical region as well as in striatum of the injured mouse brain and further explore the neuroprotective effects of selective D2R agonist quinpirole against brain injury-associated neuropathological events after brain injury.

Methods In order to test our hypothesis, a normal mouse was subjected to TBI mouse model by producing penetrating injury using a scalpel blade. A dose of 1 mg/kg of quinpirole was daily injected to the TBI mice via intraperitoneally for 7 days after producing injury. Further, immunoblots and immunohistochemistry analysis were performed for both in vivo and in vitro.

Results Our biochemical and immunohistological results demonstrated that brain injury suppresses the expression levels of D2R and deregulates the downstream signaling molecules in the cortex and striatum after TBI at day 7. Treatment with a selective D2R agonist quinpirole regulates GSK3- β /IL-1 β /Akt signaling and reduces neuroinflammation after brain injury. Concomitantly, quinpirole treatment regulated blood-brain barrier breakdown, reduced neuronal apoptosis and regulated synaptic dysfunction after brain injury. This is the first evidence, which showed that quinpirole treatment reduced secondary brain injury-induced neuropathological evidence in the cortex via D2R/Akt/GSK3- β signaling pathway. Moreover, our in vitro results demonstrated that quinpirole reversed MCM-mediated deleterious effects and significantly regulated D2R/GSK3- β /Akt level in HT22 cells.

Conclusion Our results suggest that regulation of Dopamine D2 receptor via quinpirole would be a promising therapeutic strategy against brain injury-induced neurodegeneration.

Introduction

Traumatic brain injury is a global risk factor and the leading cause of neurological disability. Recent reports elucidated that TBI triggers a variety of neurodegenerative diseases such as Alzheimer diseases and Parkinson diseases [1, 2]. Traumatic brain injuries contribute to primary injury followed by secondary brain injury. Primary brain injury is the direct mechanical force, which occurs at the time of impact on the brain. However, secondary brain injury occurs followed by inciting traumatic events leads to brain vessels and blood-brain barrier [3] disruption in the brain, which further causes extremely complex events in the TBI affected brains [4, 5]. Neuroinflammation is the major hallmark after brain injury followed by the activation of astrocytes and microglia and release pro-inflammatory cytokines and chemokines, which impair the brain endogenous ability to self-repair and eventually leads to neuronal apoptosis and neurodegenerative conditions [6] [7, 8]. Several lines of investigation provide evidence brain injuries contribute to inflammatory responses and activation of inflammatory mediators in the brain [9, 10]. Hence, the demands for the treatment of TBI as neuroprotective and neurorestorative agents is still obligatory and offer extensive exploration to treat TBI associated neuroinflammatory response and

inflammatory cytokines. Possibly restoration of blood-brain barrier integrity and neuroinflammation are the key targets to treated brain injury associated pathological events after brain injury.

Dopamine plays a major role in neurotransmission and controlling abnormal neuronal excitotoxicity and leads to dysfunction in the dopaminergic system in the brain [8, 11, 12]. Dopamine D2 receptor, a class of dopamine receptor is an important target for anti-Parkinson drugs that attenuate several neurological deficits [13]. D2R is expressed in several regions of the brain including cerebral cortex, hippocampus, and striatum [14]. A previous report has demonstrated that dopamine receptors are expressed on glial and immune cells containing [15]. Accumulative studies suggest that DA plays an important role in human and animal and the dysfunction of DA in the cortical region leads to attention deficit and hyperactivity disorder [16, 17]. Recently, a study reported that cortical Dopamine D2 receptor is involved in psychotic and mood disorder and regulates neuronal circuit [18]. The deregulation of the DA system could be a major contributing mechanism for behavior and cognitive deficits after TBI. Growing evidence reveals that Dopamine D2 receptor agonist has a protective role in regulating neuroinflammation and immune reaction that might be through inhibiting and the release of cytokines [19]. An earlier study has reported that D2R agonist quinpirole activated D2R has a positive effect on neuronal activity in cingulate cortex and striatum [12, 20]. However, to date, insufficient studies have shown the importance of D2R activation to mitigate neuropathological events in TBI associated neuropathological events.

In the present study, we sought to investigate the expression levels of D2R in the cortical region of brain which is primary target of brain injury and further extended our line of investigation to the striatum region of the injured mouse brains. We further explored the therapeutic potential of the quinpirole hydrochloride (Fig. 1a) as reported previously [21] following brain injury. Simultaneously, treatment of quinpirole at a dose of 1 mg/kg has a potential efficacy to protect neurons against brain injury-induced neurodegeneration and synaptic dysfunction via stimulation of Dopamine D2R receptor in the cortex and striatum of the injured mouse brains. Furthermore, we confirmed that microglia-mediated inflammatory mediators are involved in the D2R deregulation and quinpirole at dose of 20 μ m concentration is sufficient to stimulate D2R and regulates GSK3- β /Akt level in neuronal cell lines. This study provides evidence that treatment of quinpirole in brain-injured mouse stimulated D2R in the cortex and striatum and recovered brain damage via regulation of the GSK3- β /IL-1 β /Akt signaling pathway and inhibited neuroinflammatory response after brain injury.

Materials And Methods

Animals

Male C57BL/6N, 7 weeks of age with 25-30g weight, were obtained from Samtako Bio Korea. The animals were acclimatized in the animal care center at Gyeongsang National University, South Korea. The animals were maintained in the control environment and supply with water and food, 12/12 light cycle at 23°C, 60 \pm 10% humidity. The mice were randomly divided into three groups (n=15) after a week of acclimatization. Next, the animals were carefully handled according to the animal ethics committee

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Stab Wound Cortical Injury

The stab wound cortical brain injury mouse model was established as previously we described with modification [22, 23]. Briefly, the mice were anesthetized with rompun (0.05 mL/100g body weight) and zoletil (0.1 mL/100g body weight) and placed at stereotaxic apparatus, exposed the skull by making a mid-longitudinal incision. The dental drill was used in order to make a circular craniotomy 4mm in diameter (2mm lateral to the midline and 1mm posterior to the bregma) in the skull. For stab wound injury, sharp edge scalpel blade was inserted (3 mm; right hemisphere) in the mouse brain and kept the scalpel blade for 1 min in the mice brain and then removed slowly. The skull was covered with bone wax and tightly closed the mice skull with the silk suture. Next, The animals were placed carefully with providing continuous heating with a heating lamp until fully recovered from anesthesia and proceeded for further experiments.

Quinpirole Treatment for Mice

The treated animals were divided into the following groups

1) Saline treated control group 2) Stab Wound Cortical Injury 3) Stab Wound Cortical Injury + Quinpirole. Quinpirole was administered daily intraperitoneally (i.p) at a dose of 1 mg/kg bodyweight for 7days. For western blot (n = 8) and for confocal experiments (n = 7) mice were used. The chemical quinpirole was purchased from Tocris-Cookson (Bristol, UK).

Protein extraction

After the completion of the mice treatment, all the animals were first anesthetized and then sacrificed carefully. Immediately collected the brain after the surgery and frozen on dry ice. The brain tissue was homogenized using PRO-PREP protein extract solution (iNtRON Biotechnology) to extract protein from tissues followed by centrifugation and stored at -80°C. The samples were centrifuged at 10000×g rpm at 4 °C for 25 min. The supernatants were collected and stored at -80°C for immunoblotting.

Western Blot Analysis

The protein expression level was examined via western blot analysis as previously described [24]. In brief, equal volume of 20–30 µg of proteins (extracted from ipsilateral cortex) was mixed with 2× Sample Buffer (Invitrogen). To separate the proteins, equal volume of the proteins were run on 10% of SDS

polyacrylamide gel electrophoresis and transferred to PVDF membrane followed by blocking in 5% skim milk. The membranes were slightly washed to clear the skim milk. The primary antibody was incubated for overnight at 4°C 1: 1000, anti-Dopamine D2 receptor (D2R), anti-Glycogen synthase kinase 3 (p-GSK3- β) (Ser9), p-AKT (Ser473), anti-Glial fibrillary acidic protein beta (Anti-GFAP), anti-ionized calcium-binding adapter molecule 1 (anti-Iba-1), anti-phospho-c-Jun N-terminal kinase (p-JNK), anti-tumor necrosis factor- α (TNF- α), anti-interleukin-1 β (IL-1 β), anti-caspase-3, anti-poly (ADP-ribose) polymerase-1 (Anti-PARP-1), anti-Bax, anti-Bcl2 and anti- β -actin from Santa Cruz Biotechnology. Anti-beta actin was used as a loading control. Next day, The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies diluted in 1 \times TBST for 1-2h as appropriate, the immunoblots were developed using an ECL chemiluminescence system, according to the manufacturer's instructions (Amersham Pharmacia Biotech, Uppsala, Sweden).

