Front. Biol. 2015, 10(5): 427–438
DOI 10.1007/s11515-015-1373-z

RESEARCH ARTICLE

Targeting ERK1/2-calpain 1-NF-κB signal transduction in secondary tissue damage and astrogliosis after spinal cord injury

Xin Xin Yu[*], Vimala Bondada, Colin Rogers, Carolyn A. Meyer, Chen Guang Yu (✉)

Spinal Cord and Brain Injury Research Center, Department of Anatomy and Neurobiology, University of Kentucky College of Medicine, Lexington, KY 40536-0509, USA

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Abstract **Neuronal damage, glial inflammation, and astrogliosis/astroglial scar formation are major secondary injury mechanisms that are significant contributors to functional deficits after spinal cord injury (SCI). The objectives of the study were to evaluate the distinct roles of ERK2 vs. ERK1/2 and ERK1/2-calpain 1 – NF-κB signal transduction in the tissue damage and astrogliosis/astroglial scar formation following SCI in rats. RNAi approaches, pharmacological intervention (U0126), Western blot analysis, immunofluorescence analysis, and histological assessment were used to target ERK1/2-calpain 1-NF-κB signal transduction pathway for neuroprotection. Histological staining analysis demonstrated that selectively reducing pERK2 using ERK2 siRNA, but not inhibition of pERK1/2 with U0126, significantly reduced lesion volume and improved total tissue sparing, white matter sparing, and gray matter sparing in spinal cord two weeks after contusive SCI. An ERK1/2-calpain 1-NF-κB signal transduction pathway was involved in the astroglial scar formation after SCI. Blockade of ERK1/2 by U0126 decreased calpain 1 expression 4 h following SCI. Selective calpain 1 reduction by lentiviral shRNA attenuated astroglial NF-κB activity and astroglial scar formation after SCI in rats. Taken together, these results demonstrate the involvement of individual ERK2 and calpain 1 signaling pathways in tissue damage and astrogliosis/astroglial scar formation in animal models of SCI. Therefore, targeting individual ERK and its downstream signal transduction of calpain 1-NF-κB may provide greater potential as novel therapeutics for minimizing tissue damage and astroglial scar formation following SCI.**

Keywords calpain 1, ERK1/2, RNAi, neurodegeneration, astrogliosis, spinal cord injury

Introduction

Traumatic spinal cord injury (SCI) often causes locomotor and sensory deficits due to the damage to myelinated fiber tracts in white matter and the loss of interneurons and motoneurons in gray matter as well as sustained reactive astrogliosis/astroglial scar formation (Bramlett and Dietrich, 2007; Kwon et al., 2010; Karimi-Abdolrezaee and Billakanti, 2012; Yuan and He, 2013; Ishikawa et al., 2014). Following traumatic SCI, there is an urgent need for therapeutics to minimize the tissue damage, astroglial scar, and functional deficits. Traumatic SCI results indirect early tissue damage (primary injury) and delayed secondary gray matter and white matter loss as well as glial inflammation/glial scar formation that spread both rostral and caudal to the lesion epicenter with increasing functional deficits (Borgens and Liu-Snyder, 2012; Yiu and He, 2006). Secondary injury cascades in cells and tissues are more responsible for cell death and functional deficits over hours to weeks after the initial insult. The proper elucidation of how the spinal tissue is loss and how astroglial scars are formed following spinal cord trauma are fundamental to development of effective therapies to human SCI. Therefore, identifying the signal transduction cascade underlying secondary spinal tissue damage and astroglial scarring is of extreme importance. Excessive calpain activation and extracellular signal-regulated kinase 1 and 2 (ERK1/2) activation are two important secondary injury mechanisms

Received September 3, 2015; accepted September 11, 2015
Correspondence: Chen Guang Yu
E-mail: cyu4@uky.edu

[*]Current work location: Department of Neurology, Cleveland Clinic Foundation, Cleveland, OH, USA

(Yu and Yezierski, 2005; Zhao et al., 2007; Ray et al., 2003; Yu and Geddes, 2007; Zhao et al., 2007; Doshi and Lynch, 2009; Wall et al., 2009; Saatman et al., 2010; Sribnick et al., 2010; Yu et al., 2010).

ERK1 and ERK2 are mitogen-activated protein (MAPK) family members that are encoded by distinct genes, but share 83% homology at the amino acid levels (Cargnello and Roux, 2011). Activation of ERK1/2 by phosphorylation of threonine and tyrosine residues are associated with neuroprotection (Hetman and Gozdz, 2004), but several studies also suggest a neurodegenerative role of ERK1/2 (Zhuang and Schnellmann, 2006). SCI results in sustained activation of ERK1/2 (Yu and Yezierski, 2005; Zhao et al., 2007; Yu et al., 2010). Whether an increase in ERK1/2 activity is neuroprotective or detrimental is unclear.

Calpains are a family of Ca^{2+} -activated cysteine proteases which cleave their substrate proteins at discrete sites to modulate protein activity (Saatman et al., 2010). When excessively activated, such as following SCI, calpains are strongly implicated in the resultant neurodegeneration (Ray et al., 2003). The best characterized calpains, and those most abundant in the CNS, are the ubiquitous m- and μ-calpains. These are heterodimers consisting of a unique 80 kDa large subunit (Calpain 1 and 2) and a common 28 kDa small subunit (Calpain small subunit 1, also referred to as Calpain 4). Calpains also serve essential physiologic roles including signal transduction, cell migration, membrane fusion, and cell differentiation (Kuchay and Chishti, 2007; Liu et al., 2008; Doshi and Lynch, 2009).

