Neuroprotective Effects of the Glutamate Transporter Activator (*R*)-(-)-5-methyl-1-nicotinoyl-2-pyrazoline (MS-153) following Traumatic Brain Injury in the Adult Rat

Andréia Cristina Karklin Fontana,¹ Douglas P. Fox,² Argie Zouboulis,¹
Ole Valente Mortensen,¹ and Ramesh Raghupathi²

Abstract

Traumatic brain injury (TBI) in humans and in animals leads to an acute and sustained increase in tissue glutamate concentrations within the brain, triggering glutamate-mediated excitotoxicity. Excitatory amino acid transporters (EAATs) are responsible for maintaining extracellular central nervous system glutamate concentrations below neurotoxic levels. Our results demonstrate that as early as 5 min and up to 2 h following brain trauma in brain-injured rats, the activity ($V_{\max}$) of EAAT2 in the cortex and the hippocampus was significantly decreased, compared with sham-injured animals. The affinity for glutamate (K_M) and the expression of glutamate transporter 1 (GLT-1) and glutamate aspartate transporter (GLAST) were not altered by the injury. Administration of (*R*)-(-)-5-methyl-1-nicotinoyl-2-pyrazoline (MS-153), a GLT-1 activator, beginning immediately after injury and continuing for 24 h, significantly decreased neurodegeneration, loss of microtubule-associated protein 2 and NeuN (+) immunoreactivities, and attenuated calpain activation in both the cortex and the hippocampus at 24 h after the injury; the reduction in neurodegeneration remained evident up to 14 days post-injury. In synaptosomal uptake assays, MS-153 up-regulated GLT-1 activity in the naïve rat brain but did not reverse the reduced activity of GLT-1 in traumatically-injured brains. This study demonstrates that administration of MS-153 in the acute post-traumatic period provides acute and long-term neuroprotection for TBI and suggests that the neuroprotective effects of MS-153 are related to mechanisms other than GLT-1 activation, such as the inhibition of voltage-gated calcium channels.

Keywords: EAAT2/GLT-1; glutamate uptake; MS-153; neuroprotection; traumatic brain injury

Introduction

TRAUMATIC BRAIN INJURY (TBI) is a major cause of injury-induced mortality and morbidity, with very few options to limit acute and long-term progression of secondary injury.¹ Studies on TBI in humans and in animals have demonstrated an acute increase in tissue glutamate concentrations that remain high for up to 5 days in humans.^{2–7} The sustained elevation of extracellular glutamate levels leads to the overstimulation of the N-methyl-D-aspartate (NMDA) glutamate receptor subtype and the subsequent overloading of neurons with Ca^{2+} and Na^+ , resulting in the activation of phospholipases, endonucleases, and proteases such as calpain and eventual apoptotic and/or necrotic cell death.⁸ Clinical and experimental studies have determined that glutamate-mediated excitotoxicity is a significant contributor to acute post-traumatic neurodegenerative events.^{9–11}

The termination of glutamate neurotransmission is achieved by rapid uptake of the released glutamate by presynaptic and/or

astrocytic transporters¹² in a process that is driven by and coupled to ion gradients.^{13–15} The glutamate transporter EAAT2 / GLT-1 (human/rat homolog)¹⁶ is expressed predominantly in glia throughout the brain and spinal cord, and accounts for approximately 95% of glutamate uptake in the central nervous system (CNS),¹⁷ implicating this isoform in the maintenance of extracellular glutamate homeostasis in normal and pathological conditions.^{18–20} Developing EAAT2 as a target for neuroprotection has been explored through the discovery of compounds that can either increase EAAT2 expression or increase the activity of the transporter.^{21,22} The β -lactam antibiotic ceftriaxone increases EAAT2 expression and has been shown to decrease neuronal damage in animal models of chronic neurodegenerative disorders,^{23–25} reduce brain glutamate levels, edema, and neuronal death after TBI in the rat,^{26–29} and attenuate neuropathic pain^{30, 31} and cytokine production after spinal cord injury in the rat.³² Compounds such as riluzole,^{33–37} guanosine,^{38,39} nicergoline,⁴⁰ (*R*)-(-)-5-methyl-1-nicotinoyl-2-pyrazoline (MS-153),^{41,42}