Brain Tissue collection and sample preparation

For brain tissue collection the mice were anesthetized and transcardially perfused with saline followed by (4%) paraformaldehyde and then fixed with (4%) paraformaldehyde for 48 h. Further, the brain tissues were collected in 20% sucrose solution for 48 h and fixed vertically in OCT compound (A.O USA). The brain section was taken and cut cross-section with 14 μ m in size using a vibratome (Leica, Germany) and stored at -80°C.

Assessment of Brain Lesion Volume

For lesion volume assessment the brain tissue slides were selected and stained with cresyl violet. The images of TBI and TBI+quinpirole were taken with simple light microscope and analyzed with imagJ software. The cortical area of the tissue were selected and obtained the lesion volume of the TBI and TBI treated groups.

Immunofluorescence Staining

The tissue slides were proceeded for immunofluorescence staining as described previously with minor modification [25]. Initially, the slides were dried at room temperature and washed twice with PBS 0.01 M solution for 8-10 min. The tissue slides were incubated in proteinase-K (5 minutes) and then washed twice for 5 minutes in PBS solution. The protein was blocked for 1 h with 5% normal serum (goat/rabbit) and 0.1% Triton X-100 in 0.01M PBS solution. The tissue slides were incubated with primary antibodies (1:100) overnight at 4°C. The following antibodies were used for the immunofluorescence detection; anti-

p-GSK3- β (ser9), anti-p-AKT, anti-D2R, anti-IL1- β , anti-Caspas-3, anti-PSD-95, anti-SNAP-23, anti-claudin-5. The tissue slides were then incubated for 2 h in the secondary antibody (1:100) fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanates (TRITC) labeled secondary antibodies (anti-goat, anti-rabbit, and anti-mouse) from Santa Cruz Biotechnology. The 4', 6-diamidino-2-phenylindole (DAPI) was used for nucleus detection (8-10 min). The slides were covered with coverslips using with fluorescent mounting medium. Confocal laser scanning microscopy FluoViewer MPE-1000 (Olympus, Tokyo, Japan) was used to take the images. All the images were converted into Tiff images and measured the fluorescent intensity of the cortex and striatum via ImageJ software.

Nissl staining

To analyze the neuronal cell death and lesion after brain injury, we performed Nissl staining as we performed previously with minor modification [26]. In brief, the slides were washed with 0.01 M PBS two times for 15 min. Furthermore, the cresyl violet solution was used for 10-15 min and then washed the tissue slides with distilled water and rinsed with ethanol (70%, 95%, and 100%), placed in xylene followed by mounting medium and coverslip. The simple light microscope was used to examine the slides and taken images.

Cell culture

The Mouse hippocampal cell line HT22 and Microglial cell line BV2 were grown and maintained in Dulbecco's modified Eagle medium (DMEM) medium (Thermo Fisher Scientific, Waltham, Massachusetts, United States) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA). The final formulation comprises an additional 1% penicillin/streptomycin sulfate (Gibco, Grand Island, NY, USA). Cells were cultured in humidified cell culture incubator equipped with 5% carbon dioxide supply. Cell media was regularly replaced after every 2 days passaged. The cells were subjected to experimental procedures after confirmation of above 80% confluency.

Microglial Conditioned Media

Mouse microglial cell line BV-2 was cultured to above 80% confluency were treated with Lipopolysaccharide (1 μ g/ml) (Sigma-Aldrich, St. Louis, Missouri, United States) dissolved in

Cell culturing media. After 24 hours media was aspirated and centrifuged to remove cells and debris. The clear supernatant was collected for further biochemical analysis.

Statistical Analysis

The western blot bands results were scanned and analyzed by densitometry using sigma gel software (SPSS Inc., Chicago, IL, USA). ImageJ software (National Institutes of Health, Bethesda, MD) was used for immunohistological analysis and the obtained values were calculated as the mean \pm S.E.M. The data analysis was performed by using one-way ANOVA followed by a post-hoc analysis of variance for control and treated groups comparison. The data calculation and graphs were determined by using Prism 5 software (Graph Pad Software, In., San Diego, CA USA). The statistically significance values were considered as $p < 0.05$. * Significantly different between control and brain injury, # significantly different between brain injury and Quinpirole treated group.

Results

Quinpirole regulates D2R in cortex and striatum after brain injury

Brain injuries contribute to disrupt the striatal DA system, which disturbs DA neurotransmission [27]. Studies provide evidence that activation of astrocytic D2R has protective effect after brain injuries [28, 29]. Initially, we assessed the expression levels of D2R and to find out the effect of Quinpirole on D2R level after brain injury. Western blot results indicated the loss of D2R after brain injury, while administration of quinpirole treatment significantly regulated D2R level after brain injury at day 7 (Fig. 2a). Moreover, this effect was validated via confocal microscopy in the cortex and further extended our line of investigation to the striatum region. Consistent with our western blot, our confocal results also showed the lower expression level of D2R as compared to saline-treated mice group. However, quinpirole at a dose of 1 mg/kg significantly upregulated the level of D2R level after brain injury (Fig. 2b, c), suggesting the brain injuries are critically involved in the DA deficiency. These results clearly suggesting that quinpirole regulates the deregulated levels of D2R in the cortex and striatum region after brain injury that might be associated with synaptic deregulations.

Quinpirole reduced the activated level of astrocytes and microglia after brain injury

We further extended sought to investigate whether quinpirole treatment inhibits neuroinflammatory response in our mouse model of brain injury as D2R is expressed on glial cells. Studies reported that brain injuries contribute to astrocytes and microglia activation, which further cause deleterious effects through activation of inflammatory mediators in the brain [30, 31]. In order to detect the number of activated astrocytes and microglia in the injured mouse brain, we examined the expression of Iba-1 and GFAP through western blot analysis. Interestingly, the result indicated the higher level of Iba-1 and GFAP indicated the number of activated microglia and astrocytes in brain-injured mice as compared to the saline-treated mice group. (Fig. 3a). On the other hand, the activated glial cells were significantly reduced

in the mice brain which received quinpirole treatment. Furthermore, our immunofluorescence results clearly showed the higher expression level of astrocytes and microglial cells in injured mice brains as indicated by active GFAP and Iba-1. We observed that quinpirole treatment significantly condensed GFAP and Iba-1 immunoreactivity after brain injury (Fig. 3b, 3c). Our results clearly demonstrated that treatment of quinpirole has the potential to ameliorate brain injury-induced activated gliosis and other inflammatory mediators. This condensation may be associated with astrocytes and microglial D2R modulation with quinpirole.

Quinpirole attenuates GSK3- β /IL1- β /Akt signaling pathway after brain injury

Gliosis plays an important role in the release of pro-inflammatory cytokines such as IL-1 β and TNF- α and elevated in neurodegenerative diseases [32] [33, 34]. Dysregulation of GSK3 β is critically involved in the development and progression of neurodegenerative disease via the neuroinflammatory processes. [35]. While accumulative evidence has been demonstrated that regulation of Akt and GSK3- β attenuates neurodegeneration and neuroinflammation [36]. Consequently, for the expression level of GSK3 β /IL1- β /p-Akt, we performed a western blot analysis. Our result indicated the abnormal expression levels of p-GSK3- β phosphorylation at (Ser 9), increase expression in IL-1 β and reduced phosphorylation of p-Akt at (Ser 473) in the ipsilateral cortical region in the injured mouse brains. On the other hand, quinpirole treatment significantly regulated GSK3 β /IL1- β /Akt levels in mice brain that received injury plus Quinpirole. (Fig. 4a). Additionally, the results were validated via confocal microscopy analysis. We first examined the expression levels of p-Akt and IL1- β in the mouse cortices that received a brain injury and then further extended our observation to the striatum of the mice brain. Our results clearly demonstrated that p-GSK3 β (Ser 9) and IL1- β were significantly co-localized in the striatum region of the brain-injured mice. On the other hand, quinpirole treatment significantly regulated the level of p-GSK3 β (Ser 9), IL1- β and p-AKT signaling pathway following brain injury in cortex and striatum of the mice brain. (Fig. 4b-d). Overall, these results provide evidence that treatment of quinpirole is protective against neurodegenerative conditions via regulation of GSK3- β /IL1- β /Akt signaling pathway possibly via activation of the D2R following brain injury.

