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Short Communication

Short-Duration Treatment with the Calpain Inhibitor MDL-28170 Does Not Protect Axonal Transport in an *in Vivo* Model of Traumatic Axonal Injury

Marek Ma,[1,2] Luchuan Li,[1–3] Xinran Wang,[1,2] Diana L. Bull,[1,2] Frances S. Shofer,[1] David F. Meaney,[4] and Robert W. Neumar[1,2]

Abstract

Traumatic axonal injury is characterized by early cytoskeletal proteolysis and disruption of axonal transport. Calpain inhibition has been shown to protect axons in rodent models of traumatic brain injury. However, in these models, both white and gray matter are injured, making it difficult to determine if calpain inhibitors are directly protecting injured axons. To address this issue, we used our rat optic nerve stretch model to test the hypothesis that early calpain inhibition directly protects central nervous system (CNS) axons following stretch injury. Rats were given an intravenous bolus of the calpain inhibitor MDL-28170 (30 mg/kg) 30 min prior to unilateral optic nerve stretch, followed by a 15 mg/kg/h intravenous infusion over the next 2.5 h. Immunohistochemical analysis of optic nerves 30 min after stretch injury revealed variable increases of calpain-cleaved α-spectrin that appeared less evident in stretched nerves from drug-treated rats, although this difference was not statistically significant. Retrograde axonal transport measured by Fluorogold® labeling of retinal ganglion cells was significantly impaired after stretch injury. However, there was no difference in the number of Fluorogold-labeled cells in the vehicle vs. drug treatment groups. These results suggest that early short-duration calpain inhibitor therapy with MDL-28170 is not an effective strategy to prevent disruption of axonal transport following isolated axonal stretch injury in the CNS.

Key words: adult brain injury; animal studies; axonal injury; axonal transport; traumatic brain injury

Introduction

Worldwide, traumatic brain injury (TBI) leading to death or hospitalization is estimated to affect >10 million people annually (Hyder et al., 2007). It is predicted that by the year 2020, road traffic injuries, which are currently the leading cause of TBI, will be the third leading cause of death and disability (Murray and Lopez, 1997). Traumatic axonal injury (TAI) is thought to be a major contributor to the neurological dysfunction seen after mild TBI, as well as the high morbidity and mortality seen in severe TBI patients.

Implicated in TAI, calpains are a family of Ca^{2+}-dependent nonlysosomal proteases involved in cytoskeletal remodeling, cell-cycle regulation, signal transduction, apoptosis, and necrosis (Zatz and Starling, 2005). There are two ubiquitous isoforms, calpain I and calpain II, which differ in the Ca^{2+} concentration required for *in vitro* activity. Early calpain activity after trauma is likely caused by reversible Ca^{2+} overload either caused by disruption of the axolemma, or dysregulation of ionic transport proteins (Iwata et al., 2004; Wolf et al., 2001). Directly measuring axonal Ca^{2+} immediately after TAI *in vivo* is particularly challenging. However, isolated stretch of neurites of primary cortical neurons resulted in an immediate rise in Ca^{2+} in injured processes (Iwata et al., 2004; Staal et al., 2010). Calpain activity has been reported to be elevated within minutes after experimental TAI (Büki et al., 1999; Saatman et al., 2003). In a mouse optic nerve stretch model, diffuse calpain activity measured by immunolabeling of calpain-cleaved α-spectrin (Ab38) was detected 20–30 min post-injury (Saatman et al., 2003). This signal was typically no longer detectable at 4 h post-injury. Although a direct measure of calpain activity and elevated cytosolic Ca^{2+}, immunoreactivity for calpain-cleaved α-spectrin also signals cytoskeletal proteolysis and degradation. In addition to α-spectrin, the neurofilament triplet proteins, tubulin, and tau are known calpain substrates.