¹Department of Pharmacology and Physiology, ²Department of Neurobiology and Anatomy, Drexel University College of Medicine, Philadelphia, Pennsylvania.

and Parawixin1,^{43,44} have been reported to be neuroprotective in several models of CNS injury by increasing EAAT2 activity. In addition to reducing elevated brain glutamate levels induced by middle cerebral artery occlusion,⁴⁵ MS-153 can inhibit voltage-gated calcium channels and prevent ischemia-induced release of glutamate from nerve terminals.⁴⁶ MS-153 has been shown to affect other conditions that are believed to involve excessive glutamate signaling, such as the inhibition of morphine tolerance and dependence,⁴⁷ the attenuation of behavioral sensitization development to phencyclidine,^{48,49} an anxiolytic effect in fear conditioning,⁵⁰ and attenuation of the conditioned rewarding effects of morphine, methamphetamine, and cocaine.⁵¹ MS-153 was recently suggested to have potential as a therapeutic drug for the treatment of alcohol dependence.^{52,53}

In the present study, the hypotheses that the glutamate transporter EAAT2/GLT-1 function is altered by TBI and that treatment with an EAAT2 activator after TBI will reduce neurodegeneration were tested. Lateral fluid-percussion brain injury of moderate severity decreased the $V_{\max}$ of glutamate uptake in the cortex and hippocampus. Post-traumatic infusion of MS-153 for a period of 24 h decreased the extent of neurodegeneration, neuronal loss, microtubule-associated protein 2 (MAP-2) loss, and calpain activation. Importantly, the neuroprotective effect of MS-153 was sustained for up to 14 days after the injury. Interestingly, MS-153 could not reverse the injury-induced decrease in $V_{\max}$ of glutamate uptake in synaptosomes *ex vivo*, suggesting that the neuroprotective effects of MS-153 *in vivo* may be unrelated to its ability to increase glutamate uptake.

Methods

All of the surgical, injury, and care protocols were approved by the Drexel University Institutional Animal Care and Use Committee and are in agreement with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals.

TBI

Adult male Sprague-Dawley rats (325–411 g) were subjected to lateral fluid-percussion brain injury of 2.1–2.4 atm ($n=83$) as previously described⁵⁴; sham-injured animals ($n=24$) were surgically prepared but did not receive the injury. Table 1 lists the animals used throughout the study.

Drug administration

MS-153 was dissolved in saline and administered intravenously as a bolus dose at 5 min after the injury via the femoral vein and followed by the subcutaneous implantation of a mini osmotic pump (Model 2001D; Alze, Cupertino, CA) to deliver the drug for 24 h. Two doses of MS-153 were used; the low dose (25 mg/kg, followed by a continuous infusion of 12.5 mg/h for 23 h) and a high dose (50 mg/kg, followed by a continuous infusion of 25 mg/h for 23 h). The dose and dosing for MS-153 were based on studies in rat models of focal ischemia^{42,45} and pharmacokinetics that demonstrated that MS-153 crosses the blood–brain barrier and has a serum half-life of approximately 0.7 h.⁴⁵ Animals that survived to 7 or 14 days following injury were re-anesthetized at 24 h after the injury using isoflurane to remove the osmotic pumps.