Quinpirole regulated D2R/p-Akt and p-Gsk3- β level in HT22 cells

We further extend our line of investigation and asked whether activated gliosis could affect the expression of D2R and the downstream signaling molecules in neuronal cells. We, therefore, subjected the MCM to the HT22 cell line. The cells were collected from 24 h post MCM treatment. We co-treated HT22 cells with two different concentrations of quinpirole (10 μ M and 20 μ M). After western blot analysis, the results revealed that MCM mediated inflammatory mediators are associated with neuronal D2R deterioration and the downstream signaling molecules such as GSK3- β and the neuronal cell survival proteins Akt. Of the two different concentration 20 μ M that administration of 20 μ M reduced the toxic effect of MCM and significantly regulated D2R, which might be associated with the regulatory activity of the p-Akt and p-GSK3- β . (Fig. 5a). Additionally, the protective effect of quinpirole was validated by confocal microscopy (Fig. 5b). Co-localization of Akt and D2R result revealed that Akt and D2R

expression was significantly lowered in MCM subjected HT22 cell line. On the other hand, 20uM concentration of quinpirole was sufficient to activate D2R and the significantly increased Akt expression in HT22 cell lines. From this observation, we concluded that gliosis in the central nervous system might be associated with neuronal cell death and synaptic deregulation via affecting the level of neuronal D2R after brain injury.

Quinpirole reduced the lesion volume and restored the BBB disruption after brain injury

Previous literature has shown that traumatic brain injury contributes to sever BBB disruption, which further causes severity after brain injury [3]. We examined the BBB break down and the possible effects of quinpirole to restore BBB breakdown in our mouse model of brain injury. Our confocal microscopy of claudin-5 indicated the decreased expression level of claudin-5 in the ipsilateral cortex of the injured mice brains, while treatment with quinpirole reversed the depressed level of claudin-5 at day 7 post brain injury (Fig. 6a). It is well known that brain injury immediately causes gross tissue disruption at the site of injury. Therefore, we assessed the lesion volume at day 7. Our histological results clearly demonstrated that there was a marked increase in the lesion volume, which was significantly reversed by treatment with quinpirole (Fig. 6b). Furthermore, we observed the increase in contusion volume in brain injury mouse on day 3. It was interesting to observe that contusion volume upon treatment of quinpirole was markedly reversed in brain injury plus quinpirole treated group (Fig. 6c).

Quinpirole reduced neuronal apoptosis after brain injury

Many studies have reported the neuronal apoptosis after brain injury that occurred particularly in the perilesional area and striatum in mice brain [37, 38]. Hence, we analyzed the apoptotic markers in the ipsilateral cortex including P-JNK, Bax, Bcl-2, and PARP1 using western blot analysis (Fig. 7a). The result indicated that there was a marked increase in neuronal apoptosis in the brain-injured mice as compared to saline-treated mice. It was interesting to observe that the treatment of quinpirole significantly reduced the elevated level of P-JNK and apoptotic markers in the ipsilateral cortex of brain-injured groups as compared to alone brain-injured mice group. Moreover, the results were further validated through confocal microscopy. The immunofluorescence images indicated the increased expression level of caspase-3 in the cortex, which was expended to the striatum after brain injury. Conversely, quinpirole significantly reduced the higher expression level of caspase-3 in the cortices and striatum region in the brain injury mice group (Fig. 7b &c). Histologically, the effect of quinpirole was examined using nissl staining. To asses neuronal cell death nissl staining was performed. Our results indicated the reduced number of survival neurons of the cortex of inured mice brain, compared to normal mice brain. Likewise, treatment with quinpirole reversed this effect and significantly increase the number of survival neurons in the cortex of injured plus quin treated mice group (Fig. 7d). All these results suggested that effects of brain injury extend to the striatum region of the brain and quinpirole treatment inhibited neuronal apoptosis possibly via D2R activation the injured mouse brains.

Quinpirole attenuated synaptic dysfunction after brain injury

Previous studies reported that brain injury causes synaptic protein loss in the brain, which further leads to memory impairment [39–41]. Consequently, we examined the expression levels of synaptic proteins including SNAP-23 and PSD-95 in our mouse model of brain injury. Our western blot result indicated the decrease in synaptic protein loss in the cortical region of injured mouse brains as compared to saline-treated mice. On the other hand, treatment with quinpirole significantly restored the synaptic protein loss in the mice that receive brain injury plus quinpirole (Fig. 8a). Moreover, we performed confocal microscopy for PSD-95 in cortex and SNAP-23 in the striatum region respectively. The result indicated that brain injury remarkably decreases the synaptic protein while treatment with quinpirole significantly regulated the synaptic protein loss in the injured mouse brains (Fig. 8b & c). We determined that quinpirole have the therapeutic potential to regulate synaptic dysfunction after brain injury and it might have the ability to improve the cognitive performance of mice following brain injury.

Discussion

Brain injuries lack effect therapeutic because of the multifactorial process in the brain. Brain injuries lead to cognitive dysfunction can be prevented by targeting an important clinical option of DA-therapies [42, 43]. In the present study, we unveiled the critical role of D2R agonist quinpirole against brain injury-induced neuroinflammation, neurodegeneration, synaptic dysfunction following brain injury. The earlier study was reported that D2R^{-/-} mice increased microglia activation and inflammatory response in Parkinson disease [44]. The main objectives of the current study were to determine the neuroprotective effect of quinpirole, a specific D2R agonist in ipsilateral cortices and striatum of the injured mouse brains. This mechanistic report provides the first evidence that quinpirole attenuates several neuropathological events such as glial cell activation, neuroinflammation, neuronal apoptosis, and synaptic dysfunction via D2R/Gsk3 β /IL-1 β /Akt signaling after brain injury. This study was designed to analyze the therapeutic potential of D2R activation in cerebral cortex region as well as the striatum region of injured mice brain. Therefore, these finding correlates cortex and striatum and recommends that quinpirole activate D2R that play a key role in several neuropathological events after brain injury.

Brain injuries lead to neuroinflammation that further contributes to severe neurodegeneration in the brain. Studies reported chronic neuroinflammatory responses in the cortex and hippocampus of the injured mouse brains [9, 45]. In our mouse model of brain injury, we observed an increase in neuroinflammation as indicated by activated microglia and astrocytes in ipsilateral cortical and striatum region. Interestingly, quinpirole significantly reduced the level of inflammatory marker in the above-mentioned regions. Our results are consistent with the previous reports [46]. It is known that the regulation of p-GSK3- β via p-Akt is involved in cell survival pathway [47, 48]. The regulation of GSK3- β and Akt via D2R could be a novel therapeutics. In the present study, we evaluated the level of D2R/p-GSK3 β /p-Akt proteins that were regulated in injured mouse brain under the administration of quinpirole. Similarly, IL-1 β is also responsible to attenuate Akt and GSK3 β signaling [49, 50]. In this study, a confocal microscopy colocalization result of p-GSK3 β and IL-1 β revealed the increased expression level of p-GSK3- β and IL-1 β in injured mice brain. However, the level was normalized to the base level in the mice brain which received of quinpirole treatment. The previous study also reported the anti-neuroinflammatory effect of quinpirole

via D2R activation in ICH injury [29]. The blood-brain barrier (BBB) is a membrane, which has a central role in brain homeostasis. While disrupted BBB leads to enhance cytokines infiltration and neuronal susceptibility. Several tight junction proteins are important for the maintenance of BBB integrity, including claudin, occludin and zonula occludin (ZO-1) [51] [22, 52]. BBB breakdown in brain injuries is due to marked histopathological brain damage and tissue loss in the affected area [53]. In the current study, immunofluorescence reactivity was performed for claudin-5 and the result indicated that the claudin-5 level was markedly lowered in the ipsilateral cortical region of the mice brain that received a brain injury. Importantly quinpirole treatment restored claudin-5 level in the cortical region of the injury-affected mouse brains. The BBB breakdown restoration via quinpirole might be due to reduction of neuroinflammation and active gliosis in the brain. The previous study also observed that brain injury is critically associated with deregulated tight junction proteins [54]. Due to mark tissue disruption after brain injury [41], the Nissl stained cortical section of the injured brains indicated that there was a significant increase in lesion volume while quinpirole treatment significantly restored the lesion volume suggesting that quinpirole is important to repair the brain after injury and restore the tight junction proteins to inhibit cytokines infiltration and other blood-born biochemical agents. Next, a significant increase in the contusion volume was observed following brain, indicating the severe injury associated tissue disruption in the cortical region of the injured mouse brains. All these effects were ameliorated in the mice that received injury plus quinpirole treatment.