In both humans and animals, trauma produces a rapid elongation or deformation of axons that generally does not

Departments of [1]Emergency Medicine and [4]Bioengineering, and [2]Center for Resuscitation Science, University of Pennsylvania, Philadelphia, Pennsylvania. [3]Department of General Surgery, Shandong University Qilu Hospital, Jinan, Shandong, P. R. China.

cause primary axotomy (immediate tearing of the axon) but leads to progressive structural damage culminating in secondary axotomy (Jafari et al., 1997; Maxwell and Graham, 1997; Povlishock and Katz, 2005; Povlishock et al., 1997; Saatman et al., 2003). Impairment of axonal transport with subsequent accumulation of transported proteins and organelles, commonly visualized using antibodies targeting ß-amyloid precursor protein (ß-APP) or nonphosphorylated neurofilament, are hallmarks of TAI. A more direct measure of transport, such as retrograde transport of Fluorogold (FG, Fluorochrome, Denver, CO) after its injection into the superior colliculus, is disrupted after optic nerve stretch (Saatman et al., 2003). It is believed that post-traumatic calpain activity *within* axons contributes to transport disruption, cytoskeletal degradation, and subsequent axotomy. However, there is also robust calpain activity in neuronal somata and dendrites after TBI (Saatman et al., 2010). Trying to measure the role of axonal calpains in whole brain TBI models is complex because many supra-axonal structures are affected. In this study, we address this issue by using an *in vivo* optic nerve stretch model, in which injury is localized primarily to axons (Ma et al., 2009). We have also demonstrated that this model is amenable to therapeutic intervention, as short-duration post-injury hypothermia reduced axonal degeneration 2 weeks after nerve stretch.

Pharmacological inhibition of CNS calpain activity *in vivo* remains challenging, and no ideal agent has emerged. However, the cell-permeable MDL-28170 is one of the most well studied calpain inhibitors, has high specificity, and has ameliorated TAI in global brain injury models. A single intravenous (IV) bolus (30 mg/kg) of MDL-28170 has been shown to reduce axonal pathology in global brain injury models (Ai et al., 2007; Büki et al., 2003; Czeiter et al., 2009). It is not known whether pharmacological protection occurs at the level of the axons, supra-axonal structures, or both, as the injury model is not selective to axons. This may have important clinical implications, as certain brain injuries may have predominant axonal versus somal/dendritic effects. There are no published studies evaluating any calpain inhibitor in the optic nerve stretch model.

The goal of this study was to determine if a calpain inhibitor strategy targeting early pathological calpain activity would prevent the disruption of axonal transport in a model of isolated TAI. Using our established rat optic nerve stretch model, we tested a pharmacological strategy previously shown to protect axons in other models of TBI. We hypothesized that early intravenous therapy with the calpain inhibitor MDL-28170 would reduce α-spectrin proteolysis and axonal transport disruption after optic nerve stretch. These results could have important implications for targeting pathological calpain activity after TBI.

Methods

All animal procedures were performed in accordance with National Institutes of Health guidelines for the care and use of laboratory animals, and were approved by our Institutional Animal Care and Use Committee.

Rat optic nerve stretch

Adult male Long–Evans rats (weight 300–400 g) underwent general anesthesia with 2.5% isoflurane and were placed on a heating pad. Anesthesia was maintained via a nose cone. A femoral venous catheter was placed by cut-down for drug therapy. To receive optic nerve injury, rats were placed in a stereotactic head holder that was positioned on the injury device (modified from Gennarelli et al., 1989). The cornea was anesthetized with 0.5% proparacaine, and the eye was kept moist at all times using sterile saline. The conjunctiva was separated from the sclera using an incision extending around the entire circumference of the eye except for the medial aspect (to minimize bleeding). A flexible thin ring-shaped plastic sling, which had a continuous loop of 4-0 monofilament nylon suture secured to it at four places 90 degrees apart, was placed behind the eye. The sling had a break in its ring so that the ends could be spread apart to allow placement behind the eye, and, after placement, the two ends of the ring were tied together. The monofilament nylon loop was then attached to the optic nerve stretch device.

The head was positioned on the injury device such that the trajectory of the retro-orbital portion of the optic nerve was aligned with the stroke of the solenoid. The sling was connected to a force transducer (LFS 270; Cooper Instruments & Systems, Warrenton, VA) mounted in series with a solenoid (Lucas-Ledex, Vandalia, OH). To ensure consistent loading, a 12 g preload, measured by the force transducer, was placed on the nerve. The magnitude of the displacement was controlled by a micromanipulator that adjusts the distance traveled by the solenoid piston. The 5.5 mm magnitude of optic nerve stretch reported here refers to piston displacement. The distance the piston traveled was significantly more than the actual stretch of the optic nerve, consistent with our past experiments in the guinea pig and rat (Bain and Meaney, 2000; Ma et al., 2009). Immediately after obtaining the preload, the solenoid was triggered to produce a rapid elongation of the nerve. Force measurements were captured at 10 kHz using LabVIEW SignalExpress (National Instruments, Austin, TX). For 5.5 mm stretch, the nerve was extended to the maximum displacement, held briefly, and then returned to its original pre-injury position within 75 ms of initially activating the solenoid. The peak displacement occurred within 25 ms after triggering the solenoid, which is consistent with the timing of peak deformation that occurs within the brain during conditions that cause diffuse axonal injury (Meaney et al., 1995). Peak force ranged from ~700 to 1000 g. Animals were excluded if injury to the globe or tearing of the optic nerve was apparent by visual inspection. Approximately 30% of optic nerves tore. Twelve animals (6 in each group) were assessed for Ab38 labeling, whereas another 12 (5 in the dimethyl sulfoxide [DMSO] group and 7 in the MDL-28170 group) were scored for FG transport.