Both ERK1/2 activation and calpain activation are involved in the pathology and physiology of SCI. The challenge is to inhibit the pathological consequences of their activation but preserve their physiological functions. Small molecule calpain inhibitors have been used previously, with promising but modest results (Schumacher et al., 2000; Ray et al., 2003; Colak et al., 2009). Although some findings are encouraging and calpains are clearly druggable targets, the problems associated with many of the inhibitors (weak potency, poor aqueous solubility, and short half-life (Springer et al., 1997; Zhang et al., 2000), has precluded investigations of calpain inhibition following SCI progressing to clinical trials.

Recent studies demonstrated that the individual calpain or ERK isoform signal transduction may have distinct functions in the secondary tissue damage and glial cell proliferation (Agrawal et al., 2006; Geddes and Saatman, 2010; Yu, 2012), emphasizing importance of identifying the influence of individual ERK and calpain isoforms and their signal transduction in the pathophysiology of SCI. However, the contribution of either individual ERK or calpain isoforms or their signal transduction to the secondary tissue damage and glial scar formation is unknown. With the recent advances in siRNA methodology, distinct roles for the individual calpain or ERK isoforms have begun to emerge (Geddes and Saatman, 2010; Yu, 2012). The objectives of this study were to: 1) identify the role of selective ERK2 inhibition vs. ERK1/2 inhibition and ERK1/2-calpain 1-NF-κB signal transduction in secondary tissue damage and astrogliosis/astroglial scarring and 2) develop siRNA therapeutics for minimizing secondary tissue damage and astroglial scar formation in rat model of SCI.

Materials and methods

Animals

Female Long-Evans adult rats at 3 months, weighing 200–250 g were used for contusion injury model (Charles River, Indianapolis, IN). Animals were kept under standard housing conditions for at least 1 week following arrival. All experimental procedures were approved and carried out in accordance with the Guidelines of the US National Institutes of Health and Institutional Animal Care and Use Committee (IACUC) of the University of Kentucky.

Chemicals

Phospho-ERK1/2 antibody, ERK1/2 antibody, and ERK2 antibody were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Calpain 1 antibody was purchased from Chemicon (Temecula, CA). Phospho-NF-κB p65 antibody was purchased from abcam (Cambridge, MA). GFAP antibody was purchased from Santa Cruz (Dallas, Texas). GAPDH antibody was purchased from Sigma-Aldrich (St. Louis, MO). Lentiviral vector (lentiLox 3.7 vector) was purchased from ATCC (Manassas, VA). *In vivo* jetPEI was purchased from Polyplus Transfection, Inc. (New York, NY). Osmotic mini-pump Alzet models 1003D and 2001 were purchased from Alzet (Durect, Cupertino, CA). ON-TARGET plus Non-Targeting siRNA was purchased from Thermo Scientific-Dharmacon Products (Lafayette, CO).

U0126 administration

To determine the role of pERK1/2 in the secondary damage and its downstream target after SCI, ERK1/2 inhibitor U0126 was administered to inhibit ERK1/2 phosphorylation and calpain 1 expression in spinal cord. U0126 was intraperitoneally (IP, 10 mg/kg) or intravenously (IV, 2 mg/kg) injected to injured female rats weighing 200–250 g at 90 min pre-injury daily for 1 day or 7 days post-injury. U0126 was dissolved in DMSO at 250× their final concentration and diluted in 0.1% PBS (vehicle).

Intrathecal (IT) delivery of EXK2 siRNA

To examine the role of individual ERK 2 inhibition with ERK2 siRNA vs. ERK1/2 inhibition by U0126 in secondary damage and develop a clinically relevant siRNA therapeutic strategy for SCI, we established intrathecal delivery of ERK2

siRNA after SCI in rats. The ERK2 siRNA sequences (Sense, start 522, 5′pGCACCUCAGCAAUGAUCAUdTdT3′ and antisense 5′pAUGAUCAUUGCUGAGGUGCdTdT3′) (GenBank accession number: M64300) were selected, chemically modified, and synthesized in the "ready-to-use *in vivo*" and modification option with off-target control by Thermo Scientific-Dharmacon Products (Lafayette, CO) as described in our previous study (Yu et al., 2010). ON-TARGET plus Non-Targeting siRNA was used as the negative control siRNA (Dharmacon). The ERK2 or control siRNAs were mixed with jetPEI (Polyplus Transfection, Inc.) as previously described in www.Polyplus-transfection.com. Briefly, for 1 µg/d of siRNA (1 µl/h), 8 µg of siRNAs were diluted into 48 µl of 10% glucose solution and the volume adjusted to 96 µl with pure sterile water. 10 µl of *in vivo* jetPEI were dissolved in the same buffer for 10 min. The 96 µl of *in vivo* jetPEI solution were added to the 96 µl of siRNA solution. The 50 µl of *in vivo*-jetPEI solution were added to the 50 µl siRNA solution. Relative amounts of siRNA to carrier were 10 equivalents of PEI per siRNA. siRNA-polymer complexes were allowed to form 15 min at room temperature before infusion (www.polyplus-transfection.com). Strategies for reducing off-target effects and increasing specificity included the following: On-TARGET plus siRNA modification, BLAST analysis, comparison of two more different siRNA sequences on target gene knockdown and use of negative control siRNAs (mismatch sequence of siRNA) as described previously (www.dharmacon.com) (Yu et al., 2010, 2013).

Immediately post-injury, a small hole was made by laminectomy at L2 and the injured rats were implanted with a "pre-charged" osmotic mini-pump (Alzet) underneath the back skin and the attached tube into the lesion site, T10 level, according to the protocol described in the manufacture manual (http://www.alzet.com). The osmotic mini-pump were filled with ERK2 siRNA/jetPEI or control siRNA-jetPEI complexes for infusion at 1 µg per day at a rate of 1 µl/h (1 µg per day, 0.042 µg/µl) (Alzet models 1003D and 2001, Durect, Cupertino, CA), according to the protocol described in the manufacture's manual (http://www.alzet.com). Verification of the spinal location of the terminal end of the catheter was performed at the time the animals were euthanized by injecting methylene blue dye through the catheter. To verify pump delivery after *in vivo* use, the residual volume in the pump reservoir was measured after explantation as described in http://www.alzet.com.

