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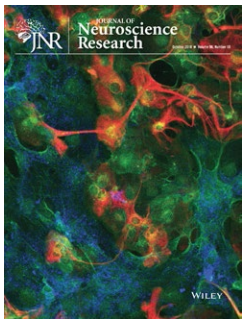
RESEARCH ARTICLE



Modification of kynurenine pathway via inhibition of kynurenine hydroxylase attenuates surgical brain injury complications in a male rat model

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Abstract

Neurosurgical procedures result in surgically induced brain injury (SBI) that causes postoperative complications including brain edema and neuronal apoptosis in the surrounding brain tissue. SBI leads to the release of cytokines that indirectly cause the stimulation of kynurenine 3-monooxygenase (KMO) and the release of neurotoxic quinolinic acid (QUIN). This study tested a KMO inhibitor, RO 61-8048, to prevent postoperative brain edema and consequent neuronal apoptosis in an in vivo model of SBI. A rodent model of SBI was utilized which involves partial resection of the right frontal lobe. A total of 127 Sprague-Dawley male rats (weight 275–325 g) were randomly divided into the following groups: Sham surgical group, SBI, SBI + DMSO, SBI + RO 61-8048 (10 mg/kg), SBI + RO 61-8048 (40 mg/kg), and SBI + RO 61-8048 (40 mg/kg) + KAT II inhibitor PF-04859989 (5 mg/kg). RO 61-8048 was administered by intraperitoneal injection after SBI. Postoperative assessment at different time points included brain water content (brain edema), neurological scoring, and western blot. SBI increased brain water content (ipsilateral frontal lobe), decreased neurological function, and increased apoptotic markers compared with sham animals. Treatment with RO 61-8048 (40 mg/kg) reduced brain water content and improved long-term neurological function after SBI. RO 61-8048 increased the expression of kynurenic acid while reducing QUIN and apoptotic markers in the surrounding brain tissue after SBI. These neuroprotective effects were reversed by PF-04859989. This study suggests KMO inhibition via RO 61-8048 as a potential postoperative therapy following neurosurgical procedures.

KEYWORDS

apoptosis, brain edema, kynurenic acid, kynurenine 3-monooxygenase (KMO), quinolinic acid, RRID:AB_2064155, RRID:AB_2714189, RRID:AB_725631, RRID:AB_725655, RRID:MGI:5651135, RRID:SCR_002798, RRID:SCR_016137, surgical brain injury

1 | INTRODUCTION

Lifesaving neurosurgical procedures often lead to unavoidable damage to neighboring, non-diseased tissue of the brain. Surgical brain injury (SBI) that is attributable exclusively to the neurosurgical procedure itself may cause postoperative complications such as brain edema, ischemia, cell death, and intracranial hematoma worsening neurological and behavioral outcomes (Manninen, Raman, Boyle, & el-Beheiry, 1999; Solaroglu et al., 2004). Although endoscopic and stereotactic brain procedures are designed to minimize tissue invasiveness, they are not free of inevitable brain injuries and complications that include brain edema and neuronal apoptosis

Abbreviations: IDO, indoleamine 2,3-dioxygenase; KAT II, kynurenine-oxoglutarate transaminase; KMO, kynurenine 3-monooxygenase or kynurenine hydroxylase; KYNA, kynurenic acid; NMDA, *N*-methyl-D-aspartate; QUIN, quinolinic acid; SBI, surgically-induced brain Injury.

Significance

Surgical brain injury (SBI) is attributable to neurosurgical procedures which may cause postoperative complications that exacerbate neurological function. While the importance of this medical problem is fully acknowledged, to date, the issue of perioperative neuroprotection specifically against SBI has not been well studied. Using a male rat model of SBI, we explored a new neuroprotective strategy targeting the kynurenine pathway. Quinolinic acid is a downstream neurotoxic target of kynurenine and product of kynurenine hydroxylase. Attenuating brain edema and apoptosis in surrounding brain tissue by selective kynurenine hydroxylase inhibition would limit postoperative complications and benefit neurological outcomes.

with delayed healing (Decq et al., 1998; Fasano & Penna, 1992; Freudenstein, Wagner, Ernemann, & Duffner, 2002). Therapeutic strategies targeting neuronal apoptosis may limit such postoperative complications.

Inhibition of the N-methyl-D-aspartate (NMDA) receptors has been proposed as a therapeutic strategy in neurodegenerative diseases, stroke, and anxiety disorders (Fan et al., 2015; Meldrum, 1985). Modulating the kynurenine pathway is a possible approach to locally antagonize NMDA receptors. Kynurenine, a metabolite of tryptophan, is synthesized by indoleamine 2,3-dioxygenase (IDO) in macrophages as an immune response (Opitz et al., 2011). Kynurenine can then be utilized in the synthesis of kynurenic acid (KYNA) in astrocytes via the enzyme kynurenine-oxoglutarate transaminase (KAT II) or in the synthesis of quinolinic acid (QUIN) in microglia via kynurenine hydroxylase (also known as kynurenine 3-monooxygenase or KMO) (Moroni, Russi, Gallo-Mezo, Moneti, & Pellicciari, 1991; Rossi, Schwarcz, & Rizzi, 2008; Stone, Forrest, & Darlington, 2012). KYNA is a competitive antagonist of NMDA and AMPA receptors at glycine and glutamate sites, respectively (Elmslie & Yoshikami, 1985). On the contrary, QUIN is an NMDA receptor agonist with excitotoxic properties (Schwarcz, Whetsell, & Mangano, 1983). We hypothesized that inhibition of KMO can increase the availability of kynurenine to be shunted toward KYNA synthesis by decreasing the formation of QUIN. This kynurenine pathway is depicted in Figure 1.

In this study, we employed a potent and selective inhibitor of KMO, RO 61-8048, to reduce QUIN and increase KYNA as a therapeutic approach to reduce SBI. We observed the effects of RO 61-8048 on brain edema and short-term and long-term neurobehavioral outcomes after SBI. We measured the expression of KYNA, QUIN, and apoptotic markers with RO 61-8048 treatment. We also conducted a mechanism study to evaluate if inhibition of KAT II would reverse the protective effects of RO 61-8048.

2 | MATERIALS AND METHODS

All the animal experiments followed protocol that was evaluated and approved by the Institutional Animal Care and Use Committee at Loma Linda University and complied with the NIH guidelines for the care and use of laboratory animals. All the animals were housed in animal care facility in 12-hr light/dark cycle, temperature and humidity controlled environment with free access to food and water. A total of 127 adult male Sprague-Dawley rats (weight, 275–325 g) were used for this study. Only male rats were used to avoid the influence of female sex hormones. Number of animals used in each group per time point and experiment is listed in Table 1.