Rectal temperature was maintained at ~36.5–37.5°C at the time of injury and then for 2 additional h. Erythromycin ophthalmic ointment was applied to both eyes in all animals, and the lids of the injured eye were sutured together for 2 days in animals surviving for 4 days, to prevent corneal drying. The contralateral uninjured eye and optic nerve were used as controls. Animals killed at 4 days received FG injections immediately after optic nerve stretch, whereas animals perfused 30 min after optic nerve stretch for Ab38 immunohistochemistry were kept anesthetized on a heating pad until perfusion.

MDL-28170 therapy

Prior to injury, a 30 mg/kg bolus of MDL-28170 (50 mg/mL DMSO; Enzo, Plymouth Meeting, PA) or equal volume of

DMSO vehicle was injected over 10–15 min. Immediately after completion of the bolus, infusion of 15 mg/kg/h MDL-28170 or equivalent volume of vehicle was initiated and continued for 2.5 h. To achieve maximum calpain inhibition at time of injury, optic nerve stretch was performed 30 min after completion of the initial IV bolus (Markgraf et al., 1998; Thompson et al., 2010).

Injection of FG into the superior colliculus

To label retinal ganglion cells (RGCs) that were capable of retrograde transport after stretch injury, animals received bilateral injections of FG into each superior colliculus. Immediately after optic nerve stretch, a midline scalp incision was made and the skull exposed by blunt dissection. Burr holes were drilled over each superior colliculus (6.8 mm posterior to bregma, 1.5 mm lateral to the sagittal suture). A total of 3 μL of 5% FG was injected over 7.5 min at two depths (3.5 and 4.3 mm). The scalp was then sutured, and animals were given 0.05 mg/kg intraperitoneal (IP) buprenorphine prior to recovering.

Immunohistochemistry for calpain cleaved α-spectrin in optic nerves

Thirty minutes after optic nerve stretch, rats were transcardially perfusion fixed with cold 1x PBS followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer. The optic nerves were exposed by dissection, removed from the head and postfixed for 2–3 h in 4% paraformaldehyde (4°C). Nerves were subsequently cryoprotected in 30% sucrose. The distal 4 mm of both the right and left optic nerves of the same animal were frozen in the same OCT block, and were cut longitudinally into 10 μm thick sections on a cryostat. Every 10th longitudinal section of the right and left optic nerve in the same animal was mounted on the same slide. After drying, slides were pretreated in 0.5% H_2O_2 and 30% methanol for 15 min at room temperature, rinsed in 1x phosphate-buffered saline (PBS), and blocked in 3% goat serum and 0.1% triton x-100. The tissue sections were incubated overnight at 4°C with Ab38 (1:20,000, kind gift from R. Siman, University of Pennsylvania), which reacts with calpain-cleaved α-spectrin. After further PBS rinses, the slides were incubated with biotinylated goat anti-rabbit secondary antibody (1:1000; Vector Labs, Burlingame, CA) for 1 h at room temperature. The enzymatic reaction was visualized using 3, 3′ diaminobenzidine tetrahydrochloride. Slides were rinsed in xylene and cover-slipped with DPX.