Glutamate uptake assays in synaptosomes

Brain- and sham-injured animals were euthanized by decapitation and tissue from the cortex and hippocampus below the impact site was dissected and immediately frozen at -20°C . When ready to perform uptake assays, frozen brain tissue was rapidly thawed and synaptosomes were prepared to examine kinetic parameters of glutamate uptake ($V_{\max}$ and K_M). These were calculated by fitting the raw data to a Michaelis-Menten equation⁴⁴; $V_{\max}$ and K_M values were normalized to percentage of sham to control for variability among different assays.⁵⁵ The kinetics of glutamate uptake in synaptosomes from brains of naïve rats in the presence of MS-153 were analyzed using a standard dose–response curve (0.1–1000 μM of MS-153, pre-incubated for 40 min). The dose–response curves from at least three independent experiments were fitted to the Hill equation. To determine the effect of MS-153 on injury-induced alterations in kinetic parameters of glutamate uptake, synaptosomes from

TABLE 1. SUMMARY OF ANIMALS USED IN THE STUDY

Study	n	Survival time	Injury status	Treatment status	Weight (g)	Apnea (sec)
Glutamate transporter activity and expression	10	5 min–2 h	Sham	NA	372 $\pm$ 18	NA
	7	5 min	Injured	NA	381 $\pm$ 17	30 $\pm$ 7
	7	15 min	Injured	NA	378 $\pm$ 13	36 $\pm$ 12
	7	30 min	Injured	NA	389 $\pm$ 11	33 $\pm$ 13
	6	2 hours	Injured	NA	387 $\pm$ 14	31 $\pm$ 9
Histology (Fluoro-Jade B, NeuN and MAP-2 loss)	4	24 h	Sham	Saline	346 $\pm$ 17	NA
	2	24 h	Sham	MS-153 (low dose)	363 $\pm$ 19	NA
	5	24 h	Injured	Saline	379 $\pm$ 13	32 $\pm$ 16
	6	7 days	Injured	Saline	388 $\pm$ 10	38 $\pm$ 26
	5	14 days	Injured	Saline	363 $\pm$ 12	37 $\pm$ 6
	4	24 h	Injured	MS-153 (low dose)	368 $\pm$ 21	26 $\pm$ 3
	7	7 days	Injured	MS-153 (low dose)	391 $\pm$ 15	34 $\pm$ 13
	6	7 days	Injured	MS-153 (high dose)	396 $\pm$ 12	28 $\pm$ 4
	5	14 days	Injured	MS-153 (high dose)	380 $\pm$ 17	40 $\pm$ 5
	4	24 h	Sham	Saline	399 $\pm$ 30	NA
Calpain activation	4	24 h	Sham	MS-153 (low dose)	393 $\pm$ 22	NA
	7	24 h	Injured	Saline	397 $\pm$ 22	30 $\pm$ 8
	8	24 h	Injured	MS-153 (low dose)	401 $\pm$ 10	29 $\pm$ 6

Apnea was recorded from the time of impact to the time of regular breathing. Statistical analyses were performed as described in the Methods. NA, not applicable; MS-153, (R)-(–)-5-methyl-1-nicotinoyl-2-pyrazoline; MAP-2, microtubule-associated protein 2.

brain-injured rats were tested for glutamate uptake in the presence of 100 μ M MS-153

Immunoblot analysis

Analysis of GLT-1 and glutamate aspartate transporter (GLAST) expression after the injury was performed in synaptosomal preparations from brain- and sham-injured animals. Lysates from synaptosomes were subjected to SDS-PAGE (4 μ g of protein per lane) and immunoblotted with antibodies against GLT-1 (1:10,000; Millipore, Billerica, CA) or GLAST (1:5,000; AbCam, Cambridge, MA); β -actin (1:1,000; Cell Signaling, Danvers, MA) was used as loading control. Detection was performed with infrared-conjugated secondary antibodies (anti-rabbit DyLight 800, green and anti-mouse DyLight 680 red; LI-COR Biosciences, Lincoln, NE) using the Odyssey Infrared Imaging System (LI-COR Biosciences). Analysis of spectrin breakdown products (SBDP) were performed in tissue lysate from brains extracted 24 h after the injury as previously described.⁵⁶