2.1 | Surgical procedure

The model of SBI was utilized as outlined in previous experiments (Jadhav, Solaroglu, Obenaus, & Zhang, 2007; Lo et al., 2007). SBI procedure involved partial resection of the right frontal lobe. The surgical procedure was performed under isoflurane anesthesia (3%

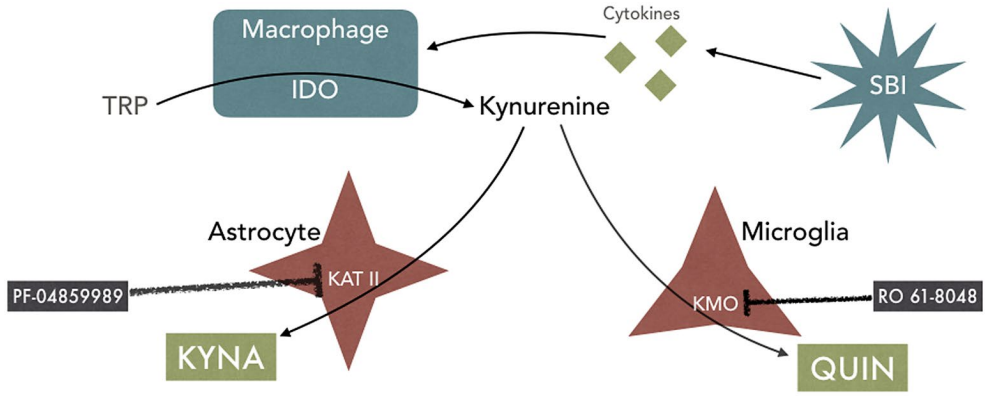

FIGURE 1 Kynurenine metabolism after SBI. Surgical Brain Injury (SBI) induces the expression of immune-mediated cytokines which activate macrophages. The activation of macrophages stimulates synthesis of kynurenine in the brain which has target receptors in microglia and astrocytes, leading to the synthesis of QUIN and KYNA, respectively. Abbreviations: SBI = Surgical Brain Injury, TRP = Tryptophan, IDO = Indoleamine 2,3 Dioxygenase, KAT II = Kynurenine Aminotransferase 2, KMO = Kynurenine 3 Monooxygenase, KYNA = Kynurenic Acid, QUIN = Quinolinic Acid

TABLE 1 Animal groups and number of animals used for the study

	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
	Time course study		Outcome study		Long-term study		Mechanism study	
Group	Animals used	Mortality	Animals used	Mortality	Animals used	Mortality	Animals used	Mortality
Sham	4	0	12	0	6	0	6	0
SBI	16	0	12	0			6	0
SBI + DMSO			12	1/13	5	1/6	6	1/7
SBI + RO (10 mg/kg)			6	0				
SBI + RO (40 mg/kg)			12	2/14	6	0	6	1/7
SBI + RO & PF							6	0
Total animals	**20**	**0**	**54**	**3**	**17**	**1**	**30**	**2**

Note: Table 1 lists the total number of animals used for the study including mortality.

isoflurane for induction for 5 min and 2% isoflurane for maintenance). After anesthetic induction, the skin was incised and a square cranial window (5 mm edge) was drilled with the left lower corner of the square toward the bregma. The dura-mater was then incised to expose underlying right frontal lobe. Partial right frontal lobectomy was performed by making 2 incisions: 2 mm lateral to the sagittal suture and 1 mm proximal to the coronal suture. Hemostasis was maintained by packing and saline irrigation after which the skin was sutured. Sham surgery included only craniotomy and replacement of the bone flap without any dural incisions. Following surgery, animals were sacrificed at different time points as indicated in the experiments below.

2.2 | Animal groups and experiments

A total of 127 Sprague-Dawley male rats (weight 275–325 g) (RRID:MGI:5651135) were used for the following experiments. Animals were randomly divided into different groups using random numbers generated by Microsoft Excel (RRID:SCR_016137). Neurobehavioral testing and western blot analysis was performed by examiners blinded to the animal groups. Sample sizes were calculated for all groups assuming a type I error (false positive) rate = 0.05 and power = 0.8 on a two-sided test. Based on the previous studies (Jadhav et al., 2007; Lo et al., 2007), mean values and standard deviation, as well as the expected % change in the means (a change of ~2% for brain water content measurements, and 15%–20% for western blot), we concluded that a sample size of 6 rats/group/time point are needed for brain water content measurement and western blot. The following 4 experiments were performed.

2.2.1 | Experiment 1

This experiment characterized the endogenous time-course expression of QUIN and KYNA in the perisurgical brain tissue after SBI. For time-course experiments, animals were randomly divided into Sham or SBI groups. SBI animals were sacrificed at different time points including 6, 12, 24, and 72 hr after SBI procedure. Brain samples were collected for western blot to measure KYNA and QUIN expression.

2.2.2 | Experiment 2

This experiment evaluated outcomes after SBI with RO 61-8048 treatment. For outcome study, animals were randomly divided into the following groups: Sham, SBI, SBI + DMSO, SBI + RO 61-8048 (10 mg/kg) and SBI + RO 61-8048 (40 mg/kg). Dimethyl sulfoxide (DMSO) was used to re-constitute RO 16-8048. The SBI + DMSO group received 100 µl of 100% DMSO by intraperitoneal injection at 1 min, 30 min, and 6 hr after SBI. The percent and volume of DMSO was deemed safe and chosen based on previous study (Cozzi, Carpenedo, & Moroni, 1999). The treatment RO 61-8048 (10 mg/kg) and (40 mg/kg) (Catalog # S8172, Selleckchem, Houston, TX, USA) dissolved in 100 µl of 100% DMSO was administered by intraperitoneal injection at 1 min, 30 min, and 6 hr after SBI. The dose and route of RO 61-8048 administration was based on a previous study in rat model of cerebral ischemia (Cozzi et al., 1999). Neurological function was evaluated at 24 hr and 72 hr after SBI. Animals were sacrificed at 24 hr and 72 hr to collect brain samples for brain water content measurement.

2.2.3 | Experiment 3

This experiment evaluated long-term outcomes after SBI with RO 61-8048 treatment. Rats were randomly divided into the following groups: Sham, SBI + DMSO, and SBI + RO 61-8048 (40 mg/kg). The SBI rats were administered either DMSO or RO 61-8048 using the same regimen as described above in Experiment 2. Long-term neurological function was evaluated using Morris water maze test (Bromley-Brits, Deng, & Song, 2011; Lekic et al., 2010) at 4 weeks after SBI.

2.2.4 | Experiment 4

This experiment evaluated the effect of administration of KAT II inhibitor along with RO 61-8048 treatment. For mechanism study, animals were randomly divided into the following groups: Sham, SBI, SBI + DMSO, SBI + RO 61-8048 (40 mg/kg), and SBI + RO 61-8048 (40 mg/kg) + KAT II inhibitor PF-04859989 (5 mg/kg). The KAT II inhibitor PF-04859989 (5 mg/kg) (Catalog # PZ0250, Sigma Aldrich, St. Louis, MO, USA) was administered by intravenous injection 15 min before SBI. The dose and route for PF-04859989 administration were chosen based on previous publication (Linderholm et. al., 2016). Neurological function was evaluated at 24 hr after SBI after which brain samples were collected for western blot.

2.3 | Brain water content measurement

The animals were sacrificed under deep anesthesia at 24 hr and 72 hr after the SBI procedure. Immediately after sacrifice, the brain was resected and divided into six parts on ice which included frontal ipsilateral, frontal contralateral, parietal ipsilateral, parietal contralateral, cerebellum, and brain stem. These parts were weighed immediately (wet weight), placed in a drying oven (105°C) for 48 hr and then re-weighed (dry weight), as previously described (Xi, Hua, Keep, Younger, & Hoff, 2002). The percentage of brain water content was calculated as [(wet weight − dry weight)/wet weight] × 100 as previously described (Sherchan et al., 2017).