Increasing evidence revealed the occurrence of neuronal apoptosis after brain injury [55]. Our results indicated the increase expression neuronal apoptotic markers including Bax, PARP1 and decrease the expression of Bcl-2 an anti-apoptotic protein. Administration of quinpirole significantly reduced the levels of the apoptotic markers in the cortex and striatum of the mice brain. Further study supported the notion that D2/D3 receptor agonist quinpirole protects against apoptosis induced neurodegeneration via JNK dependent mechanism [56]. Our results are consistent with the previous that D2R agonist reduced neuronal cells apoptosis [57]. Further study provides evidence that D2R agonist reduced oxidative stress, neuroinflammation, and apoptosis in endothelial cells [58]. In order to know the dead and damaged neuron and morphology of the brain tissue, we performed the morphological analysis after brain injury. The Nissl stained section of the ipsilateral cortex result indicates the decreased level of survival neurons; however, quinpirole reversed this toxic effect and increased the survival neuron after brain injury. Previous study demonstrated that activation of D2R via agonist activates neuroprotective signaling pathways and enhance cell survival [57].

Synaptic protein loss is associated with brain injury and leads to the cognitive deficit and neurotransmission impairment in the cortex, hippocampus, and striatum [59, 60]. Therefore, we further examined the synaptic protein markers after brain injury. Our western blot and the immunofluorescent result revealed the decreased expression level of synaptic markers including PSD-95 and SNAP-23 in the cortex and striatum of brain injury mice as compared to control and treated. D2R that control synaptic transmission and are important for several brain functions, such as learning and working memory [61]. Another study provides evidence that activation of D1R/D5R protects against Amyloid- β oligomers-mediated synapse dysfunction [62]. These results suggested that brain injuries contribute to D2R

suppression which further activates deleterious signaling pathway in later stage after brain injuries and treatment of quinpirole may have a neuroprotective effect via stimulation of D2R in injured brains.

Conclusion

This study demonstrates that D2R is critically involved in the neurodegenerative conditions in the cortical region of the brain after brain injury and regulation of D2R might be a therapeutic strategy to attenuate brain injury-associated neuropathological events. Herein, we unveiled the critical role of D2R agonist quinpirole having the potential to attenuate several neuropathological processes via D2R/GSK3- β /IL-1 β /p-Akt signaling in the injured mouse brains (Fig. 9). Further study is required to elucidate the molecular mechanism of neuroprotective effects of quinpirole via stimulation of D2R receptor in brain injury associated neuropathological events.

Abbreviations

IL-1 β : Interleukin-1 β ; TRITC: Tetramethyl rhodamine isothiocyanate; CNS: Central nervous system; DAPI: 4', 6'-Diamidino-2-phenylindole; 2'7'-Dichlorodihydrofluorescein diacetate; PARP-1: Poly polymerase-1; P-JNK: Phospho-C-jun N-terminal Kinase; FITC: Fluorescein isothiocyanate; PSD-95: Post-synaptic density-95; SNAP23: Synaptosomal-associated protein-23; SD: Standard deviation; MCM: Microglial Conditioned Media; i.p: Intraperitoneally; Iba-1: Ionized calcium-binding adapter molecule 1; GFAP: Glial fibrillary acidic protein ; DMEM: Dulbecco's modified eagle medium; FBS: Fetal bovine serum; D2R: Dopamine D2 Receptor; PBS: Phosphate buffer saline.

Declarations

Consent for publication

NA

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Acknowledgments

NA

Conflicts of Interest

The authors declare no conflict of interest.

Availability of data and materials

The authors hereby state that the datasets generated in this study will be available upon reasonable request to the corresponding author.

Authors' contributions

SIA designed, managed the experimental work, and wrote the manuscript. MGJ performed nissl staining. MWK analyzed the data. NBA performed in vitro experiment. MOK is a corresponding author, reviewed and approved the manuscript, and holds all the responsibilities related to this manuscript.

Ethics approval

All the experiments with animal and other experimental protocols and procedures were approved (Approval ID: 125) by the Ethics Review Committee of the Gyeongsang National University, Republic of Korea

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Figures

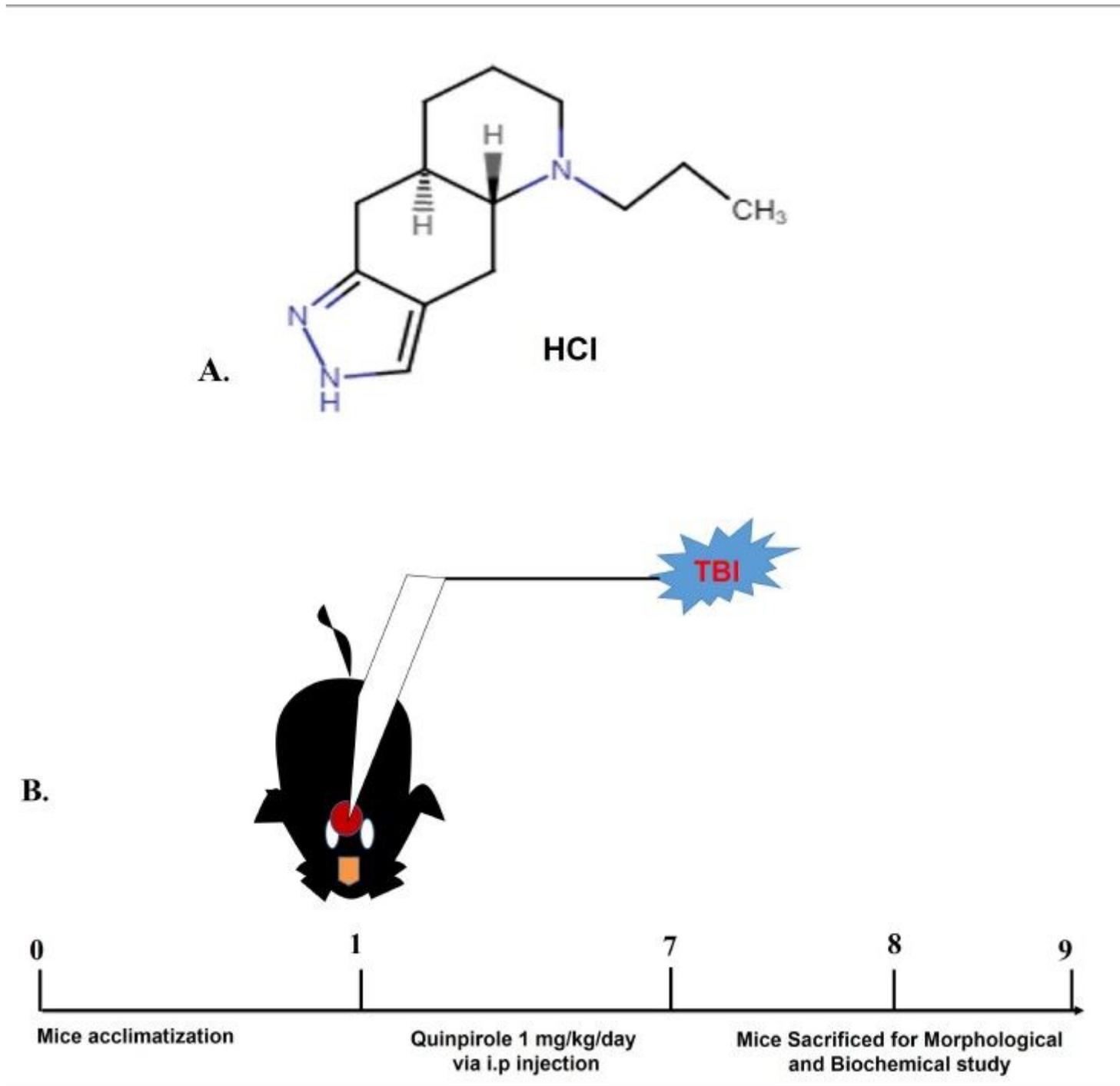


Figure 1

1 Chemical structure of Quinpirole hydrochloride. (b) Schematic representation of the brain injury procedure and treatment schedule in mice.