Histological analysis

Brain- and sham-injured animals were anesthetized, transcardially perfused with heparinized saline followed by 10% formalin, and brains processed for histologic evaluation as previously described.⁵⁷ Coronal sections (40 μ m) were taken between approximately 1.5 mm and 6 mm posterior to bregma, reflecting the rostral-caudal extent of the injury. Staining and quantification of Fluoro-Jade B (FJ-B) reactivity (0.001%; Chemicon; Millipore), neuronal nucleus protein immunoreactivity (NeuN, 1:10,000, clone A60; Millipore), and loss of MAP-2 (1:5,000, clone AP-20; Sigma-Aldrich, St. Louis, MO) were performed as previously described.^{58–60}

Statistical analyses

Animal weights at the time of injury, duration of apnea immediately after injury, glutamate uptake kinetics, GLT-1/GLAST expression and SBDP generation in immunoblots, and FJ-B counts were compared using a one-way analysis of variance (ANOVA) followed by appropriate *post hoc* analyses for comparisons between groups. NeuN cell counts were compared using a two-way ANOVA followed by a Neuman-Keuls *post hoc* test, using the right hemisphere values as control, as they were similar to both hemispheres in sham animals (not shown). Loss of MAP-2 immunoreactivity was compared using a Student's *t*-test. All analyses were performed using GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, CA), with $p < 0.05$ considered significant.

Results

Three brain-injured rats (out of 83) died immediately post-injury (3.75 %). Surviving animals exhibited an apnea period of 22–60 sec with no statistical differences among groups (Table 1). Importantly, comparison of animal weights and apnea times across the groups of animals used for the various outcomes did not detect any differences (Table 1).

TBI decreased glutamate uptake activity

Lateral fluid-percussion brain injury of moderate severity resulted in a significant decrease of glutamate uptake ($V_{\max}$) in synaptosomes obtained from the site of maximal cortical injury ($F[4,26] = 10.13$; $p < 0.001$; Fig. 1A) and the hippocampus below

FIG. 1. Effect of lateral fluid-percussion brain injury on glutamate uptake and GLT-1 expression. $V_{\max}$ (nmol.mg⁻¹.min⁻¹) of ³H-L-glutamate uptake was measured in synaptosomes prepared from the injured cortex (A) and hippocampus (B) of brain-injured or sham-injured rats as described in the Methods. (C) Representative immunoblots illustrating GLT-1, glutamate aspartate transporter (GLAST) and actin expression from brain-injured or sham-injured rat synaptosomes. (D) Quantitative densitometric analysis of immunoblots. Bars represent means and standard deviation, * $p < 0.05$.

FIG. 2. Effect of (*R*)-(-)-5-methyl-1-nicotinoyl-2-pyrazoline (MS-153) on Fluoro-Jade B (FJ-B) reactivity in the cortex and hippocampus following lateral fluid-percussion brain injury. Representative photomicrographs of FJ-B-stained sections from the cortex (**A-C**) and area CA3 of the hippocampus (**D-F**) from sham-injured (**A, D**), vehicle-treated brain-injured (**B, E**) and MS-153-treated brain-injured rats (**C, F**). Note the absence of FJ-B (+) profiles in sham-injured animals (**A, D**). Scale bar in panel **D** represents 100 μ m for all panels. Quantitative analyses are illustrated in graphs (**G**) and (**H**) 1 day post-injury, (**I**) and (**J**) 7 days post-injury, and (**K**) and (**L**) 14 days post-injury. The dose and dosing of MS-153 are described in the Methods. All values are presented as means and standard deviation. $##p < 0.01$ and $###p < 0.001$, compared with sham values; $*p < 0.05$ and $**p < 0.01$, compared with saline-treated values.