2.4 | Short-term neurological evaluation

Neurological examination was performed on animals using modified Garcia test (21 point scoring system), beam balance test, and forepaw placement test.

2.4.1 | Modified Garcia test

The modified Garcia test consisted of evaluation of seven parameters for the SBI model (Garcia, Wagner, Liu, & Hu, 1995; Mcbride, Wang, Sherchan, Tang, & Zhang, 2015). These parameters included: (a) Spontaneous activity: animals were placed in a large cage and observations were made on the animal's level of activity for 5 min. (b) Symmetry in the movement of all four limbs: the animal was raised by the tail and limb symmetry was noted. (c) Forepaw outstretching: the animal was raised by the tail and made to walk on forelimbs evaluating movement on both sides. (d) Climbing on a 12-inch platform inclined at 45 degrees: animal was placed on the inclined plane and ability to climb was noted for 1 min. (e) Body proprioception: the side of the animal was stimulated with a cotton swab. Differences in response were noted. (f) Response to whisker stimulation: animal's whiskers were stimulated with a cotton swab. Differences in response were noted. (g) Lateral turning: animal's ability to walk in a straight line or tendency to turn toward one side was evaluated. Each of the parameters was given a score ranging from 0 or 1 to 3. The scores of the seven parameters were added to a maximum score of 21. Higher scores indicated better function.

2.4.2 | Beam balance test

The test examined the animal's ability to walk on a beam (100cm x 2.25cm diameter) connected by two platforms at the ends. Each animal received a score ranging from 0 to 4 with higher scores indicating better performance (Mcbride et al., 2015; Zausinger, Hungerhuber, Baethmann, Reulen, & Schmid-Elsaesser, 2000). The scoring was as follows: 5, animal reached platform within 25 s; 4, animal reached platform between 25 and 40 s; 3, animal moved halfway to a platform and stayed on the beam for at least 25 s; 2, animal moved less than halfway on the beam and stayed on the beam for at least 25 s; 1, animal did not move and stayed on the beam for 40 s; and 0, animal fell off the beam in less than 25 s.

2.4.3 | Foot-fault test

The test assessed locomotor function of the animals. Animal was placed on an elevated horizontal ladder and trained to cross the device. Animal was allowed to walk for 2 min across the ladder. A video recording was obtained for each animal during the 2 min of walking across the ladder. The number of paw misplacements or foot faults through the ladder rungs and the total number of steps was counted. The percent of foot faults was calculated.

2.5 | Long-term neurological evaluation

2.5.1 | Morris water maze test

The test evaluated long-term spatial learning and memory function as previously described (Bromley-Brits et al., 2011; Lekic et al., 2010). The rats were allowed to swim and find a submerged platform in a metal pool with water made opaque with non-toxic tempera paint. Each rat performed 4 trials a day for 5 days followed by day 6 in which the platform was removed. The swim path and swim distance was recorded by an overhead camera which was linked to a computer tracking system (Noldus Ethovision, Tacoma).

2.6 | Western blot analysis

Western blot analysis was performed as previously described (Sherchan et al., 2016; Zhou et al., 2011). Animals under deep anesthesia were infused with 100 ml/100 g of ice-cold 0.1 mmol Phosphate Buffered Solution (PBS) through the left ventricle. Brains were then removed and flash frozen using liquid nitrogen and stored at −80°C until analysis. Brain samples from the right frontal perisurgical region were homogenized in Ripa lysis buffer (Santa Cruz Biotechnology, Dallas, TX, USA) for protein extraction as previously

described (Hasegawa, Suzuki, Altay, & Zhang, 2011). After homogenization, samples were centrifuged at 14,000 *g* at 4°C for 30 min. The supernatants were collected and protein concentration in the samples was determined using a detergent compatibility assay using a spectrophotometer (Bio-Rad, Irvine, CA, USA). Equivalent amounts of protein (50 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes. The membranes were then incubated overnight with respective primary antibodies which are detailed in the primary antibody reporting table (Table 2). Membranes were also probed with anti-β actin as loading controls. The membranes were then incubated with appropriate secondary antibodies (1:4,000) (all from Santa Cruz Biotechnology, Dallas, TX, USA) for 1 hr at room temperature. The bands were then visualized using ECL Plus Chemiluminescence kit (Amersham Biosciences, Arlington Heights, IL, USA). The target band was identified by using a molecular weight maker. The band density of the western blot bands was quantified using Image J software (National Institutes of Health, Bethesda, MD, USA) and when two bands were detected for a target protein; both bands were selected to measure the density using Image J software. Representative western blot bands were cut and shown in the figures. Results were expressed as density relative to β-actin as loading control and normalized to average value of the sham group as previously described (Hasegawa et al., 2011). First, the band density for each animal was determined relative to β-actin. Next, the average value of the band density for sham group was determined. Lastly, the band density for each animal including the sham animals was divided by the average value of sham density.

2.7 | Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 6.0e (GraphPad Software, La Jolla, CA, USA, RRID:SCR_002798). Quantitative data a presented as mean ± *SD*. Statistical differences among groups were analyzed using one-way ANOVA followed by Tukey post hoc test to determine differences among groups at each time point for brain edema, foot-fault, and western blot data. One-way ANOVA Kruskall-Wallis with Dunn's post hoc test was used to analyze non-parametric data such as neuroscore and beam balance score. Morris water maze test was evaluated using one-way repeated measures ANOVA with Tukey post hoc test. *P* values less than 0.05 were considered statistically significant.

3 | RESULTS

All 28 sham rats survived. Six rats out of 99 SBI rats died within 6 hr post-surgery with an overall mortality rate of 6.06% in the SBI group (Table 1). The mortality in SBI animals was related to intraoperative bleeding and poor postoperative recovery. The mortality rate was not significantly different between all the groups. All animals were included for the study except the animals that died.

3.1 | Time course expression of QUIN and KYNA after SBI

The expression of QUIN and KYNA were measured at 6, 12, 24 and 72 hr after SBI. Our results indicate that levels of QUIN

TABLE 2 Primary antibody reporting

Antibody name	Structure and host	Catalog number	MW	Concentration	RRID
Polyclonal Antibody to Quinolinic Acid (QA)	IgG, Polyclonal antibody preparation, Host Rabbit	Cloud Clone Corp., Houston, TX, USA PAK552Ge01	68 kDa	1:200	NA
Polyclonal Antibody to Kynurenic Acid (KYNA)	IgG, Polyclonal antibody preparation, Host Rabbit	Cloud Clone Corp., Houston, TX, USA PAD718Ge01	86 kDa	1:200	NA
Anti-Bcl-XL antibody	IgG, Monoclonal Antibody, Host Rabbit	Abcam, Cambridge, MA, USA ab32370	26 kDa	1:1,000	RRID:AB_725655
Anti-Bcl-2 antibody	IgG, Monoclonal Antibody, Host Rabbit	Abcam, Cambridge, MA, USA ab59348	26 kDa	1:1,000	RRID:AB_2064155
Anti-Bax antibody	IgG, Monoclonal Antibody, Host Rabbit	Abcam, Cambridge, MA, USA ab32503	21 kDa	1:1,000	RRID:AB_725631
β-Actin antibody	IgG, Monoclonal Antibody, Host Mouse	Santa Cruz Biotechnology, Dallas, TX, USA sc-47778	43 kDa	1:4,000	RRID:AB_2714189

Note: Table 2 lists the details of primary antibodies used for western blot experiments for the study. IgG, Immunoglobulin; MW, Molecular weight.