For semiquantitative assessment of axonal Ab38 labeling, 200 x images of the region with the most intense Ab38 labeling in every 10th longitudinal section were captured by charge-coupled device (CCD) camera attached to a Leica DM4500B (Buffalo Grove, IL). Camera and microscope settings were kept constant. The region of greatest Ab38 labeling was usually 1–2 mm from the chiasm, which is consistent with a study by Saatman and associates (2003). The scorer, who was blinded to both treatment and injury, used Adobe Photoshop to convert images to gray scale, followed by applying the invert filter so that more Ab38 staining translated to higher intensity. NIH ImageJ was then used to measure the integrated density along a line drawn perpendicular to the long axis of the nerve and across the region with the most intense Ab38 labeling. The sum of the integrated density from every 10th longitudinal section was divided by the sum of the lengths of the drawn line (width of the nerve sections). The mean value obtained from the uninjured nerve was subtracted from the injured side of the same animal. The higher the number, the more intense the staining in the injured nerve versus the contralateral uninjured nerve.

Assessment of FG axonal transport in RGCs

Four days after optic nerve stretch, rats were re-anesthetized and transcardially perfusion fixed as described previously. Retinas were removed from the eye and postfixed for 2–3 h in 4% paraformaldehyde (4°C). After washing with 1x PBS, retinal flat mounts were blocked in 10% goat serum, 1% bovine serum albumin (BSA), and 0.2% triton x-100 in 1x PBS for 1 h at room temperature. Retinas were incubated overnight at 4°C with TUJ1 (1:1000; Covance, Princeton, NJ), which labels RGCs. After further washes, retinas were incubated in Alexa 488 goat anti-mouse (1:1000; Molecular Probes, Eugene, OR) overnight at 4°C. Retinas were washed and cover-slipped with Fluoromount™.

After TUJ1 immunohistochemistry, the retina was flat mounted and viewed with a fluorescent microscope (Leica DM4500B). FG was imaged using the A4 filter cube. Images of labeled RGCs were taken in black and white (and subsequently colorized) at 200 x magnification in 12 standard fields: 1/6, 3/6, and 5/6 of the retinal radius from the center of the retina in each quadrant. FG-positive RGCs were counted by a blinded observer using Adobe Photoshop and NIH ImageJ.

Statistical analysis

The Ab38 signal intensities in the DMSO and MDL-28170 groups were compared using the Student's t test. To compare FG counts, analysis of variance in repeated measures was performed. A p-value < 0.05 for main effects and < 0.2 for the interaction space were considered statistically significant. Pairwise comparisons were specified *a priori* and were accomplished using the t test with pooling of the variance. Significance levels for pairwise comparisons were adjusted for by using the Bonferroni correction. There were two preplanned comparisons: injured versus uninjured retinas in DMSO-treated animals, and DMSO versus MDL-28170 injured retinas. For these two pairwise comparisons, a $p < 0.025$ was considered significant. Data are presented as means and standard deviations. All data were analyzed using SAS statistical software (version 9.2, SAS Institute, Cary, NC).

Results

Effect of MDL-28170 administration on calpain-mediated α-spectrin degradation

Thirty minutes after optic nerve stretch, the most intense Ab38 labeling was seen 1–2 mm from the optic chiasm (Fig. 1). In this region of intense signal, it was difficult to identify individual Ab38 labeled axons, whose diameters are $\sim$1 μm, in a 10 μm thick section. Ab38 signal was not seen in the contralateral unstretched nerve.

Based on semiquantitative analysis, injured nerves from animals treated with DMSO have variable amounts of Ab38 labeling 30 min after injury relative to contralateral uninjured controls (intensity difference = 7.6 ± 10.7). In contrast, Ab38 labeling in injured nerves from MDL-28170 treated rats was