FIG. 3. Effect of (*R*)-(-)-5-methyl-1-nicotinoyl-2-pyrazoline (MS-153) on NeuN immunoreactivity following lateral fluid-percussion brain injury. Representative photomicrographs illustrating NeuN immunoreactivity in the cortex from the left (injured) hemisphere (A, C) and the right hemisphere (B, D) at 24 h after injury in vehicle-treated (A, B) and MS-153-treated (C, D) brain-injured rats. Scale bar in panel C represents 100 μ m for all panels. Analyses of NeuN(+) counts in the cortex at 1 (E), 7 (F) and 14 days (G) after injury. Bars represent mean and standard deviation. # p < 0.01; ## p < 0.001.

the site of impact ($F[4,30]=2.8$; $p < 0.05$; Fig. 1B); this decrease was observed as early as 5 min in the cortex and 15 min in the hippocampus, and was sustained up to 2 h post-injury, compared with sham-injured animals. The $V_{\max}$ of glutamate uptake was not altered in the corresponding regions in the hemisphere contralateral to the impact site, compared with values for sham-injured rats (not shown). Additionally, the affinity for glutamate (K_M) was unchanged in the brain-injured samples, compared with their sham-injured counterparts (not shown). The decrease in $V_{\max}$ was not accompanied by changes in the expression of

GLT-1 or GLAST in cortical (Fig. 1C, 1D) or in hippocampal synaptosomes (not shown).

MS-153 decreased neurodegeneration after TBI

There was no FJ-B(+) reactivity visible in the cortex (Fig. 2A) and hippocampus (Fig. 2D) of sham-injured animals. However, at 24 h following brain injury, FJ-B(+) cells were observed within the site of maximal injury in the cortex (Fig. 2B, 2C) and the pyramidal layer of area CA3 of the hippocampus (Fig. 2E, 2F) in both saline-

FIG. 4. Effect of (*R*)-(-)-5-methyl-1-nicotinoyl-2-pyrazoline (MS-153) on microtubule-associated protein 2 (MAP-2) immunoreactivity and calpain activation following lateral fluid-percussion brain injury. **(A)** Representative photomicrographs illustrating showing MAP-2 immunoreactivity in sham-injured rats (upper panel) and in saline-treated, brain-injured rats after traumatic brain injury (middle panel) and MS-153-treated, brain-injured rats (lower panel). Arrow represents the site of maximal cortical injury as denoted by a loss of MAP-2 immunoreactivity. **(B)** Quantitative analysis of MAP-2 loss. * $p < 0.05$. **(C)** Representative immunoblots depicting spectrin breakdown products (SBDPs) in lysates of cortex (upper panel) and hippocampus (lower panel) from sham- and brain-injured animals. Note the presence of the 150–145 kDa doublet in brain-injured samples. **(D)** Quantification of SBDPs in cortex (upper panel) and hippocampus (lower panel). # $p < 0.05$ and ## $p < 0.01$, compared with sham values; * $p < 0.05$, compared with saline-treated values.

(Fig. 2B, 2E) and MS-153-treated animals (Fig. 2C, 2F). Quantification of FJ-B (+) profiles at 1 day post-injury revealed that MS-153 administration decreased the number of FJ-B (+) cells in the cortex ($F[2,10] = 37.2$; $p < 0.01$) by ~47% (Fig. 2G) and in the hippocampus ($F[2,11] = 37.5$; $p < 0.01$) by ~33%, compared with saline-treated brain-injured animals (Fig. 2H). Interestingly, the reduction in FJ-B (+) cells remained evident at both 7 (Fig. 2I, 2J) and 14 days post-injury (Fig. 2K, 2L). Increasing the dose of MS-153 by 100% did not increase the extent of neuroprotection, with a reduction in neurodegeneration in the cortex of ~47 and ~49% ($F[3, 19] = 14.8$; $p < 0.01$; Fig. 2I, 2J) with low and high doses of

MS-153, respectively, and ~54% and ~63% in the hippocampus ($F[3,19] = 7.6$; $p < 0.05$; Fig. 2I, 2J). After 14 days following injury and treatment with the high dose of MS-153, a reduction in neurodegeneration of ~55% was observed in the cortex ($F[2, 12] = 39.7$; $p < 0.0001$; Fig. 2K) and of ~39% in the hippocampus ($F[2, 12] = 14.3$; $p < 0.05$; Fig. 2L).