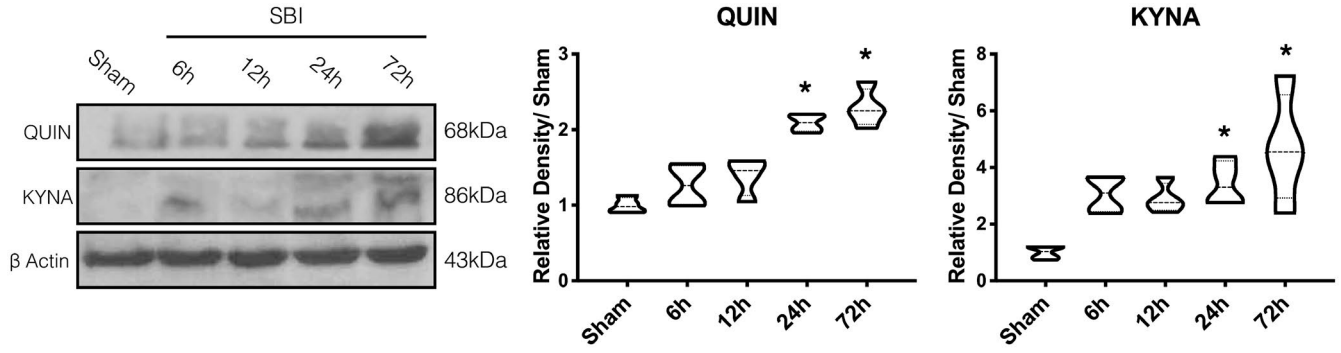

FIGURE 2 Time course study expression of QUIN and KYNA after SBI. Data expressed as mean ± *SD*. *N* = 4 per group. Differences among groups were assessed with one-way analysis of variance (ANOVA) and Tukey post hoc test. * denotes significance versus Sham, Abbreviations: KYNA = Kynurenic Acid, QUIN = Quinolinic Acid

(one-way ANOVA; Tukey test; $F_{(4,15)}$ = 27.49; n = 4; p < 0.0001 for 24 and 72 hr vs. Sham) and KYNA (one-way ANOVA; Tukey test; $F_{(4,15)}$ = 6.652; n = 4; p = 0.0029 at 24 hr and p = 0.0011 at 72 hr vs. Sham) were significantly increased at 24 hr and 72 hr after SBI (Figure 2).

3.2 | Brain edema and neurobehavioral function 24 hr after SBI improved with RO 61-8048 treatment

We tested two doses of RO 61-8048 treatment for the 24 hr outcome study. Brain water content was significantly increased in the right frontal lobe near the resection site 24 hr after SBI, and RO 61-8048 (40 mg/kg) treatment significantly reduced brain edema in the right frontal lobe 24 hr after SBI compared to SBI + DMSO group (one-way ANOVA; Tukey test; $F_{(4,25)}$ = 9.506; n = 6; p = 0.0041) (Figure 3a). RO 61-8048 (40 mg/kg) treatment significantly increased Garcia neuroscore compared to SBI + DMSO group (one-way ANOVA; Kruskal–Wallis with Dunn's post hoc; n = 6–12; p = 0.0162) (Figure 3b). There was no significant difference in beam balance score after SBI with RO 61-8048 treatment compared to SBI and SBI + DMSO groups (one-way ANOVA; Kruskal–Wallis with Dunn's post hoc; n = 6–12; p = 0.3746 and p = 0.1351) (Figure 3c). RO 61-8048 (10 mg/kg) and (40 mg/kg) both significantly reduced the percent of foot-faults on the contralateral side 24 hr after SBI compared to SBI + DMSO group (one-way ANOVA; Tukey test; $F_{(4,49)}$ = 23.8; n = 6–12; p = 0.0026 and p < 0.0001) (Figure 3d). Additionally, SBI + DMSO group did not show any significant changes in brain water content, Garcia score, beam balance, or foot-fault when compared to the SBI only group.

3.3 | Brain edema and neurobehavioral function 72 hr after SBI improved with RO 61-8048 treatment

Based on the results of the 24 hr study, we used RO 61-8048 (40 mg/kg) dose for the 72 hr study. Treatment with RO 61-8048 (40 mg/kg) significantly (one-way ANOVA; Tukey test; $F_{(3,20)}$ = 18.58; n = 6; p = 0.0068) reduced brain edema in the right frontal lobe near the resection site 72 hr after SBI (Figure 4a). RO 61-8048 (40 mg/kg) significantly increased Garcia neuroscore (one-way ANOVA; Kruskal–Wallis with Dunn's post hoc; n = 6; p = 0.0285), improved beam balance score (one-way ANOVA; Kruskal–Wallis with Dunn's post hoc; n = 6; p = 0.0281), and reduced percent of foot-faults on the contralateral side 72 hr after SBI compared to SBI + DMSO group (one-way ANOVA; Tukey test; $F_{(3,20)}$ = 40.61; n = 6; p < 0.0001) (Figure 4b–d).

3.4 | RO 61-8048 administration improved long-term neurological function after SBI

In the water maze test, SBI + RO 61-8048 group traveled a significantly shorter swim distance to find the submerged platform compared to SBI + DMSO group during block 3 testing (One-way repeated measures ANOVA; Tukey test; n = 5–6; p = 0.011) (Figure 5a,b). Further, the sham and SBI + RO 61-8048 groups showed improved swim distance in blocks 3 and 4 compared to sham in blocks 1 and 2 (One-way repeated measures ANOVA; Tukey test, n = 5–6; p < 0.05) (Figure 5b).

3.5 | Protective effects of RO 61-8048 were reversed with KAT II inhibitor, PF 04859989

We administered the established KAT II inhibitor PF-04859989 to determine if effects of RO 61-8048 treatment would be reversed. The beneficial effects of RO 61-8048 (40 mg/kg) on improved neurological function were reversed with administration of KAT II inhibitor, PF 04859989 evaluated 24 hr after SBI. The foot-fault scores in SBI + RO group was reversed with PF 04859989 administration, and the neurological function in SBI + RO +PF group were all statistically insignificant versus SBI group as indicated by Garcia score (one-way ANOVA; Kruskal–Wallis with Dunn's post hoc; n = 6; p > 0.9999 vs. SBI), beam balance (one-way ANOVA; Kruskal–Wallis with Dunn's post hoc; n = 6; p > 0.9999 vs. SBI), and foot-fault scores (one-way ANOVA; Tukey test; $F_{(4,25)}$ = 30.87; n = 6; p = 0.7057 vs. SBI) (Figure 6a–c).