Lentiviral calpain 1 shRNA construction and intraspinal gene transfer

To identify the calpain 1 downstream target astroglial NF-κB activation and astrogliosis/scar formation, intraspinal gene transfer of lentiviral calpain 1 shRNA was performed after contusive SCI in rats. Calpain 1 siRNA sequences (Sense, start 5′-GCACAAUCAGAGCACUUUAUC-3′) and mismatch control siRNA (5′-GCAGAAUCACAGCA-CUUUAUC-3′) were selected, chemically modified, subjected to BLAST analysis, and synthesized in the modification option with off-target control by Thermo Scientific-Dharmacon Products (Lafayette, CO) as described in our previous study (Yu et al., 2013). Construction of calpain 1 shRNA vectors (lentiLox 3.7 vector), production of lentiviral calpian 1 shRNA (LV-calpain1 shRNA) or control shRNA viruses, and validation of calpain 1 knockdown were performed as described in our previous study (Yu et al., 2013). LV-calpain1 shRNA or control shRNA were administered by convection enhanced delivery (CED) system (Quintessential Stereotaxic Injector, Stoelting CO, Wood Dale, IL, USA, www.stoeltingco.com) at T10. Intraspinal diffusion of LV-calpain1 shRNA or LV-control shRNA was verified using Western blot and fluorescent microscopy.

Contusion SCI model

Female Long-Evans rats, weighing 200–250 g (3 months old) received a spinal contusion injury. Contusion SCI was produced following a T10 laminectomy using Infinite Horizons impactor device (Yu et al., 2010; Yu et al., 2013; Yu et al., 2014). A moderate injury (180 kdyn setting for rats) was produced. No significant differences in impact parameters (actual force, displacement, and velocity) were found between treatment and control groups (data not shown), indicating similar injuries to all animals. The surgical procedure and post-operative care were similar to that described in our previous studies (Yu et al., 2010; Yu et al., 2013; Yu et al., 2014). Female rats were used due to their shorter urethra which facilitates post-injury manual bladder expression.

Spinal cord tissue processing

For Western blot, animals were euthanatized by pentobarbital (100 mg/kg for rat, ip injection) and decapitation. A 5-mm spinal cord centered on lesion site was removed and snap-frozen on dry ice, then stored at −80°C. For lesion/tissue assessment and immunofluorescence staining, following anesthesia, the rats were transcardially perfused with ice-cold saline followed by phosphate-buffered 4% paraformaldehyde. The spinal cords were removed, cut into blocks according to the segments, and post fixed with the same fixative overnight. The tissue blocks were processed for frozen/cryostat sections.

Assessment of total tissue sparing, gray matter sparing, and white matter sparing

The fixed spinal cord was prepared for histological staining assessment as previously described (Rabchevsky et al., 2002; Yu and Geddes, 2007; Yu et al., 2013; Yu et al., 2014). Spinal cords were serially cryosectioned at a thickness of 20µm.

Every fifth section was mounted onto gelatin-coated slides and stored at −20°C. A modified eriochrome cyanine staining (EC staining) protocol for myelin that differentiates both white matter and cell bodies was used to visualize spared spinal tissue. Area measurements in lesion, gray matter, white matter, and total spinal tissue and calculation of total tissue sparing, white matter sparing, and gray matter sparing in transverse sections of the injured cords were performed as previously described (Rabchevsky et al., 2002; Yu and Geddes, 2007).

Western blots analysis

The spinal cord sampleswere homogenized in a RIPA lyses buffer and sonicated as described previously (Yu et al., 2010; Yu et al., 2013).The protein samples were obtained by microcentrifugation at 14000 rpm for 10 min. Protein quantities were determined using the BCA method. Western blotting was performed as described in our previous studies (Yu et al., 2010; Yu et al., 2013) using LI-CDR Odyssey infrared imaging system (Lincoln, NE). Briefly, spinal cord protein samples were loaded on SDS-PAGE gels and electrotransfered to nitrocellulose membranes. Blots were probed with primary polyclonal antibody against specific target (1:200 or 1:1000) and reprobed with monoclonal antibody against GADPH (1:1000, Sigma). Blots were then incubated with infrared-labeled anti-rabbit or anti-mouse secondary antibodies (1:5000). All blots were visualized and analyzed on the Li-Cor Odyssey infrared imaging system (see http://biosupport.licor.com/docs/whatsnew/Western_07737.pdf for a detailed protocol). This system provides for improved quantitation of western blots, as compared to X-ray film or chemiluminescent-based imaging systems. The primary antibodies used in this study include antibodies against calpain 1 (Chemicon, Temecula, CA), pERK1/2, ERK1/2, and ERK2 (Cell Signaling Technology, Inc. Danvers, MA), and GAPDH (Sigma-Aldrich, St. Louis, MO).