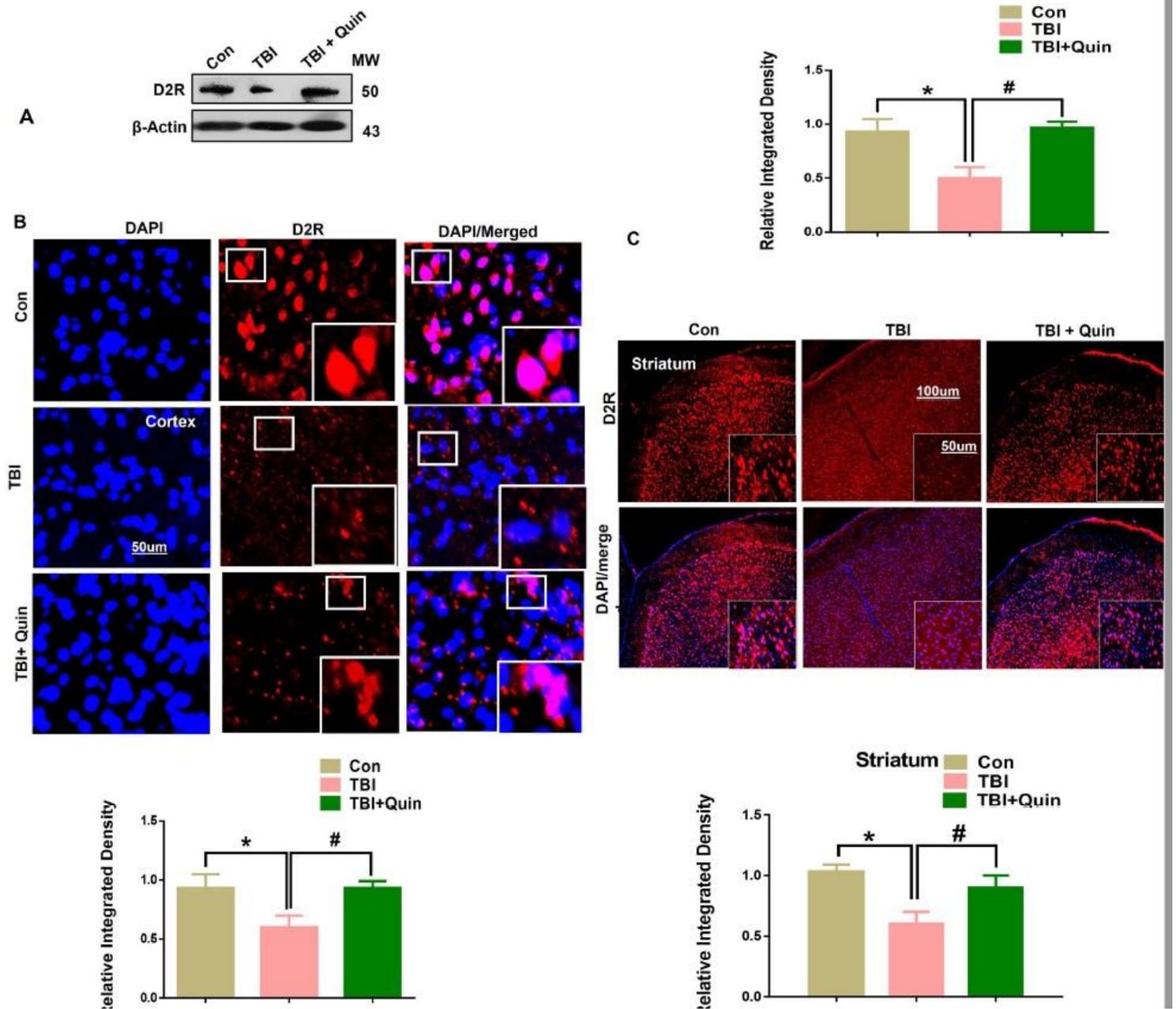


Figure 2

Quinpirole activates D2R level after brain injury. (a) Representatives are the western blot and histogram analysis in the mice brain. β -Actin was used as a loading control. Western blot bands were quantified via SigmaGel software (b and c) Shown are the immunofluorescence reactivity of D2R in the cortex and striatum of control, brain injury and brain injury plus quinpirole treated mice group. Magnification 10 \times . n = 5. Data were calculated from three independent experiments. Quantitative analysis of the confocal images were done through ImageJ software. Values are the Mean \pm SEM. One-way ANOVA followed by post-hoc analysis. The statistically significance values were considered as p < 0.05. * Significantly

different between control and brain injury, # significantly different between brain injury and Quinpirole treated group.

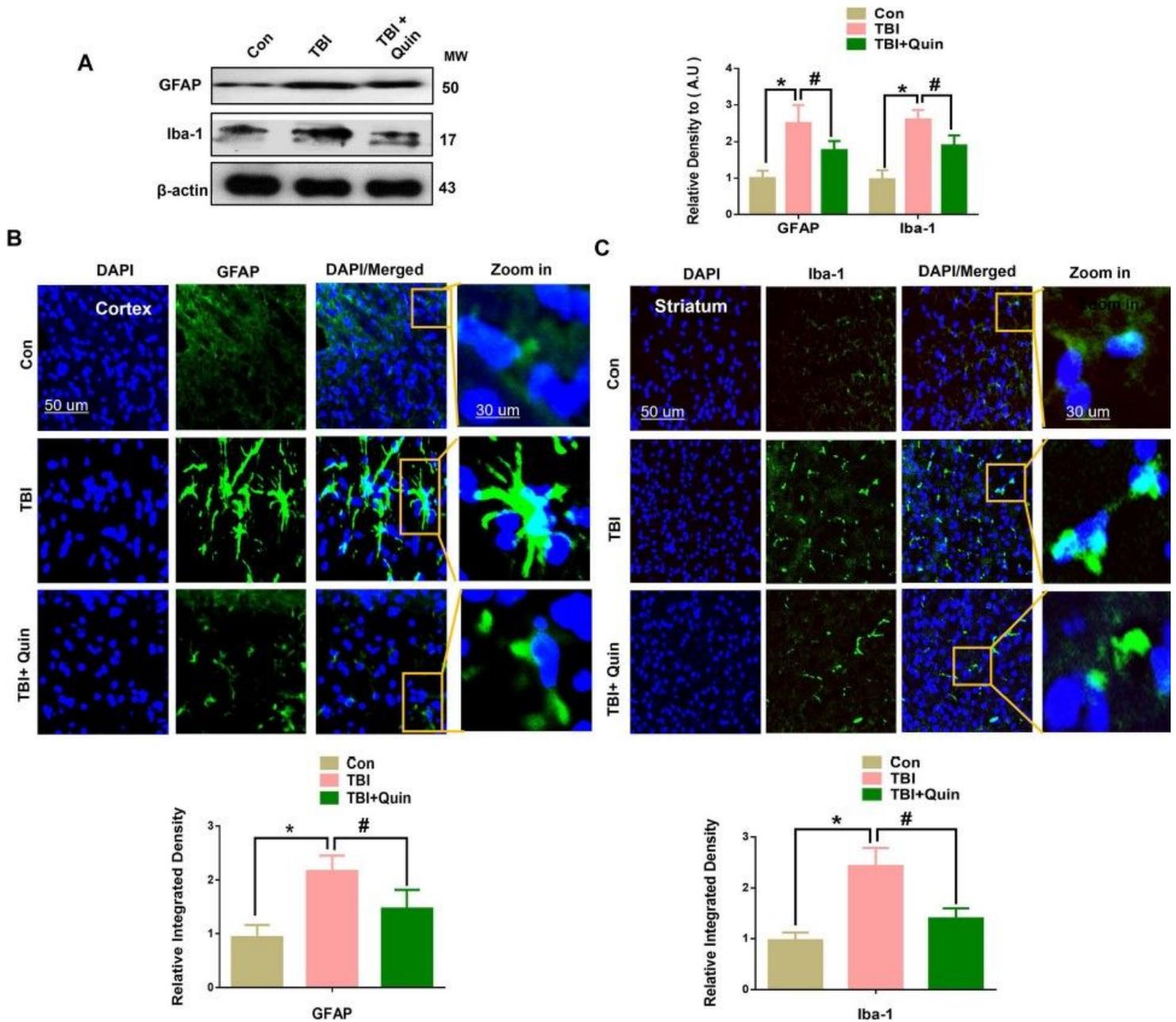


Figure 3

Quinpirole reduces the activation of astrocytes and microglia after brain injury. (a) Representatives are the western blot analysis and histogram for expression level of GFAP and Iba-1 in brain injury and brain injury plus quinpirole treated group. β-actin was used as loading control. (b and c) Confocal microscopy images of GFAP and Iba-1 in different experimental group of brain injury mice. For immunohistological analysis the ImageJ software was used. Magnification 10×. n = 5. Data were calculated from three independent experiments. Quantitative analysis of the confocal images were done through ImageJ software. Values are the Mean ± SEM. One-way ANOVA followed by post-hoc analysis. The statistically

significance values were considered as $p < 0.05$. * Significantly different between control and brain injury, # significantly different between brain injury and Quinpirole treated group.