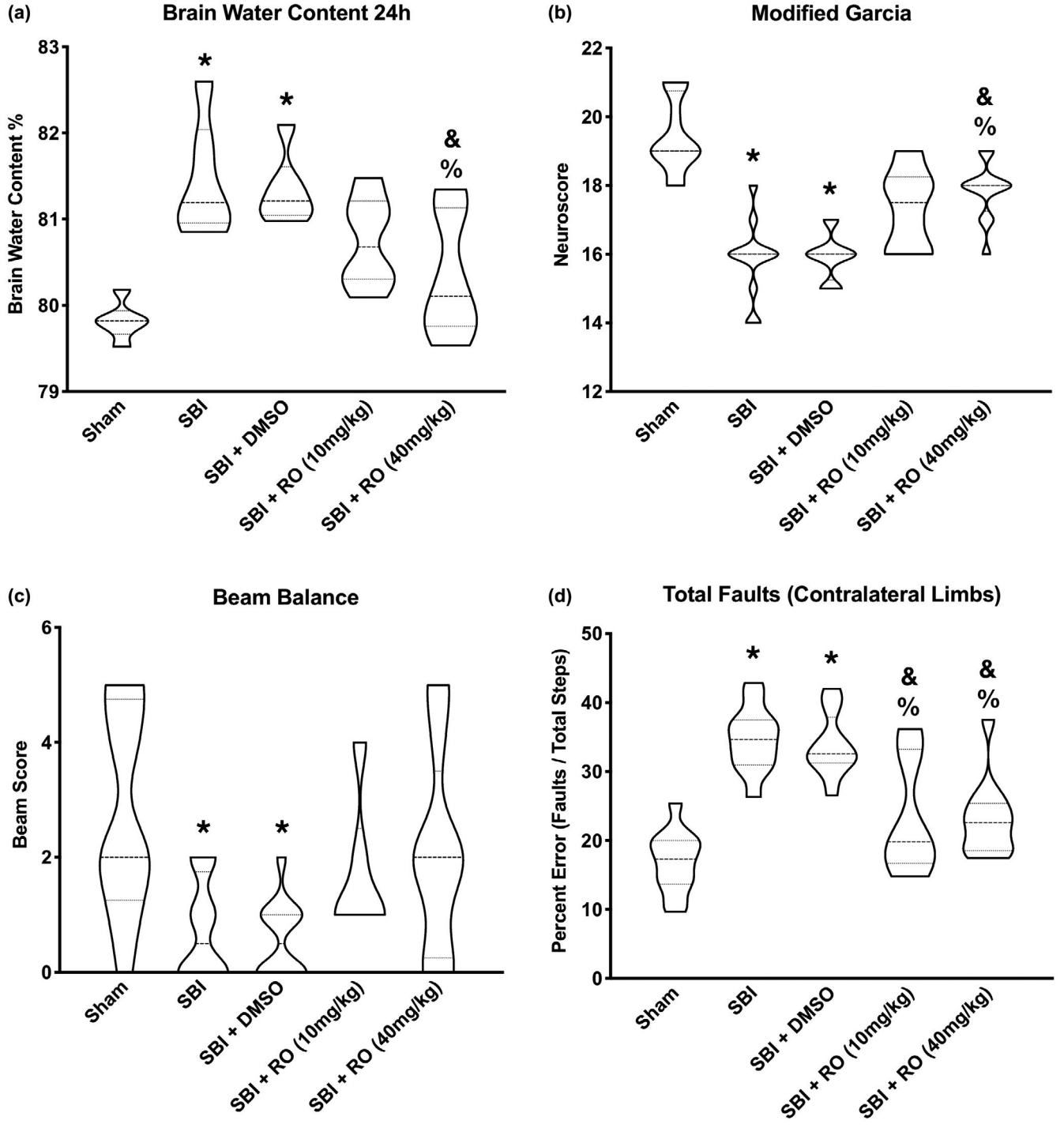

FIGURE 3 Brain edema in right frontal lobe and neurobehavioral function 24 hr after SBI with RO 61-8048 treatment. Data expressed as mean ± *SD*. (a) *N* = 6 rats per group. Differences among groups were assessed with one-way analysis of variance (ANOVA) and Tukey post hoc multiple comparison test. (b–d) The 24 hr behavior data included animals from 24 and 72 hr outcome groups. *N* = 12 rats per group and *N* = 6 for SBI + RO (10 mg/kg). Differences among groups were assessed with one-way ANOVA and Tukey post-hoc test for d. b and c were assessed with ANOVA Kruskall-Wallis test with Dunn's post hoc. * denotes significance versus Sham, & denotes significance versus SBI, % denotes significance versus SBI + DMSO. Abbreviations: RO = RO 61-8048, RF = Right Frontal, LF = Left Frontal, RP = Right Parietal, LP = Left Parietal, C = Cerebellum, BS = Brain Stem. RO = RO 61-8048