Immunofluorescence staining analysis

The fixed spinal cords were serially cryosectioned at 20 µm. Immunofluorescence staining was performed using Cell signaling Technology protocols as previously described (Yu et al., 2014). Briefly, spinal cord sections were double-immunolabeled with polyclonal antibody against pNFkBp65 (1:300, Abcam, Cambridge, MA) or monoclonal antibody against GFAP (1:200, Santa Cruz, Dallas, Texas) followed by incubation with Alexa Fluor 594 or 488 conjugated secondary antibodies. The GFAP-positive signal (marker for astrogliosis/astroglial scar formation) and NF-κBp65-positive/GFAP-positive/hoechst-positive signal (marker for astroglial nuclear NFκBp65 activity) at 2 mm rostral and caudal to the lesion epicenter were analyzed using a DSU fluorescent microscopy image system (8 sections separated 100 µm apart per animal, n = 6 animals per group). The analysis of GFAP expression and NF-κB activation at the 2 mm rostral and caudal border of the lesion site were chosen because a growth-inhibiting environment at the injury site blocks injured fibers to penetrate the caudal border of the lesion site. For each section, four areas (shown in Fig. 2C) were imaged and the total fluorescent signal (histogram) from all four areas were calculated. For each animal, the calculated total fluorescent signal for GFAP expression or astroglial nuclear NFκBp65 activity was averaged. The average signal per section of all animals in each group was then averaged to obtain a mean value per section.

Experimental groups

For U0126 treatment, female Long-Evans rats were randomly assigned to the following groups: (A) U0126 plus SCI and (B) Vehicle plus SCI. Western blot analysis was used to measure protein levels of ERK1/2 phosphorylation and calpain 1 at 4 h post-injury (n = 4/group). Histological (EC staining) analysis was used to assess tissue sparing 1 week post-injury (n = 4/group).

For ERK2 siRNA treatment, female Long-Evans rats were randomly assigned to the following groups: (A) SCI plus ERK2 siRNA and (B) SCI plus control siRNA. Western blot analysis was used to measure protein levels of ERK1/2 phosphorylation at 2 days post-injury (n = 4/group). Histological (EC staining) analysis was used to assess tissue sparing 2 week post-injury (n = 4/group).

For effect of lentiviral shRNA pretreatment on NF-κB activation and astrogliosis/astroglial scar formation after contusive SCI in rats: Female Long-Evans rats were randomly assigned to the following groups: (A) Sham (laminectomy without injury), (B) LV-Control shRNA plus SCI, and (C) LV-calpain 1 shRNA plus SCI. Immunofluorescence staining and confocal microscopy were used to observe whether LV-calpain 1 shRNA intraspinal administration results in reduction in astroglial scar formation and NF-κB expression in astrocytes of spinal cord at 6 weeks post-injury (n = 6/group).

Statistical analysis

Statistical analysis was performed using StatView (SAS Institute, Cary, NC). Data are presented as mean±S.E.M. Group differences were evaluated by t-test or repeated measures ANOVA and Bonferroni post hoc test. Null hypotheses were rejected at the $p < 0.05$ level.

Results

Effects of U0126 administration on pERK1/2 activation and calpain 1 expression after SCI in rats

U0126 pretreatment (IP 10 mg/kg and IV 2 mg/kg at 90 min prior to injury) significantly decreased levels of ERK1/2

phosphorylation (Fig. 1) and calpain 1 expression (Fig. 2) in spinal cordat 4 h in spinal cord following SCI in rats, compared to vehicle-pre-treated animals (*$p < 0.5$, ANOVA and Bonferroni post hoc test). The results suggest that U0126 administration reduced contusion injury-induced pERK1/2 activation and calpain 1 expression in spinal cord after SCI in rats.

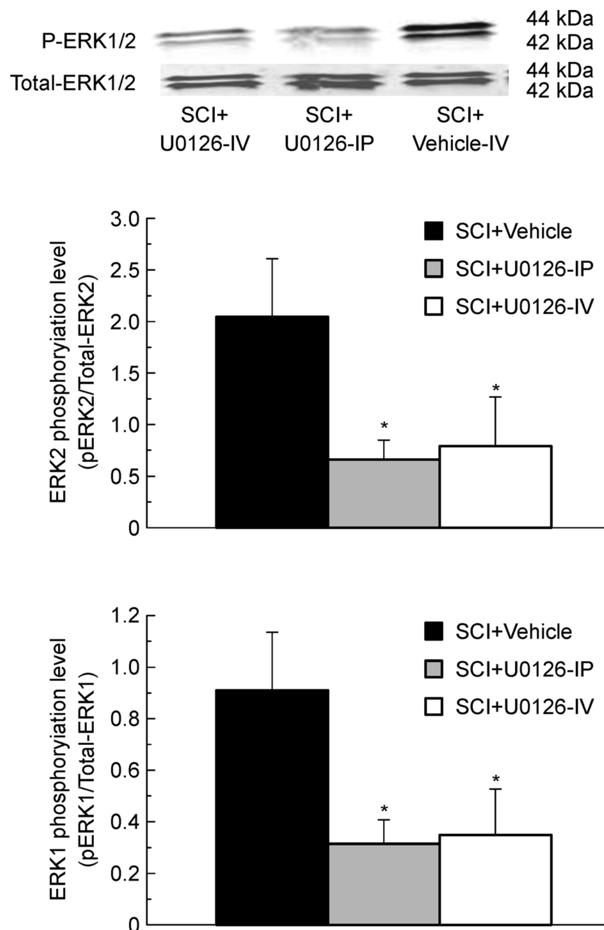

Figure 1 Effect of U0126 on ERK1/2 phosphorylation in rat spinal cord after contusive SCI. U0126 ip injection (10 mg/kg, gray bar) or iv injection (2 mg/kg, empty bar) 90 min prior to SCI resulted in inhibition of ERK1/2 phosphorylation in the spinal cord 4 h post-injury using Western blotting with antibodies specific for phospho-ERK1/2 (top panel) or total ERK1/2 (bottom panel). Data are presented as the mean±S.E.M. and analyzed with repeated measures ANOVA followed by Bonferroni post-hoc analysis. Differences were considered statistically significant at $p < 0.05$. *$p < 0.05$, compared to vehicle-treated animals, $n = 4$/group.