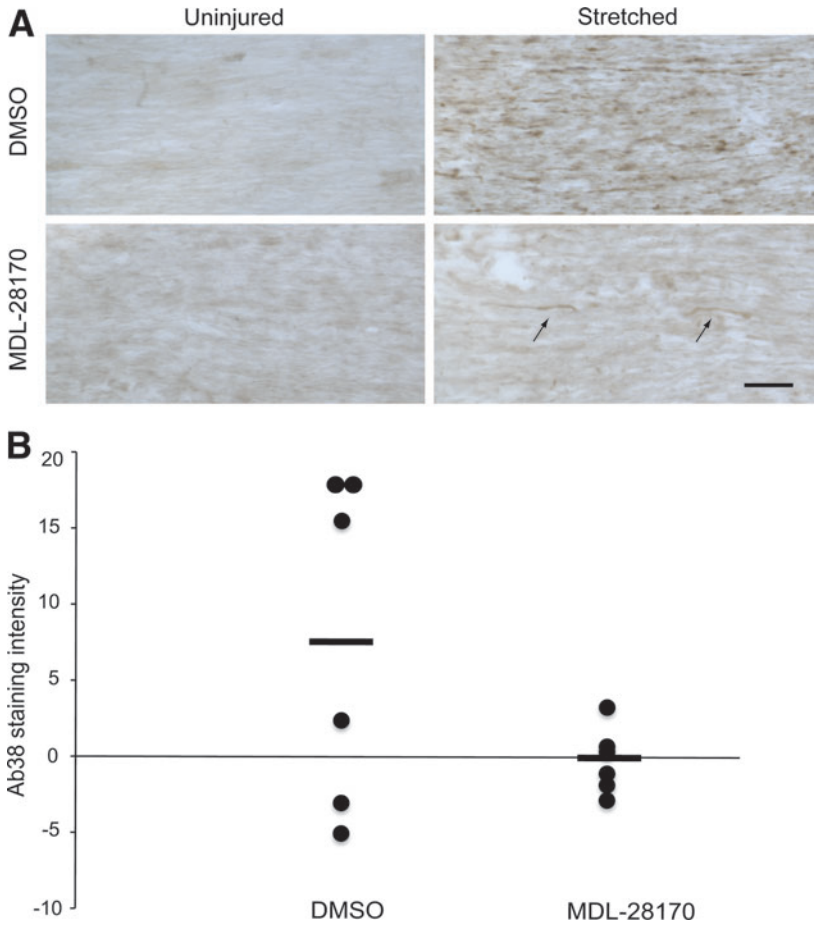

FIG. 1. Effect of intravenous MDL-28170 on calpain-mediated α-spectrin degradation 30 min after optic nerve stretch. **(A)** Ab38 immunolabeling for calpain-cleaved α-spectrin proteolytic fragments in 10 μm thick longitudinal sections of optic nerves. Animals received MDL-28170 or dimethyl sulfoxide (DMSO) vehicle alone prior to unilateral optic nerve stretch. Images near the optic chiasm were captured at 400 x magnification. Arrows highlight discrete Ab38-labeled axonal profiles in an animal treated with MDL-28170, whereas in regions of intense Ab38 labeling in DMSO animals, labeling is more diffuse. Scale bar = 20 μm. **(B)** Semiquantitative assessment of Ab38 staining intensity. Horizontal bar represents the group mean. Color image is available online at www.liebertonline.com/neu

similar to the contralateral nerve in all cases (intensity difference = -0.2 ± 2.2). However, the mean intensity of staining was not statistically different between treatment groups ($p = 0.11$).

FG-cells in the retina

As a functional outcome, we wanted to determine if the disruption of retrograde transport after optic nerve stretch could be ameliorated with calpain inhibition. Transport of FG injected into the superior colliculus has been previously reported to be impaired after optic nerve stretch in mice (Saatman et al., 2003). In our model, there was a significant *injury* effect ($p < 0.0001$) but not a *treatment* effect (MDL-28170 vs. DMSO, $p = 0.74$) or interaction (injury*treatment, $p = 0.87$). We detected a significant decrease in the number of fluorogold labeled RGCs after optic nerve stretch in animals administered DMSO, indicating disruption of retrograde axonal transport ($p = 0.002$; Fig. 2). However, pre-injury short-duration MDL-28170 therapy was not protective relative to DMSO-injured retinas ($p = 0.93$). The percentage decrease in FG-labeled RGCs after optic nerve stretch (comparing injured to its contralateral uninjured nerve) was $66 \pm 30\%$ in DMSO-treated rats versus $64 \pm 25\%$ in MDL-28170 treated rats.

Discussion

Overall, we were unable to demonstrate statistically significant inhibition of early post-injury calpain activity or long-term protection of axonal transport with intravenous MDL-28170 pre-treatment in our rat optic nerve stretch injury model. Unlike the effects demonstrated in other models of TBI, our results suggest that early calpain inhibitor therapy with MDL-28170 might have limited potential in directly preventing disruption of axonal transport caused by isolated axonal stretch injury.