MS-153 increased neuronal survival

To determine whether reduction of FJ-B reactivity was associated with neuroprotection, the number of viable NeuN (+) neurons

was counted in the cortex. A decrease in NeuN(+) cell number was observed in the cortex below the site of impact (Fig. 3A, 3C), compared with the hemisphere contralateral to the site of impact (Fig. 3B, 3D).

At 1 day post-injury, an increase of $\sim 104\%$ in the number of NeuN (+) cells was observed in MS-153-treated, brain-injured animals, compared with their vehicle-treated counterparts (Fig. 3E). A two-way ANOVA revealed an injury effect (hemisphere, $F[1,12]=52.2$; $p<0.001$), a treatment effect ($F[1,12]=1.4$; $p<0.05$) and an interaction effect ($F[1,12]=8.7$; $p<0.05$; Fig. 3E). *Post hoc* analysis indicated a treatment effect on the left hemisphere ($p<0.05$) but not on the right hemisphere ($p>0.05$). At 7 days, significant hemisphere ($F[1,34]=31.5$; $p<0.001$) and treatment effects ($F[2,34]=4.5$; $p<0.05$) were observed in the absence of an interaction between treatment and hemisphere ($F[2,34]=0.5$; $p>0.05$). *Post hoc* analysis revealed a dose-dependent effect of MS-153 on NeuN (+) quantification in the left hemisphere: the low dose increased the number of NeuN (+) neurons by $\sim 49\%$ ($p<0.05$) and the high dose by $\sim 85\%$ ($p<0.01$), compared with vehicle-treated animals. No treatment effect was observed in the right hemisphere ($p>0.05$; Fig. 3F). At 14 days after the injury, a two-way ANOVA revealed a significant hemisphere effect ($F[1,18]=53.1$; $p<0.001$), a treatment effect ($F[1,18]=53.6$; $p<0.001$) and an interaction effect ($F[1,18]=82.1$; $p<0.001$; Fig. 3G). *Post hoc* analysis indicated a treatment effect on the left hemisphere ($p<0.05$) but not on the right hemisphere ($p>0.05$).

MS-153 decreased MAP-2 loss after TBI

Previous studies have reported that MAP-2 immunoreactivity was profoundly diminished in the cortex and hippocampus in brain-injured animals by 10 min and remains diminished up to 7 days after injury.⁶¹ MAP-2 loss is linked to the apoptotic process and is a powerful indicator of the extent of damage caused by brain injury.^{62,63} Figure 4A illustrates representative coronal sections from sham- (upper image) and brain-injured rats (middle and bottom images), with loss of MAP-2 reactivity depicted by the arrow in brain-injured animals treated either with saline (middle image) or MS-153 (bottom image), representing the area of maximal cortical injury. Quantification revealed that MS-153 significantly de-

creased the loss of MAP-2 staining, compared with saline-treatment by $\sim 41.2\%$ ($t[8]=2.3$; $p<0.05$; Fig. 4B).

MS-153 decreased calpain-mediated spectrin proteolysis in the hippocampus

Calpain activation has been implicated in the acute neurodegenerative events following lateral fluid-percussion brain trauma.⁵⁶ Figure 4C displays spectrin proteolysis in brain-injured tissue at 24 h after injury. Injured samples from the cortex and hippocampus exhibit the typical calpain-mediated spectrin breakdown products at 150 and 145kDa, which were minimally present in sham-injured samples. The relative optical density of both 150 and 145 kDa bands in the injured animals (white bars) was increased, compared with sham-injured animals (black bars, Fig. 4D). In the cortex, the 150kDa band was increased by $\sim 166\%$ ($F[2,21]=5.7$; $p<0.05$) and the 145kDa band by $\sim 138\%$ ($F[2,21]=5.1$; $p<0.05$), compared with shams. Even though MS-153 treatment does not significantly reduce SBDPs in cortex (compared with saline), SBDP expression in the MS-153-treated injured cortex was no longer significantly different from sham-injured animals. In the hippocampus, the 150kDa band was increased by $\sim 95\%$ ($F[2,21]=7.9$; $p<0.01$) and the 145kDa band by 136% ($F[2,21]=10.8$; $p<0.001$), compared with sham-injured values. Quantification revealed that MS-153 treatment (gray bars) attenuated spectrin breakdown in hippocampus of both 150kDa ($F(2,21)=7.9$; $p<0.05$), and 145kDa ($F(2,21)=10.8$; $p<0.01$) bands.