Effects of U0126 administration on neuronal/axonal damage after SCI in rats

Histological staining analysis demonstrated that IV administration of U0126, but not IP administration, resulted in slight and non-significant improvements in total tissue sparing and white matter sparing in spinal cord at 7 days after moderate contusive SCI in rats, compared to vehicle-pretreated animals (Fig. 3). This dada suggest that ERK1/2 inhibition by U0126 results in modest reduction in tissue damage after SCI in rats.

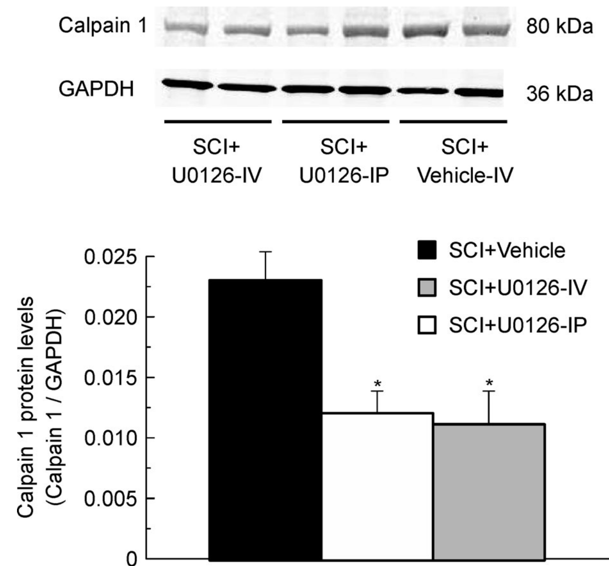

Figure 2 Effect of U0126 on calpain 1 expression in spinal cord after contusive SCI in rats. U0126 ip injection (10 mg/kg, gray bar) or iv injection (2 mg/kg, empty bar) 90 min prior to SCI resulted in reduction in calpain 1 expression in the spinal cord 4 h post-injury using Western blot blotting with antibody specific for calpain 1. Data are presented as the mean±S.E.M. and analyzed with repeated measures ANOVA followed by Bonferroni post-hoc analysis. Differences were considered statistically significant at $p < 0.05$. *$p < 0.05$, compared to vehicle-treated animals, $n = 4$/group.

Effects of ERK2 siRNA on pERK1/2 activation after SCI in rats

Western blot analysis demonstrated that post-injury ERK2 siRNA intrathecal treatment (ERK2 siRNA/jetPEI via minipump, 1.0 µg/d for 2 days) reduced the levels of both ERK2 protein (B) and ERK2 phosphorylation (C) in spinal cord 2 days following contusive SCI using antibody specific for ERK2 or pERK1/2 (Fig. 4, $p < 0.05$, *t*-test). The ERK2 siRNA does not alter pERK1 levels in spinal cord using Western blot with antibody against pERK1 (D).

Effects of ERK2 siRNA on neuronal/axonal damage after SCI in rats

Histological assessment showed that post-injury administration of ERK2 siRNA/jetPEI via minipump (1.0 µg/d for 7 days) resulted in significant increases in total tissue sparing (A), total white matter sparing (B) and total gray matter sparing (C) as well as tissue sparing (D) and white matter sparing (E) at 1 to 4 mm rostral and 1 to 2 mm caudal to the injury epicenter following contusion injury to the spinal cord,

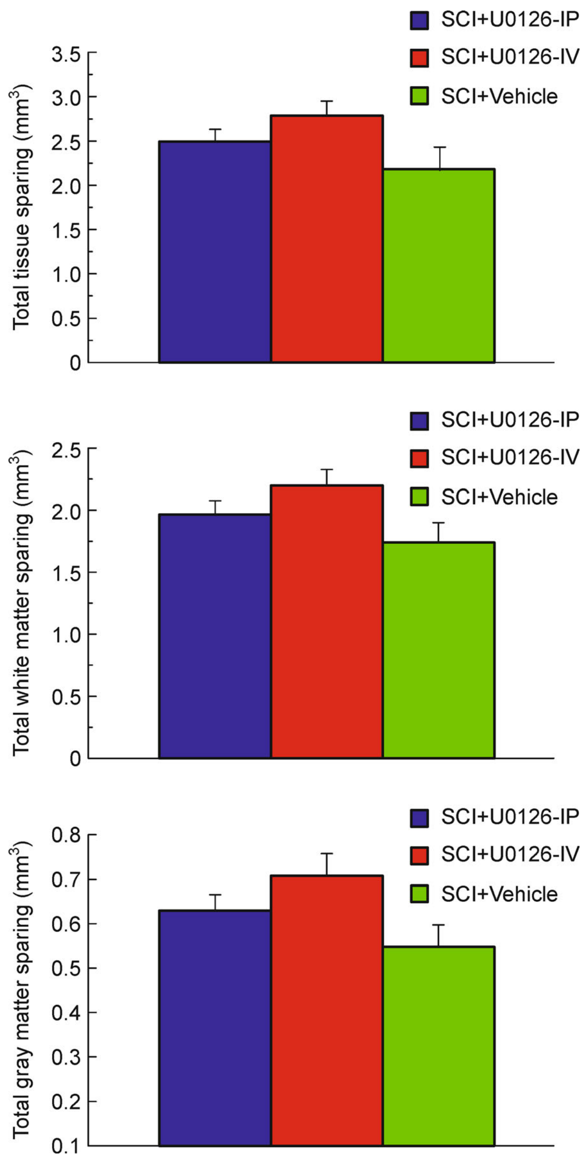

Figure 3 Effects of U0126 on tissue sparing 1 week following moderate contusive SCI in rats. U0126 IV administration, but not IP administration, starting at 90 min pre-injury, then once daily for 7 days post-SCI (180 kdyn) resulted in a modest non-significant increase in total tissue sparing and white matter sparing 1 week post-injury compared to vehicle-treated animals. Data are presented as the mean±S.E.M. and analyzed with repeated measures ANOVA followed by Bonferroni post-hoc analysis. Differences were considered statistically significant at $p < 0.05$, $n = 4$/group.

compared with control siRNA treated group (Fig. 5, $*p < 0.05$, ANOVA and Bonferroni post hoc test). These results suggest that selective inhibition of ERK2 with ERK2 siRNA significantly reduces white matter and gray matter damage in spinal cord after SCI in rats.