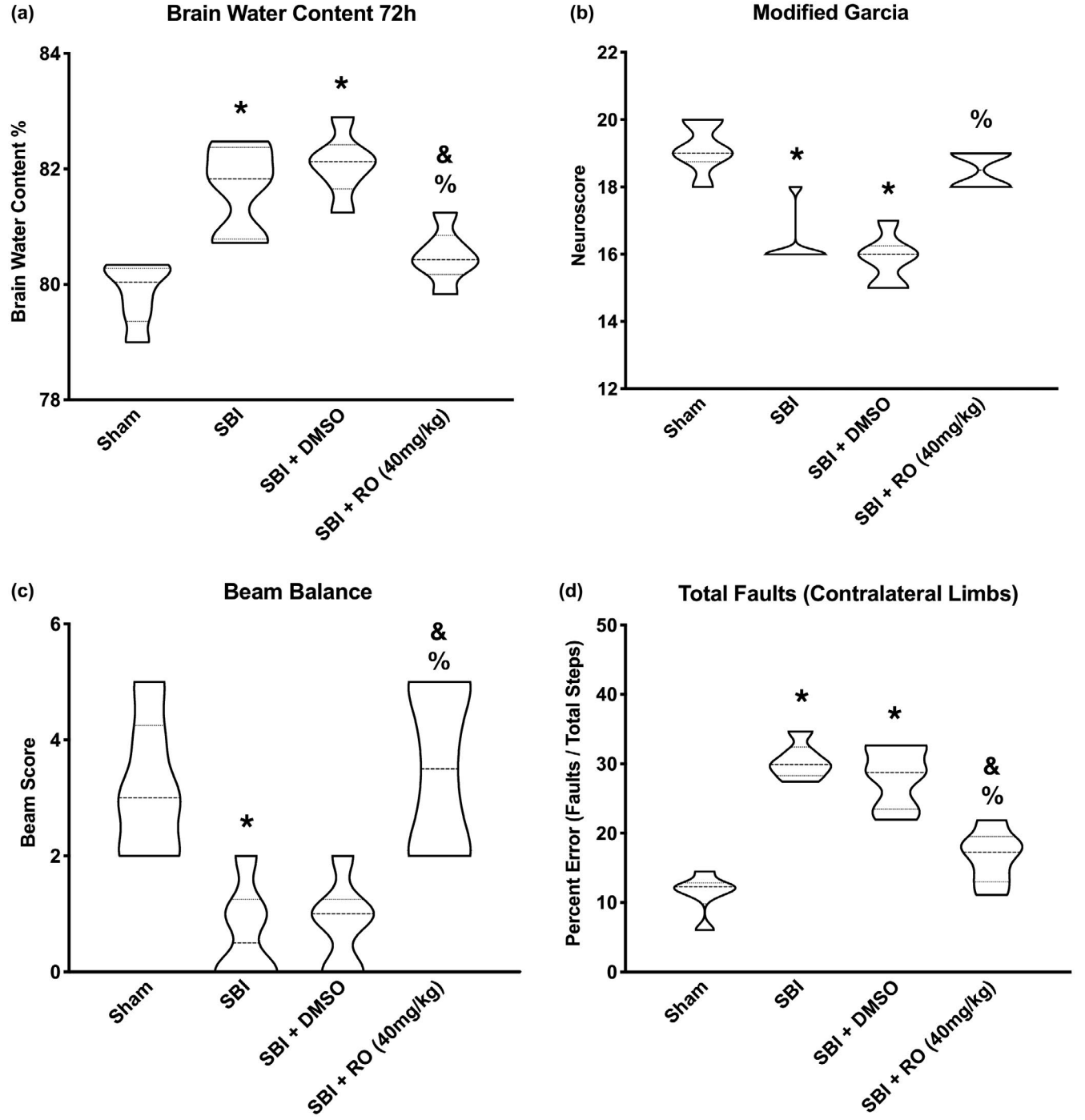

FIGURE 4 Brain edema in right frontal lobe and neurobehvioral function 72 hr after SBI with RO 61-8048 treatment. Data expressed as mean ± *SD*. (a) *N* = 6 rats per group. Differences among groups were assessed with one-way analysis of variance (ANOVA) and Tukey post hoc multiple comparison test. (b–d) Differences among groups were assessed with ANOVA- Kruskall-Wallis test with Dunn's post hoc one-way ANOVA and Tukey post hoc test. * denotes significance versus Sham, & denotes significance versus SBI, % denotes significance versus SBI + DMSO. Abbreviations: RO = RO 61-8048, RF = Right Frontal, LF = Left Frontal, RP = Right Parietal, LP = Left Parietal, C = Cerebellum, BS = Brain Stem, RO = RO 61-8048

3.6 | RO 61-8048 treatment increased KYNA expression and decreased apoptotic markers after SBI which was reversed with the KAT II inhibitor, PF 04859989

Western blot results showed that treatment with RO 61-8048 (40 mg/kg) significantly reduced the expression of QUIN (one-way ANOVA; Tukey test; $F_{(4,25)}$ = 18.65; *n* = 6; *p* = 0.0010) (Figure 7a,b) while increasing KYNA expression (one-way ANOVA; Tukey test; $F_{(4,25)}$ = 28.7; *n* = 6; *p* = 0.0006) 24 hr after SBI (Figure 7a,c). The administration of KAT II inhibitor, PF 04859989, significantly reversed increase in KYNA expression seen with RO 61-8048 treatment. Furthermore, treatment with RO 61-8048 (40 mg/kg) significantly increased the expression of anti-apoptotic markers BCLxl (one-way

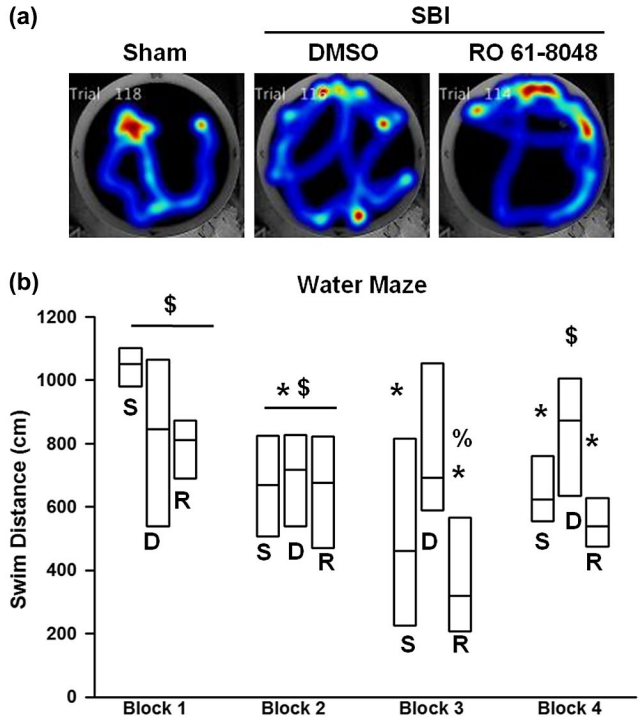

FIGURE 5 Long-term neurobehavioral function evaluation 4 weeks after SBI. Morris water maze test showing (a) Heat map traces of swim path and (b) Swim distance in Morris water maze test performed 4 weeks after SBI in Sham, SBI + DMSO and SBI + RO (40 mg/kg) groups. Data expressed as mean ± *SD*. *N* = 5–6 per group. * denotes significance versus Sham, % denotes significance versus SBI + DMSO in block 3, $ denotes significance versus SBI + RO in block 3. S = Sham, D = SBI +DMSO, R = SBI +RO 16-8048 (40 mg/kg)

ANOVA; Tukey test; $F_{(4,25)}$ = 17.96; n = 6; p = 0.0016) and BCL2 (one-way ANOVA; Tukey test; $F_{(4,25)}$ = 28.97; n = 6; p = 0.0005) while decreasing the expression of pro-apoptotic BAX (one-way ANOVA; Tukey test; $F_{(4,25)}$ = 14.2; n = 6; p < 0.0074). This effect was significantly reversed with PF 04859989 administration (Figure 7a,d–f, respectively).

4 | DISCUSSION

The neural kynurenine pathway metabolizes the amino acid tryptophan to generate a number of neuroactive metabolites that can be either neuroprotective or neurotoxic (Campbell, Charych, Lee, & Möller, 2014). KMO in microglia metabolizes kynurenine, as part of an immune response, to upregulate the downstream production of QUIN. QUIN has been identified as neurotoxic due to its excitotoxin abilities, as an NMDA agonist, which contributes to neuronal apoptosis and energy depletion (Guillemin, 2012). In addition, studies have shown that QUIN likely forms a complex with iron, and electron transfer from this complex to oxygen results in the formation of reactive oxygen species (Goda, Kishimoto, Shimizu, Hamane, & Ueda, 1996; Stipek, Stastny, Platenik, Crkovska, & Zima, 1997). QUIN–iron complexes display significant pro-oxidant characteristics that may lead to the accumulation for lipid peroxidation and could further have implications for QUIN neurotoxicity.

In this study, we focused on understanding the contribution of kynurenine pathway to perisurgical site injury after SBI. Various cytokines and inflammatory mechanisms are activated following SBI (Sherchan et al., 2016; Wang et al., 2017), which can stimulate KMO and thereby upregulate the downstream release of the neurotoxic QUIN. We first measured the endogenous expression of QUIN and KYNA in the perisurgical brain tissue following SBI. We observed that QUIN, a product of kynurenine via KMO, increased significantly in the surrounding perisurgical site brain tissue following SBI. This neurotoxic signal was associated with an increase in apoptotic marker BAX and decreased expression of anti-apoptotic BCLxL and BCL2 at the perisurgical site. Given that the expression of QUIN was