Calpain inhibition in TBI

Intravenous MDL-28170 has been demonstrated to penetrate into the parenchyma of uninjured brains as well as having neuroprotective properties after ischemic and traumatic brain injury (Markgraf et al., 1998; Thompson et al., 2010). The blood–optic nerve barrier is similar in structure to the blood–

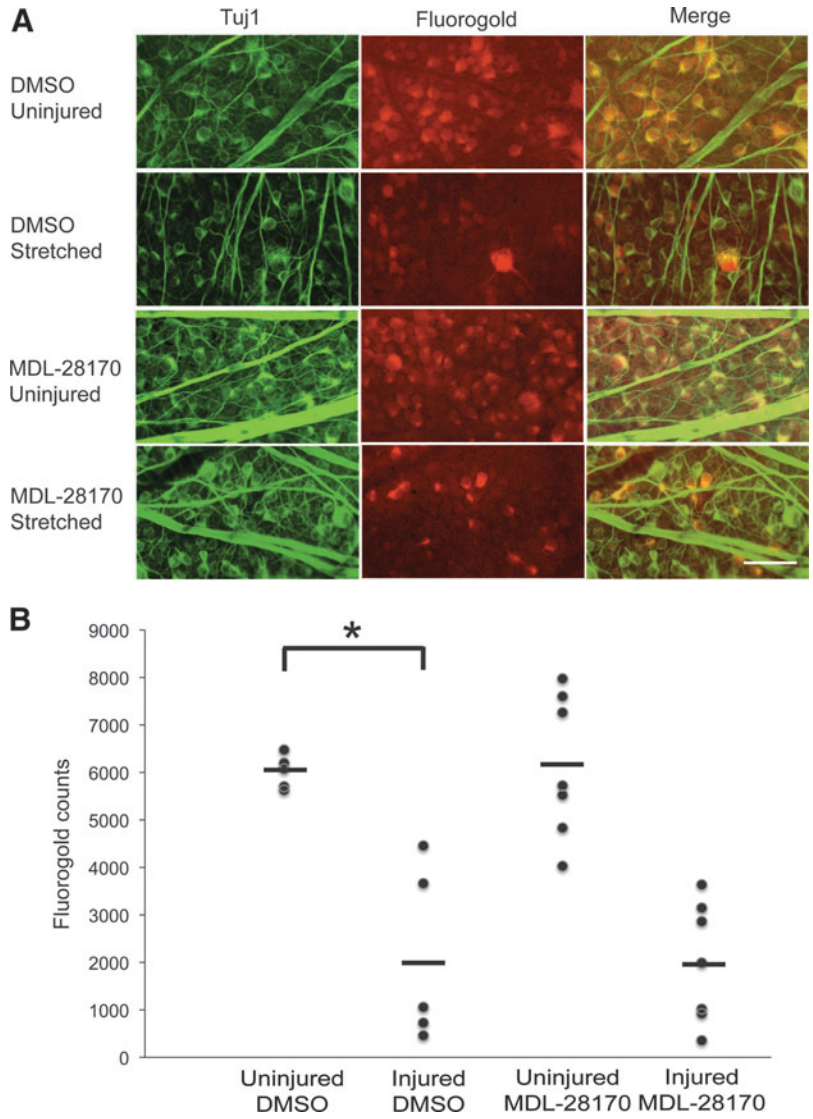

FIG. 2. Effect of intravenous MDL-28170 on axonal transport disruption 4 days after optic nerve stretch. **(A)** Representative images captured at 200 x of retinal flat mounts immunolabeled for tuj1 antibody (neuron-specific class III β-tubulin), a marker of retinal ganglion cells (RGCs, green). Fluorogold is pseudocolored red. Animals received MDL-28170 or dimethyl sulfoxide (DMSO) vehicle alone prior to unilateral optic nerve stretch and fluorogold injection. Scale bar = 50 μm. **(B)** Quantification of RGCs that were able to transport Fluorogold from the superior colliculus after unilateral optic nerve stretch. Horizontal bar represents the group mean. *$p = 0.002$ when comparing uninjured and injured DMSO retinas. Color image is available online at www.liebertonline.com/neu

brain barrier (Zhao et al., 2007). A single 30 mg/kg IV dose of MDL-28170 30 min prior to impact head acceleration injury in rats reduced ß-APP and RMO-14 staining in the corticospinal tract and medial longitudinal fasciculus 2 h post-injury (Büki et al., 2003). RMO-14 is believed to indicate pathological proteolysis or dephosphorylation of neurofilament side-arms. Czeiter and associates (2009) subsequently used the same dosing strategy, which resulted in improved axolemmal integrity in the corticospinal tract 2 h post-impact head acceleration injury. Longer-term protection was also achieved with a single 30 mg/kg IV dose of MDL-28170. Given 30 min prior to fluid percussion injury in rats, there was preservation of compound action potential and decreased ß-APP labeling of corpus callosum up to 7 days after injury (Ai et al., 2007).