These above data suggest that calpain 1 is the downstream target of ERK1/2 activation and selective ERK reduction/inhibition by ERK2 siRNA, but not pERK1/2 inhibition by U0126, significantly reduces secondary tissue damage in spinal cord after SCI.

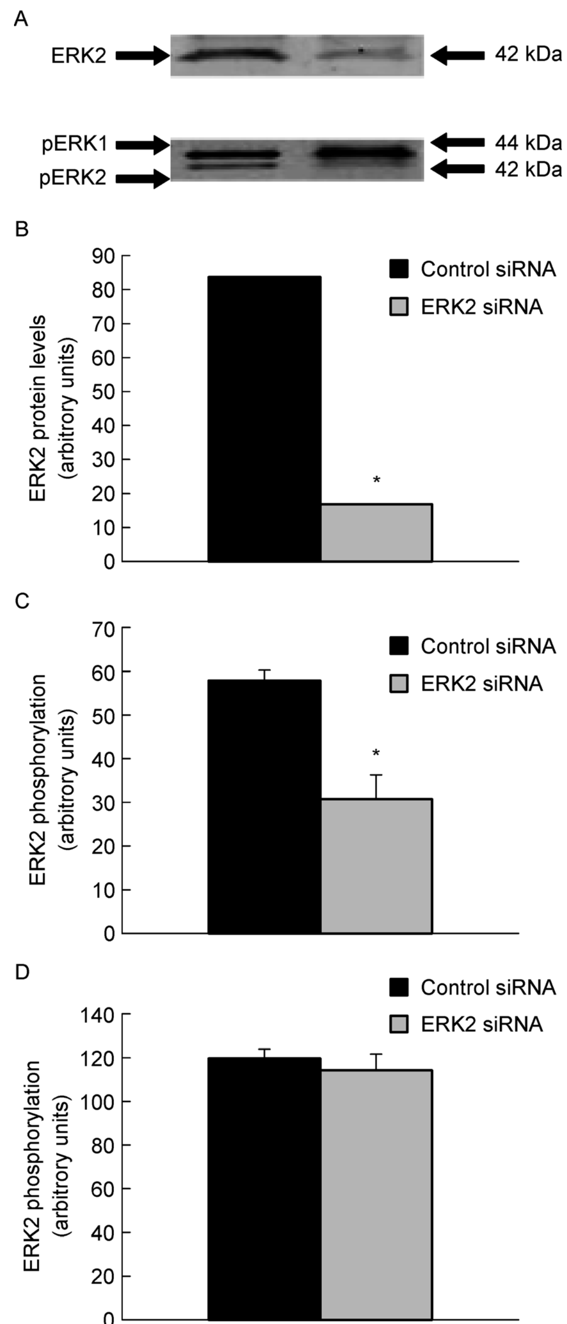

Figure 4 Effect of ERK2 siRNA on expression of ERK2 and pERK1/2 in the spinal cord 2 days after contusive SCI (A, B, C &D). Postinjury ERK2 siRNA intrathecal treatment knocked down the levels of ERK2 protein (A top panel and B) and both ERK2 and ERK1 phosphorylation (A bottom pane, C & D) 2 days following contusive SCI using Western blotting with antibody specific for ERK2 or pERK1/2. These ERK2 siRNAs do not alter pERK1 levels using Western blot with antibody against pERK1 (A bottom panel and D). Data were presented as mean±S.E.M. and analyzed by *t*-test, $*p < 0.05$, $n = 4$/group. ERK2 siRNA was mixed with jetPEI and administered by intrathecal infusion 30 min postinjury via minipump for 2 days (1.0 μg/day) after contusive spinal cord injury (IH impactor, 180 kdyn setting) in female Long Evans rats.