MS-153 increased glutamate transporter activity only in naïve brain preparations

The (*R*)-enantiomer of MS-153, but not the (*S*)-enantiomer, increased glutamate uptake in synaptosomes prepared from naïve rat brains in a dose-dependent manner, with an ED_{50} value of $32.3 \pm 0.4 \mu M$ (Fig. 5A). Figure 5B illustrates glutamate uptake saturation curves displaying classical Michaelis-Menten kinetics with MS-153 increasing the V_{max} of glutamate uptake by $\sim 140\%$, compared with phosphate-buffered saline-treated synaptosomes from naïve brains, with no effect on the affinity for the substrate glutamate (K_M). The V_{max} of glutamate transport in synaptosomes obtained from the cortex at 2 h after injury was decreased by 82% in

FIG. 5. Effect of (*R*)-(-)-5-methyl-1-nicotinoyl-2-pyrazoline (MS-153) on glutamate uptake in synaptosomes *ex vivo*. (A) Glutamate uptake in synaptosomes from naïve rat brains following incubation with increasing concentrations of the active (*R*)- or inactive (*S*)-enantiomer of MS-153. (B) Graph illustrates kinetic analysis of L-glutamate uptake in synaptosomes from either naïve rat brains or injured-brains pre-incubated with 100 μM MS-153. Values for V_{max} and K_M are presented in the table; K_M was not statistically different between the groups. *** $p<0.001$ drug, compared with phosphate-buffered saline buffer; ### $p<0.001$ injured, compared with naïve values.

the presence of phosphate-buffered saline, and 76% in the presence of MS-153 (100 μ M, table in Fig. 5B). A two-way ANOVA revealed an injury effect ($F[1,19]=56.7$; $p<0.001$), a treatment effect ($F[1,19]=233.7$; $p<0.001$) and an interaction effect ($F[1,19]=47.7$; $p<0.001$; Fig. 5B). *Post hoc* analyses revealed that MS-153 significantly increased $V_{\max}$ in naïve brain synaptosomes ($p<0.001$), but not in synaptosomes from injured brains. Naïve values of glutamate transport were not different from sham values (not shown).

Discussion

Lateral fluid-percussion brain injury of moderate severity resulted in a decrease in glutamate uptake activity in synaptosomes from the injured cortex and hippocampus, with no alterations in either the affinity for glutamate or the expression of GLT-1 or GLAST. This supports the idea that the elevated extracellular concentration of glutamate that has been reported following brain injury is partially a result of diminished glutamate uptake and clearance.⁹

In the present study, we did not observe changes in the total levels of GLT-1 and GLAST up to 2 h post-injury. In the hours/days following brain trauma in the rat⁶⁴ or in human,^{65,66} GLT-1 expression has been reported to decrease in the cortex and hippocampus. Further, antisense knockdown of GLT-1 exacerbates neuronal damage following contusive brain trauma in the rat.⁶⁷ The decrease in $V_{\max}$ of glutamate uptake activity in the absence of down-regulation of total levels of GLT-1 that was observed in our study suggests that the transporter may be internalized. One mechanism of rapid (within minutes) intracellular trafficking of sodium-dependent neurotransmitter transporters like GLT-1 is linked to the activation of protein kinase C (PKC),⁶⁸ which is activated post-TBI.⁶⁹ The internalization model for the decrease in activity also may explain the inability of MS-153, when incubated with synaptosomes *ex vivo*, to restore $V_{\max}$ of glutamate uptake in brain-injured samples. Future studies will confirm whether GLT-1/EAAT2 is internalized as a result of the injury using cross-linking assays.⁷⁰