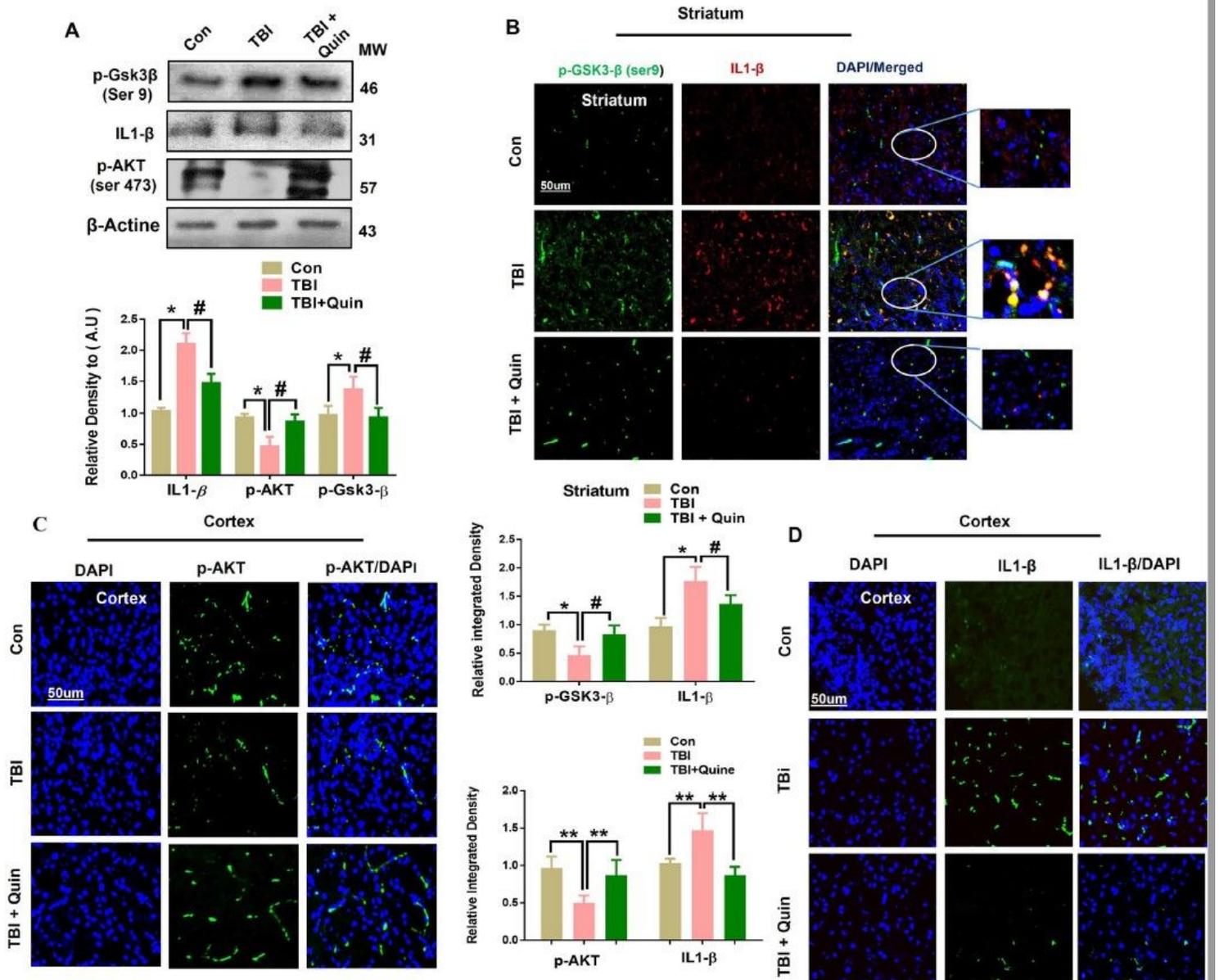


Figure 4

Quinpirole treatment reduced neuroinflammation via p-Akt /p-GSK3- β signaling after brain injury. (a) western blot analysis for p-Akt, p-GSK3- β , and IL1- β in mice brain. (b and c) showing are the double immunofluorescence of p-GSK3- β (ser9) (FITC-label, green) and IL1- β (TRITC-label, red) in the striatum region and Immunofluorescence reactivity of p-Akt (ser9) in cortex after brain injury. (d) Images of confocal microscopy for IL1- β in cortex. Magnification 10 \times . n = 5. Data were calculated from three independent experiments. Quantitative analysis of the confocal images were done through ImageJ software. Values are the Mean \pm SEM. One-way ANOVA followed by post-hoc analysis. The statistically significance values were considered as $p < 0.05$. * Significantly different between control and brain injury, # significantly different between brain injury and Quinpirole treated group.

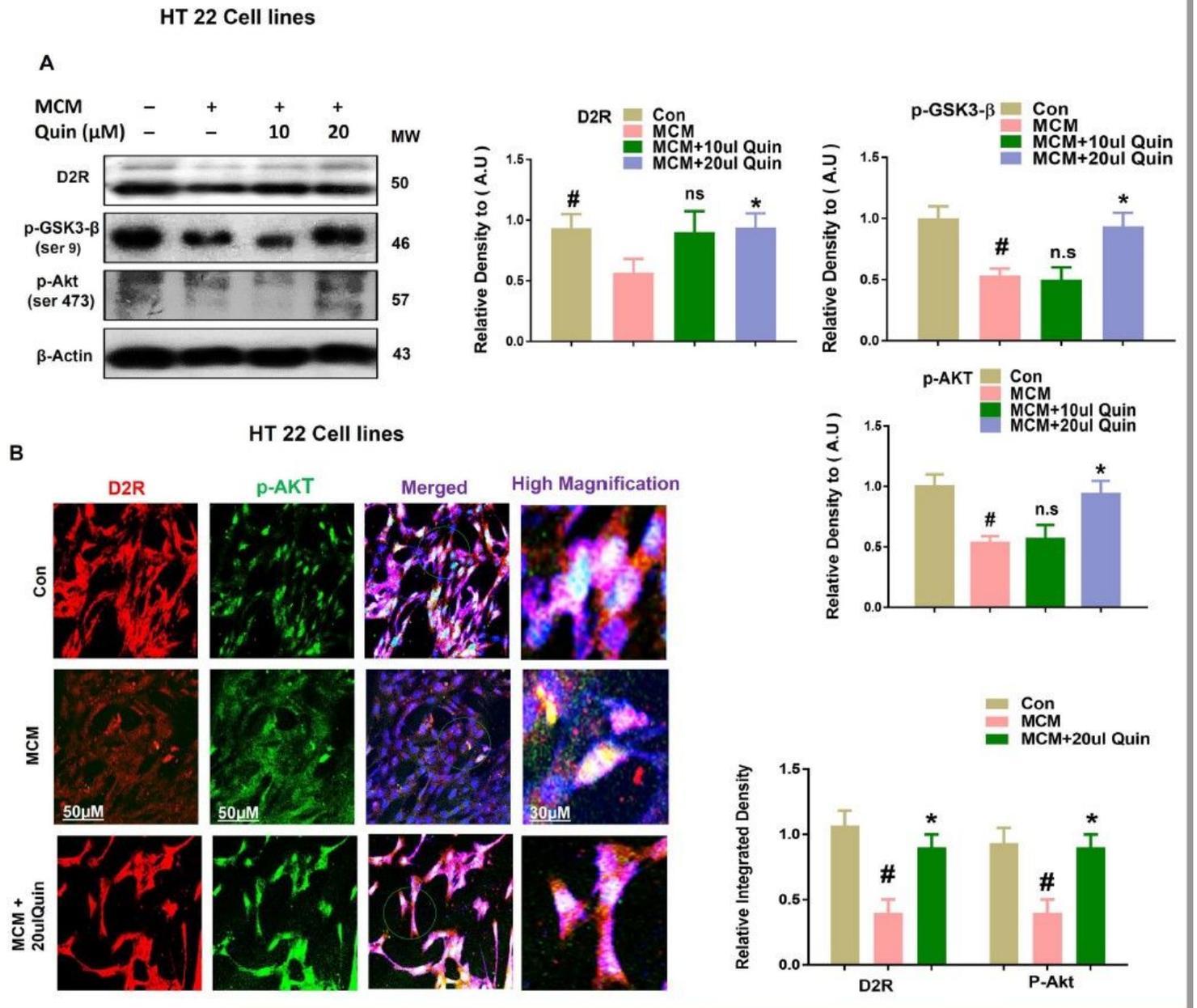


Figure 5

Quinpirole regulated the BBB related Claudine-5 level and lesion volume after brain injury. (a) Shown are representative confocal microscopy for claudin-5, an immunofluorescence reactivity in the injury site of mice brain. (b) Nissle stained images showed the lesion volume of brain injury and brain injury plus quinpirole. (c) Lesion of brain injury and brain injury plus Quinpirol treated groups after brain surgery. Magnification 10 \times . n = 5. Data were calculated from three independent experiments. Quantitative analysis of the confocal images were done through ImageJ software. Values are the Mean \pm SEM. One-way ANOVA followed by post-hoc analysis. The statistical significance was considered as $p < 0.05$. * Significantly different between control and brain injury, # significantly different between brain injury and Quinpirole treated group.