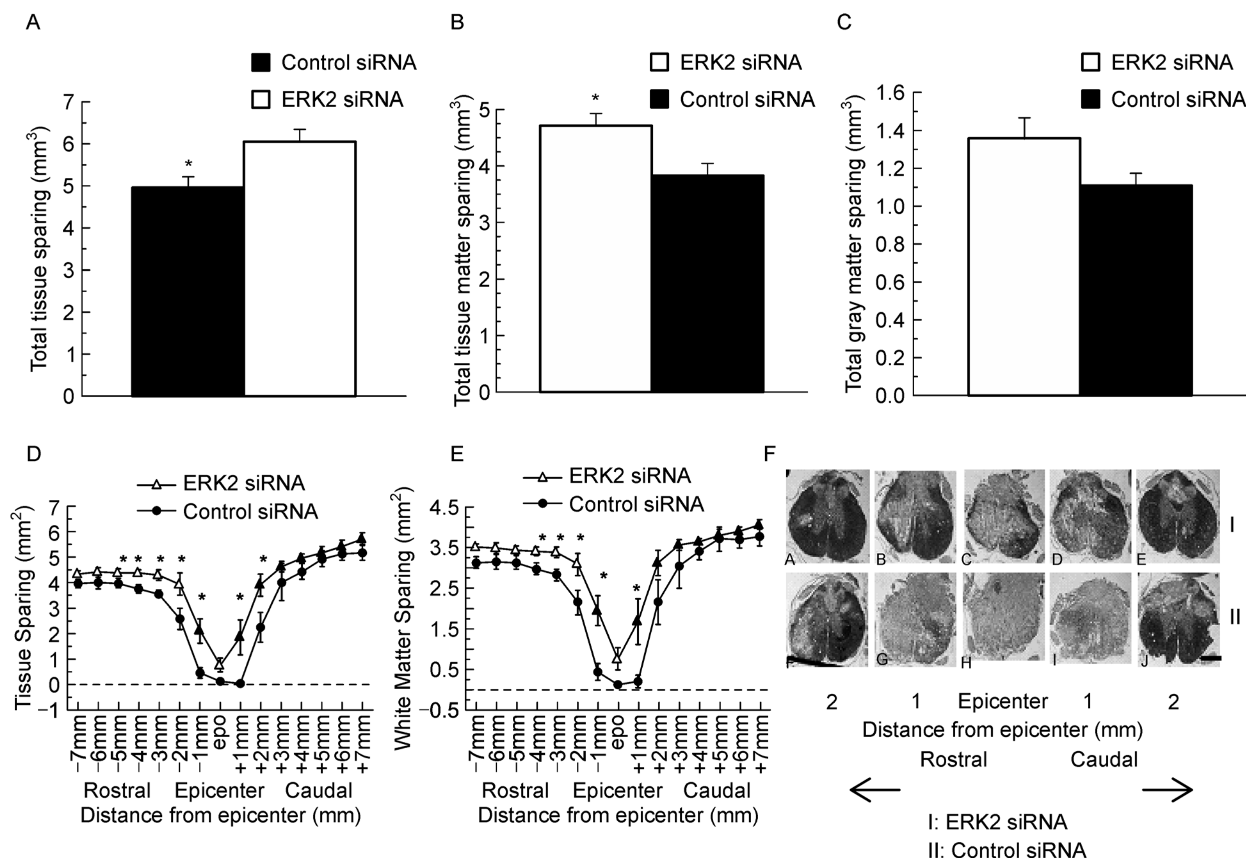

Figure 5 Effects ofERK2 siRNA intrathecal treatment on tissue damage 2 weeks after contusive SCI. Histological assessment showed that postinjury administration of ERK2 siRNA/jetPEI via minipump (1.0 μg/d for 7 days) resulted in a significant increase in total tissue sparing (A), tissue sparing at 1 to 4 mm rostral and 1 to 2 mm caudal to the injury epicenter (D), total white matter sparing (B), and white matter sparing at 1 to 4 mm rostral and 1 mm caudal to the injury epicenter (E) following contusion injury to the spinal cord, compared with control siRNA treated group. (F): Photomicrographs of representative transverse spinal cord section 2 weeks following severe contusion SCI at the epicenter and in 1-mm increments rostral and caudal to the lesion epicenter. The sections were stained with eriochrone cyanine for myelin. Scale bar: 500 μm. Contusive SCI was produced using the Infinite Horizons impactor, 180 kdyn setting, at T10. No significant differences in parameters were found between treatment and controls. Data are presented as mean±S.E.M. and analyzed by *t*-test (A, B, and C) or repeated measures ANOVA & Bonferroni post-hoc test (D & E). $*p < 0.05$, $n = 7$/group.

Effects of LV-calpain 1 shRNA on astroglial NF-κB activation and astroglial scar formation after SCI

Immunofluorescence staining analysis of spinal cord sections demonstrated that intraspinal administration of LV-calpain 1 shRNA at 7 days preinjury resulted in significant decreases in nuclear pNF-κBp65 expression in astrocytes (Fig. 6) and astrogliosis/astroglial scar formation (GFAP expression, Fig. 7, $*p < 0.05$, $n = 6$ per group) at the rostral and caudal border of the lesion site 6 weeks after contusive SCI compared with the administration of LV-control shRNA. These results suggest that reducing calpain 1 inhibits nuclear NF-κB activation and astrogliosis and astroglial scar formation in spinal cord following traumatic SCI in rats.

Discussion

The main goal of the present study was to acquire a better understanding of the distinct function of individual ERK and their signal transduction in the secondary tissue damage and astrogliosis/astroglial scarring in order to develop new signal transduction therapies for neuroprotection against SCI.

Distinct function of ERK1 and ERK2 in secondary tissue damage after SCI by ERK2 siRNA administration vs. U0126 administration

Traumatic SCI causes sustained ERK1/2 activation (Yu and Yezierski, 2005, Zhao et al., 2007; Yu et al., 2010). Our previous study has established the key role of ERK2 in the long term pathological and function outcome after contusive SCI in rats (Yu et al., 2010). However, it is still unclear if ERK1 and ERK2 play distinct functions in secondary tissue damage and functional recovery after SCI (Hetman and Gozdz, 2004, Zhuang and Schnellmann, 2006). Increasing evidence suggests that ERK1 and ERK2 may have distinct functions (Yu, 2012), with ERK2 contributing to secondary neural damage (Yu et al., 2010), while ERK1 is neuroprotective