Trying to tease apart the role of axonal calpains in *in vivo* whole brain TBI models is complex, as the traumatic event may proximately damage nuclei, dendritic fields, nerve tracts, glia, and vasculature (Ma et al., 2009). The interplay between the various brain structures does not allow the investigator to isolate the specific mechanism of TAI. Direct injury to neuronal cell bodies and their dendritic fields, glial cells, or vasculature, as well as elevated intracranial pressure and secondary ischemia, could have secondary effects on axons. These limitations can be significantly overcome in the optic nerve stretch model. The optic nerve shares similar properties with white matter tracts within the brain, including poor regenerative capacity, myelin sheath structure, and the presence of oligodendrocytes (Cho et al., 2005; Hirano and Lena, 1995).

Axonal transport disruption after optic nerve stretch

The characteristics of axonal injury and degeneration seen after optic nerve stretch mimic those found in established whole brain models of axonal injury, as well as in human brain after TBI (Ma et al., 2009). Axonal swellings and bulbs are hallmarks of TAI, and are thought to result from primary axonal transport disruption. In a murine optic nerve stretch model, ~60% of axons failed to transport FG from the superior colliculus (Saatman et al., 2003). Retrograde axonal transport of FG was also impaired after experimental closed head injury in mice (Creed et al., 2011).

We were unable to show an improvement of retrograde axonal transport of FG after unilateral optic nerve stretch in animals given MDL-28170. Maxwell and Graham (1997) demonstrated in the guinea pig optic nerve stretch model that there was a dramatic loss of microtubules throughout axons 15 min post-injury. As microtubules are known calpain substrates, an attractive hypothesis is that calpains proteolyze microtubules, resulting in transport disruption. Our short-duration intense therapy was targeted toward these early events. It may be necessary to inhibit calpains for a longer duration.

Limitations

We attempted to maximize the total dose of MDL-28170 by giving a 30 mg/kg bolus followed by 15 mg/kg/h infusion for 2.5 h. However, it is possible that the resulting MDL-28170 concentration in the optic nerve axons was not sufficient to inhibit pathologic calpain activity. Based on available evidence, we do not believe a higher dose would be feasible in our model. During the initial MDL-28170 bolus, ~15–20% of rats had transient bradycardia and respiratory difficulty, and deaths occurred when we tried to increase the infusion rate. These adverse effects were not seen when DMSO was administered alone. In rabbits, a 60 mg/kg IV infusion of MDL-28170 over 10 min has been reported to be lethal (Neumar et al., 1998). MDL-28170 doses >30 mg/kg IP were lethal in mice (Üçeyler et al., 2010).

The results of this study do not demonstrate a statistically significant decrease of Ab38 labeling 30 min after optic nerve stretch in animals treated with MDL-28170 versus DMSO alone. However, half of the animals in the DMSO group had high Ab38 intensity in the stretched nerve, whereas all the animals in the MDL-28170 group had similar intensity in the stretched and contralateral uninjured nerve. The variability of pathological calpain activity in the DMSO group highlights a limitation of the model, which makes it more difficult to find a statistically significant treatment effect.

Conclusion

There is growing evidence that inhibiting calpains in experimental TBI models protects against axonal pathology. However, it is not known where in the neuron these inhibitors exert their effects. We attempted to address this question by using a well-studied calpain inhibitor in an *in vivo* model of isolated axonal stretch injury. Using this model, we were not able to detect a therapeutic benefit of short duration intravenous MDL-28170 therapy. It is believed that after mild TBI, long white matter tracts are preferentially injured, and this loss of "connectivity" may underlie the persistent symptoms seen in this group of patients. Axonal injury is offered as an explanation as to why some patients after trauma are comatose even when their brains lack mass lesions (Gennarelli et al., 1982). Therefore, continued work is needed to determine if mechanisms of axonal degeneration differ between isolated white matter injury and predominantly gray matter or mixed injury. If significant differences exist, then it might be necessary to individualize therapeutic strategies to target the appropriate injury pathway.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Marek Ma, M.D.
Department of Emergency Medicine
University of Pennsylvania
3400 Spruce Street, Ground Ravdin
Philadelphia, PA 19104

E-mail: mamarek@uphs.upenn.edu