Another possible explanation for the decrease in $V_{\max}$ by the injury is reversal of glutamate transport. Under physiological conditions, the conventional transport direction is inward and dependent on the Na^+ and K^+ gradients; however, in conditions when extracellular Na^+ and intracellular K^+ decrease, glutamate can be transported in the outward direction.^{71–73} Indeed, it has been reported that reversal of glutamate transport via GLT-1 is an important event in glutamate release following ischemia.^{74,75} It is well established that ion gradients that drive glutamate uptake and the enzymes, such as the Na^+/K^+ -ATPase, that maintain these ion gradients, are compromised in the traumatically-injured brain.^{76,77} Importantly, massive increases in extracellular potassium and the indiscriminate release of glutamate has been reported following concussive brain injury.⁷⁸ Nonetheless, because we assessed accumulation of radiolabeled glutamate into synaptosomes *ex vivo*, it is not possible to determine if the lower $V_{\max}$ is a result of reversal of transport.

The lack of an effect of MS-153 on synaptosomal glutamate uptake in samples from injured brains suggests that the neuroprotective effects of MS-153 seen in our study may be attributed to cellular mechanisms other than a direct effect on GLT-1 activity. MS-153 can block voltage-gated calcium channels through a mechanism that is protein kinase C (PKC)-dependent⁴⁶; MS-153 inhibits the translocation and activation of PKC- γ that leads to

the suppression of the calcium channel current.⁴⁵ Calcium channel blockers such as emopamil⁷⁹ and nimodipine⁸⁰ have been shown to be neuroprotective in TBI. Further, the decrease in calpain-mediated spectrin proteolysis would suggest that the effects of MS-153 are mediated via a reduction of calcium influx into injured cells.⁴⁶ Several classes of compounds including NMDA receptor antagonists⁸¹ and compounds that target calcium influx,^{79,82} have been shown to ameliorate cellular damage and neurologic deficits, as well as cognitive decline and motor impairments caused by TBI. In models of brain injury, glutamate receptor antagonists, such as kynurenic acid,⁸³ NBQX, and CGS 19755,⁸⁴ have been reported to partially ameliorate cytoskeletal injury with preserved immuno-labeling of MAP-2 and to reduce the accumulation of calpain-cleaved spectrin byproducts. However, these compounds have been associated with substantial adverse effects, making them less attractive for translation to patients.^{85,86} As a result, there currently are no approved pharmacological treatments available for directly halting the deleterious effects of glutamate excitotoxicity.^{87,88}

In conclusion, moderate TBI induced by lateral fluid-percussion trauma resulted in a decreased rate of glutamate uptake in the injured hemisphere, and the GLT-1 activator MS-153 reduced neurodegeneration and neuronal loss for up to 14 days. Whereas MS-153 increased the rate of glutamate uptake in synaptosomes from naïve rat brain, it did not restore the injury-induced decreased activity of GLT-1. However, the lack of involvement of GLT-1 in the neuroprotective effects of MS-153 is based on the *ex vivo* synaptosomal assays and cannot completely rule out the contribution of glutamate transport activation *in vivo* to the neuroprotective effect of MS-153. Currently, there is a lack of approved pharmacological treatments for directly reducing the effects of excitotoxicity after acute brain trauma. Also, it is worth noting that MS-153 attenuates excitotoxic effects without blocking glutamate receptors, a process known to cause severe adverse effects.¹⁹ To elucidate the role of enhanced GLT-1 activity in modifying pathological outcomes following TBI and to pursue its promise as a therapeutic target in TBI, better tools and more selective GLT-1 compounds would be required.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Andréia Cristina Karklin Fontana, PhD
Department of Pharmacology and Physiology
Drexel University College of Medicine
245 N. 15th Street
Philadelphia, PA 19102
E-mail: andreia.mortensen@drexelmed.edu