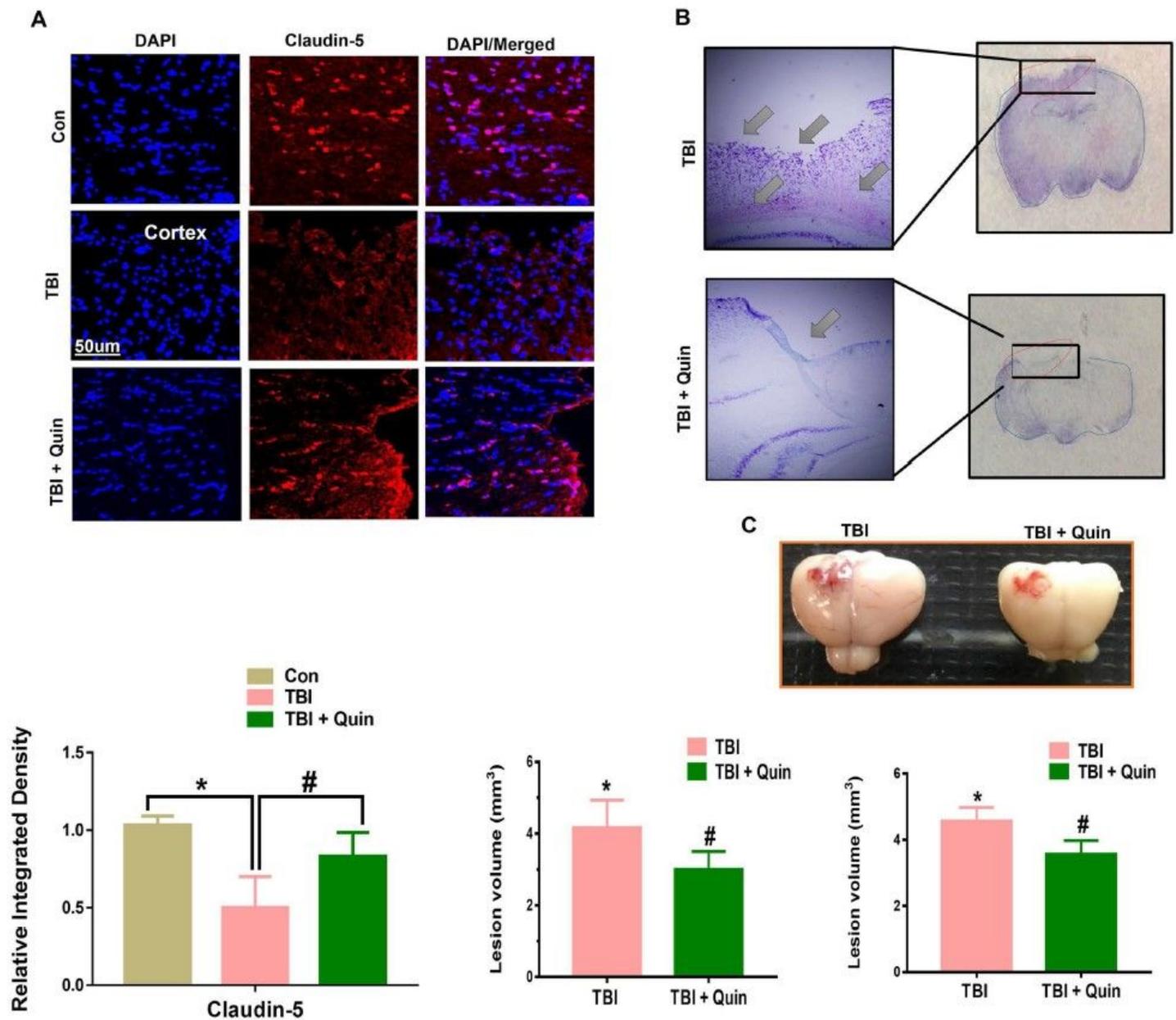


Figure 6

Quinpirole regulated the BBB related Claudine-5 level and lesion volume after brain injury. (a) Shown are representative confocal microscopy for claudin-5, an immunofluorescence reactivity in the injury site of mice brain. (b) Nissle stained images showed the lesion volume of brain injury and brain injury plus quinpirole. (c) Lesion of brain injury and brain injury plus Quinpirol treated groups after brain surgery. Magnification 10 \times . n = 5. Data were calculated from three independent experiments. Quantitative analysis of the confocal images were done through ImageJ software. Values are the Mean \pm SEM. One-way ANOVA followed by post-hoc analysis. The statistical significance was considered as $p < 0.05$. * Significantly different between control and brain injury, # significantly different between brain injury and Quinpirole treated group.

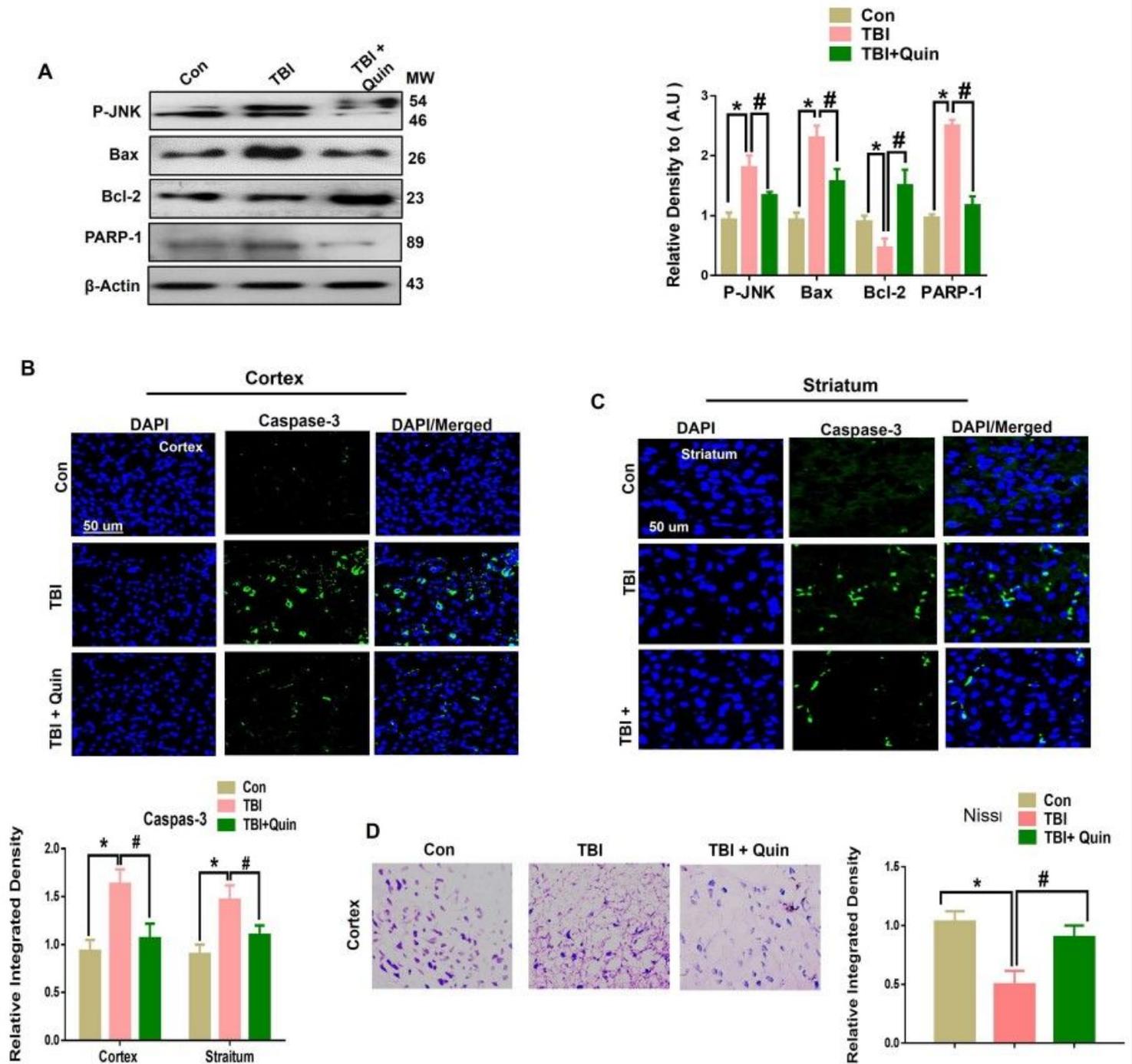


Figure 7

Quinpirole inhibited brain injury-induced neuronal apoptosis in mice brain. (a) Representatives are the immunoblots and histogram analysis of Bax, Bcl-2 and PARP-1 result in mice brain. (b and c) Showing are the immunofluorescence reactivity for cl-caspase-3 in cortex and striatum. ImageJ software was used for immunohistological analysis. (d) immunohistochemistry for histological analysis. Magnification 10 \times . n = 5. Data were calculated from three independent experiments. Quantitative analysis of the confocal images were done through ImageJ software. Values are the Mean \pm SEM. One-way ANOVA followed by post-hoc analysis. The statistically significance values were considered as p < 0.05. * Significantly

different between control and brain injury, # significantly different between brain injury and Quinpirole treated group.