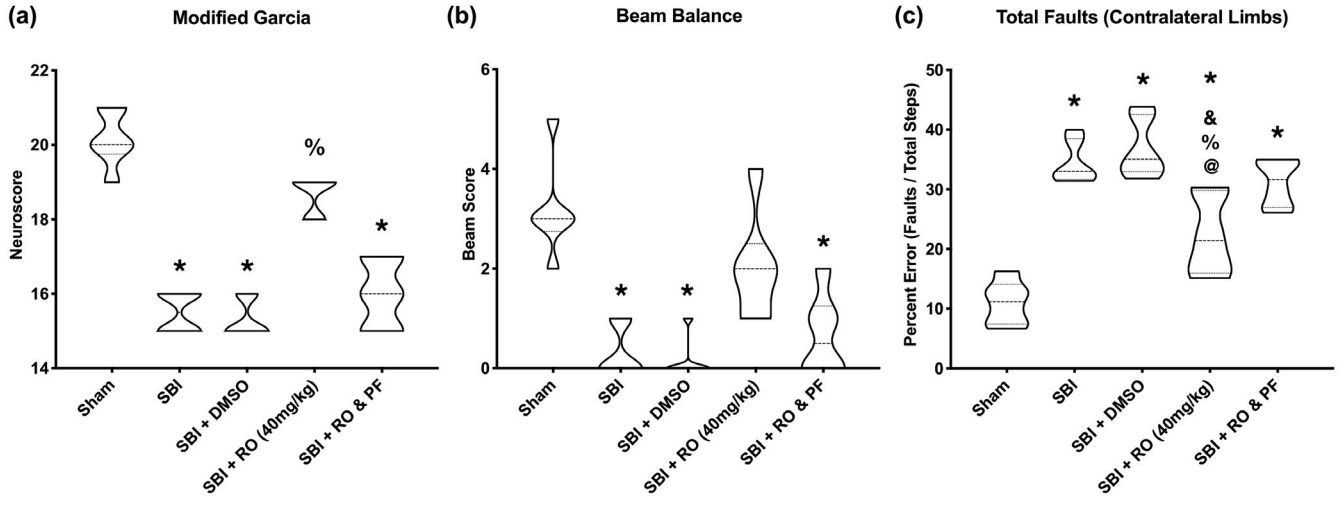

FIGURE 6 Neurobehavioral function assessment in the mechanism study groups 24 hr after SBI. Data expressed as mean ± *SD*. *N* = 6 per group. (a and b) Differences among groups were assessed with ANOVA- Kruskall-Wallis test with Dunn's post hoc test. (c) One-way analysis of variance (ANOVA) and Tukey post hoc test was used for analysis. * denotes significance versus Sham, & denotes significance versus SBI, % denotes significance versus SBI + DMSO, @ denotes significance versus SBI + RO & PF. Abbreviations: RO = RO 61-8048, PF = PF-04859989

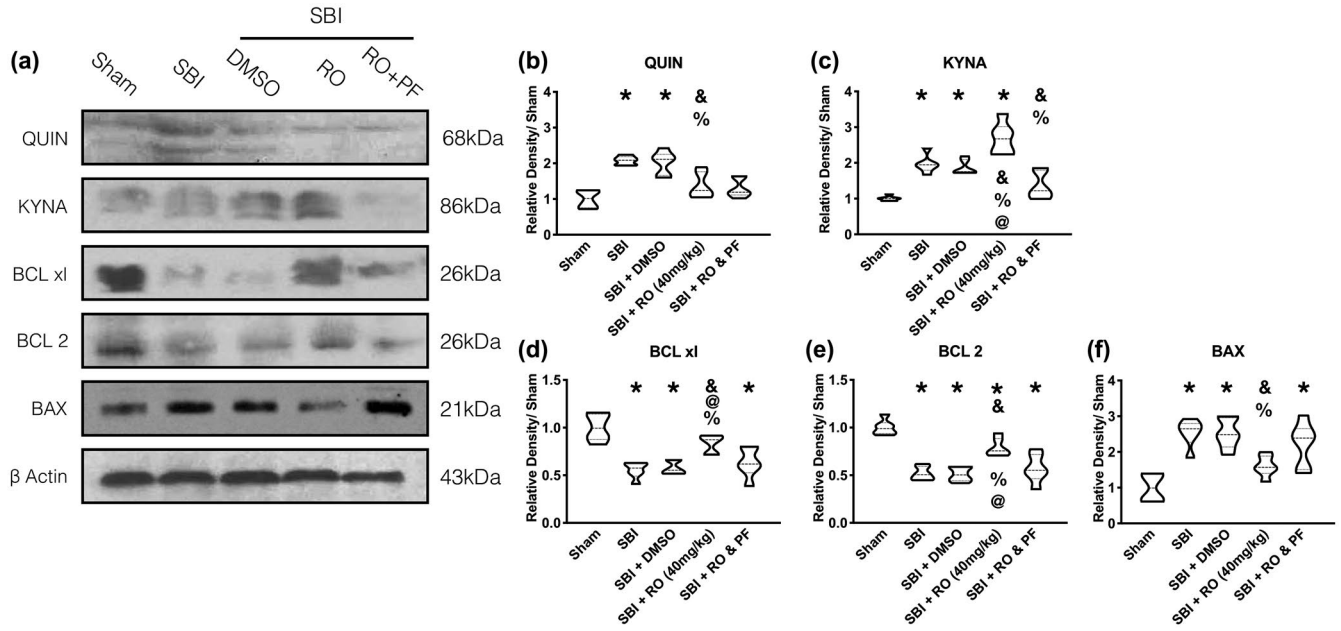

FIGURE 7 Western blot evaluation in the mechanism study groups 24 hr after SBI. Data expressed as mean ± *SD*. *N* = 6 per group. Differences among groups were assessed with one-way analysis of variance (ANOVA) and Tukey post hoc test. * denotes significance versus Sham, & denotes significance versus SBI, % denotes significance versus SBI + DMSO, @ denotes significance versus SBI + RO & PF. Abbreviations: RO = RO 61-8048, PF = PF-04859989

increased after SBI, higher levels of QUIN possibly contributed to the suppression of anti-apoptotic proteins after SBI.

Next, we determined whether inhibition of KMO would be beneficial in SBI model, particularly in terms of reducing postoperative brain edema and apoptosis at perisurgical site of injury. We observed that administration of KMO selective inhibitor, RO 61-8048 improved outcomes in SBI rats. We initially tested two doses of RO 61-8048, and the outcome study showed that RO 61-8048 (40 mg/kg) significantly reduced brain edema at the perisurgical injury site and improved neurological function in SBI rats. We therefore, continued to use RO 61-8048 (40 mg/kg) dose for rest of the study. Inhibition of KMO was associated with a decrease in pro-apoptotic BAX and increase in anti-apoptotic BCLxL and BCL2 expression at the perisurgical injury site after SBI. Additionally, we performed long-term evaluation in SBI rats at 4 weeks after injury. Our results showed that treatment with RO 61-8048 (40 mg/kg) improved long-term neurological function in SBI rats. We did not observe any evidence of systemic adverse effects based on behavioral evaluation and systemic evaluation during sacrifice up to 4 weeks after SBI. Furthermore, the overall mortality rate in SBI group was 6.06% which is within the expected mortality rate up to 10% in this model.