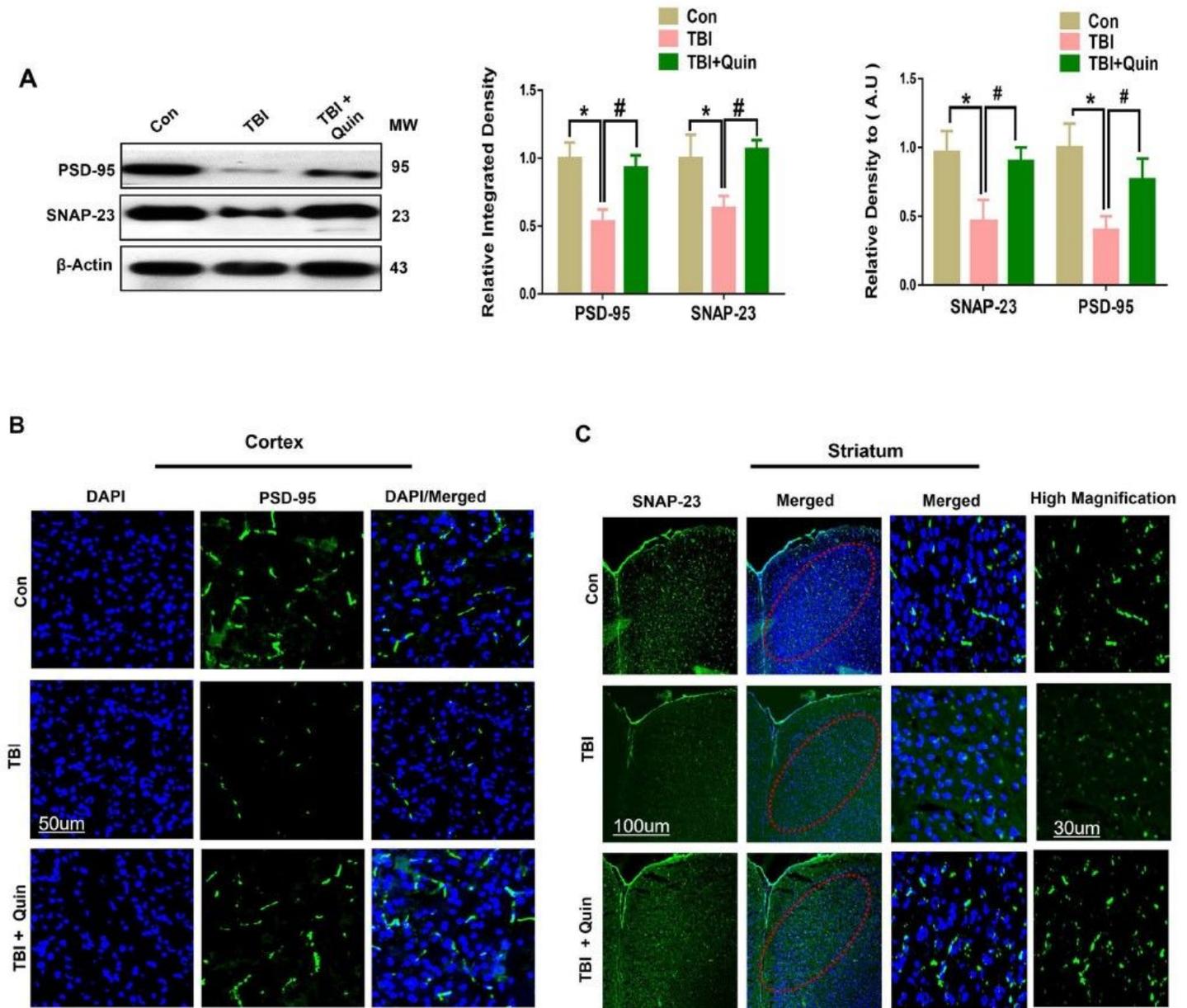


Figure 8

Quinpirole re-established synaptic protein loss after brain injury. (a) Western blot and histogram analysis for PSD-95 and snap-23 in mice brain. β -Actin was used as a loading control. The level of protein bands was quantified by using sigma gel. (b, c) Confocal microscopy was applied for SNAP-23 in striatum while PSD-95 was examined in cortices of mice brain. $n = 5$. Data were calculated from three independent experiments. Values are the Mean \pm SEM. One-way ANOVA followed by post-hoc analysis. The statistically significance values were considered as $p < 0.05$. * Significantly different between control and brain injury, # significantly different between brain injury and Quinpirole treated group.

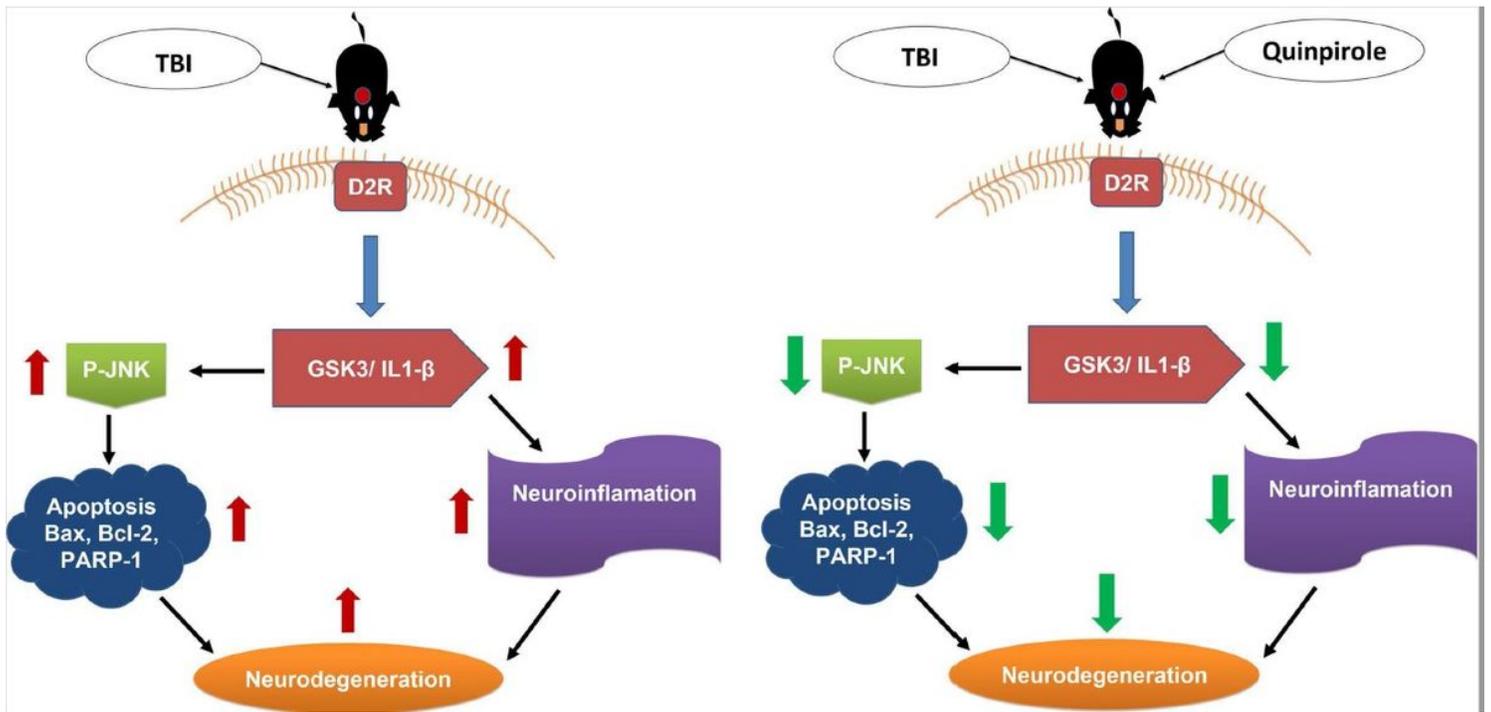


Figure 9

Schematic representation of the proposed mechanism of neuroprotection of Quinpirol against brain injury-induced neuroinflammation and neurodegeneration via D2R/ GSK3-β/ IL-1β/p-Akt signaling in the injured mouse brains.