This is the first study that describes the potential neuroprotective effects of KMO inhibitor, RO 61-8048, in reducing brain edema and improving neurological behavior in a rat model of SBI. We next sought to determine the possible protective mechanism of RO 61-8048 treatment. While RO 61-8048 does not cross the blood-brain barrier well, previous study showed that inhibition of peripheral KMO with the administration of JM6, a pro-drug of RO 61-8048, increased peripheral kynurenine which was efficiently transported to the brain and converted to the neuroprotective metabolite KYNA in mice (Zwilling et al., 2011). We observed that RO 61-8048 treatment decreased the expression of QUIN while increasing KYNA after SBI. The inhibition of KMO with RO 61-8048 treatment decreased the downstream formation of QUIN in the kynurenine pathway. Therefore, inhibition of KMO increases the availability of kynurenine that may be metabolized by the enzyme KAT II thereby increasing KYNA expression in SBI rats treated with RO 61-8048. In the brain, KAT II has been reported to be expressed in astrocytes. In its natural state, KAT II is an enzyme that increases the expression of KYNA, a neuroprotective competitive antagonist of NMDA (Elmslie & Yoshikami, 1985; Moroni et al., 1991; Stone, 2000). The antagonism of NMDA has been linked to protection in neurodegenerative diseases (Rossi et al., 2008; Stone et al., 2012), which has been attributed to its role in reducing the expression of pro-apoptotic BAX by increasing anti-apoptotic inhibitor BCL2 (Fan et al., 2015). Likewise, our results showed that RO 61-8048 treatment reduced pro-apoptotic markers and increased anti-apoptotic markers following SBI.

KYNA works as a neuroprotective NMDA receptor antagonist at the glycine site of the receptor. Kynurenine has also been hypothesized to activate GPR35, a Gi-protein coupled receptor, which decreases cAMP and calcium levels, thereby reducing the release of excitatory amino acids from glia and inflammatory mediators from leukocytes (Moroni, Cozzi, Sili, & Mannaioni, 2012; Wang et al., 2006). In line with previous studies that have suggested KYNA to have neuroprotective effects in response to neuronal insults in cerebral ischemia and neurodegenerative disorders (Cozzi et al., 1999; Meldrum, 1985; Rossi et al., 2008; Stone et al.,

2012), our results suggest that rats treated with KMO inhibitor RO 61-8048 displayed improved neurological function and reduced brain edema. The protective effects of RO 61-8048 can possibly be attributed to an increase in KYNA levels while decreasing QUIN formation. This is supported by our mechanism study in which SBI rats were administered a KAT II inhibitor, PF 04859989 in addition to RO 61-8048 treatment. We observed RO 61-8048 treatment improved neurological function in SBI rats and decreased apoptotic markers in the perisurgical brain tissue, which was reversed with PF 04859989 administration. Furthermore, RO 61-8048 treatment increased expression of KYNA which was reversed with administration of KAT II inhibitor, PF 04859989. Accordingly, previous study showed that administration of JM6, a pro-drug of RO 61-8048, prevented neurological deficits and synaptic loss in a mouse model of Alzheimer's disease (AD). Furthermore, the study showed that JM6 increased KYNA levels in the brain in wild-type (WT) mice and transgenic AD mouse model (Zwilling et al., 2011). These findings demonstrate that the protective mechanism of RO 61-8048 treatment possibly can be attributed to increased levels of KYNA in exchange for decrease in QUIN expression. While our study shows the beneficial effects of RO 61-8048 after SBI was contributed at least in part by increase in KYNA, we did not explore further mechanisms that could contribute to the protective effects of KMO inhibition.

This study has some limitations. First, we did not rule out potential mechanisms how KMO inhibition can provide beneficial effects against SBI-induced injury. Previous study showed that kynurenate can inhibit glycine binding site with much higher affinities than for glutamate binding sites of NMDA receptors (Kessler, Terramani, Lynch, & Baudry, 1989). It has been reported that KYNA is a broad-spectrum antagonist of ionotropic excitatory amino acid receptors at supraphysiological concentrations and can also competitively block the glycine coagonist site of the NMDA receptor at endogenous brain levels (Zwilling et al., 2011). While our study shows the beneficial effects of RO 61-8048 after SBI was contributed at least in part by increase in KYNA, we did not explore the link between KMO inhibition and alteration of NMDA receptor activation or glutamatergic system which could also play a role in protective effects of RO 61-8048. Second, from our study we were able to show that inhibition of KMO with RO 61-8048 shunts the kynurenine pathway toward an increase in KYNA. A shortcoming of our study is that we did not trace the kynurenine pathway further upstream to see whether KMO inhibition increases the availability of tryptophan which could shift toward the production of serotonin and that could lead to serotonin toxicity. Third, we used 100% DMSO to reconstitute RO 61-8048 and 100 μl was administered based on previous publication in which the KMO inhibitor attenuated symptoms of cerebral ischemia in rodents (Cozzi et al., 1999). We did not observe significant difference in brain edema and neurological outcomes between SBI and SBI + DMSO groups up to 72 hr which was the end time-point of this study. Additionally, we did not observe any gross abnormalities or signs of organ toxicity during postmortem examination. Fourth, we used only male rats for the study and we did not evaluate sex differences in SBI outcomes, which is a limitation of the study. Fifth, significant anatomical differences exist between humans and rats, and the geometry of resection in a rat model is relatively simple and therefore, not intended to mimic any specific human neurosurgical procedure. Rather, this model serves as a reproducible and predictable model of the neuronal damage that may be a complication of routine neurosurgical procedures. Additionally, we did not perform neurobehavioral assessment that can measure subtle frontal lobe behavioral deficits. In our study, beam balance results were not significantly improved in the treated rats after 24 hr, in part, due to the model's testing of gross sensorimotor loss and low sensitivity for fine motor loss that can result from perisurgical edema (McBride et al., 2015).

Overall, this study indicates that kynurenine pathway contributes to injury mechanisms after SBI. Increased KMO activation and QUIN expression potentially contribute to perisurgical site injury. The clinical significance of this study is the use of specific treatment that targets kynurenine pathway. NMDA pathway can be targeted by many other treatments that can yield similar results; however, modulation of the kynurenine specific pathway is unique in that the pathway is activated primarily in areas subject to damage or degeneration (Stone et al., 2012). In this respect, a KMO inhibitor will have immense advantages over other treatments that can block all nicotinic or glutamate receptors through central and peripheral tissues where they are physiologically important.

DECLARATION OF TRANSPARENCY

The authors, reviewers, and editors affirm that in accordance to the policies set by the Journal of Neuroscience Research, this manuscript presents an accurate and transparent account of the study being reported and that all critical details describing the methods and results are present.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Conceptualization, G.Z., P.S., and J.H.Z.; *Supervision*, J.T. and J.H.Z.; *Writing – Original Draft*, G.Z., P.S. and Q.L. performed the experiments and drafted the manuscript. All authors critically analyzed the manuscript and data analysis.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplementary Figure 1. Brain edema in different brain regions A) 24 hours and B) 72 hours after SBI with RO 61-8048 treatment. Data expressed as mean ± SD. N = 6 rats per group. Differences between groups were assessed with a one-way analysis of variance (ANOVA) and Tukey's multiple comparison test. * denotes significance vs. Sham, & denotes significance vs SBI, % denotes significance vs. SBI + DMSO. Abbreviations: RO = RO 61-8048, RF = Right Frontal, LF = Left Frontal, RP = Right Parietal, LP = Left Parietal, C = Cerebellum, BS= Brain Stem.

Supplementary Figure 2. Foot-faults in limbs ipsilateral to the surgical lesion at 24 hours after SBI. Data expressed as mean ± SD. N = 6 rats per group. Differences between groups were assessed with a one-way analysis of variance (ANOVA) and Tukey's multiple comparison test. There was no significant difference in ipsilateral foot-faults among the groups. Abbreviations: RO = RO 61-8048.

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