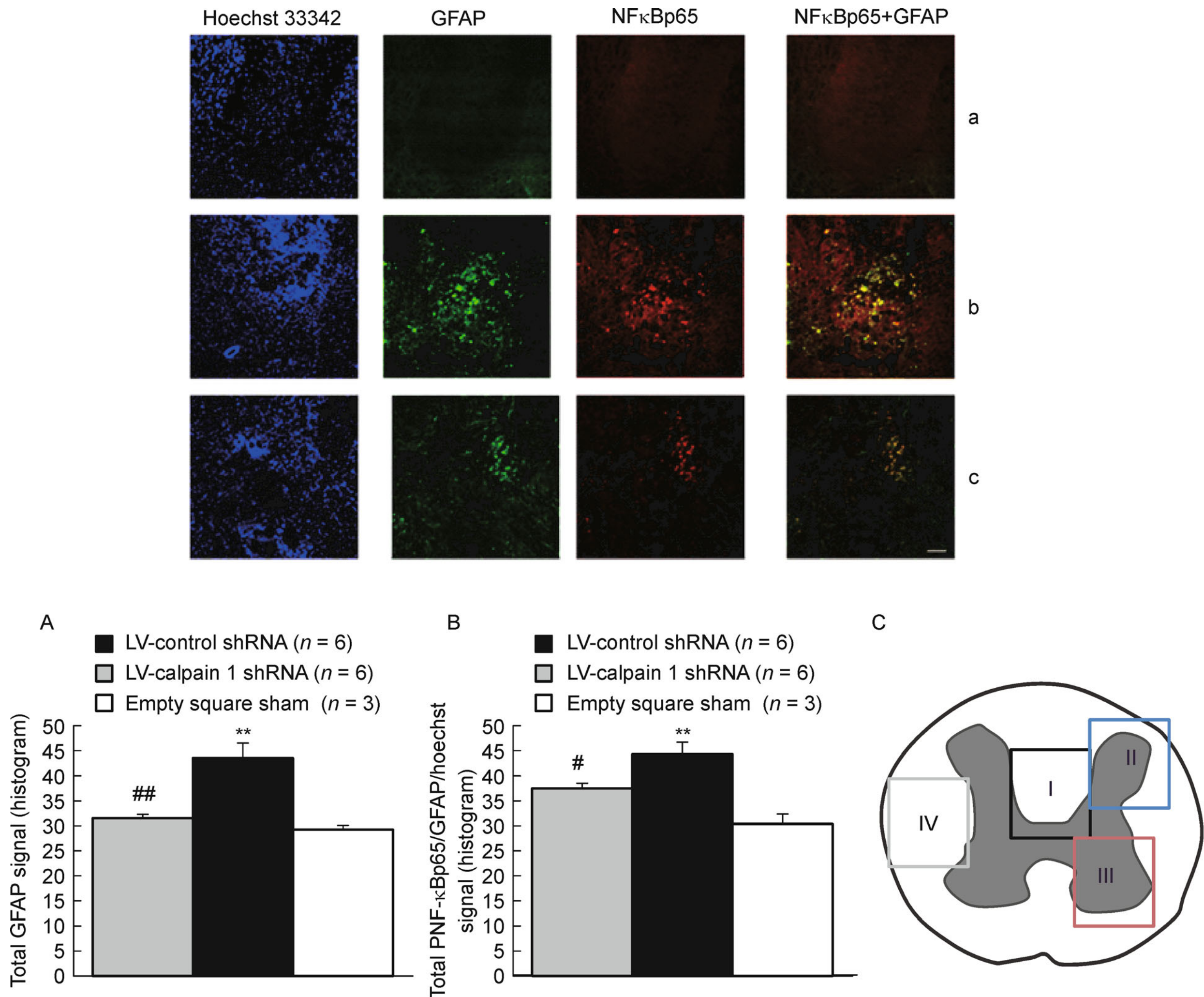

Figure 6 Effects of lentiviral-calpain 1 shRNA on astroglial NF-κB activation and astrogliosis/astroglial scar formation 6 weeks after contusion SCI in rats. The spinal tissues at 2 mm rostral and caudal to the lesion epicenter were collected from sham (a), injured spinal cord-treated with LV-calpain 1 shRNA (c) or injured spinal cord-treated with LV-control shRNA (b). The cross-sections were immunofluorescence-stained with antibody against pNF-κBp65 (red) or GFAP (green). The yellow color visualized in the merged images represented colocalization of two proteins. Blue, Hoechst 33342 nuclear stain. Scale bar: 50 μm. Quantification of fluorescence signals for immunofluorescence staining of GFAP (marker for astroglial scar formation) and for triple immunofluorescence staining of nuclear pNFκBp65 plus GFAP in the spinal cord sections was performed. In contrast to the sham-operated group (a), increased expression of GFAP (astrogliosis/astroglial scar formation, A) and astroglial nuclear activity of pNF-κBp65 (B) after SCI with pretreatment of LV-control shRNA were evident by their upregulated nuclear pNF-kBp65 and/or GFAP labeled immunoreactivity at 6 weeks post-injury (b). LV-calpain 1 shRNA intraspinal pretreatment (c, gray bar in A and B) significantly decreased astrocyte proliferation (A) and nuclear pNF-κBp65 activity in astrocytes (B) 6 weeks postinjury, compared to LV-control shRNA-pretreated group (b, dark bar in A and B). SCI condition is described in Material and Method Section. Four designated areas (I: dorsal central area, II: dorsal horn area, III: ventral horn area, and IV: lateral area) in each section were imaged and the total fluorescent signal (histogram) from all four areas were calculated. Data are presented as mean±S.E.M. and analyzed with repeated measures ANOVA followed by Bonferroni post-hoc analysis, #$p < 0.05$, ##$p < 0.01$, compared to SCI + LV control shRNA and **$p < 0.01$, compared to sham, $n = 6$/group. Differences were considered statistically significant at $p < 0.05$.

against secondary damage (Agrawal et al., 2006; Nakazawa et al., 2008; Liu et al., 2009). In the present study, one objective was to apply specific ERK2 siRNA and ERK1/2 inhibitor U0126 to investigate distinction of the specific ERK2 inhibition vs. both ERK1 and ERK2 inhibition in secondary tissue damage after acute SCI. The present study demonstrated that selective ERK2 inhibition by ERK2 siRNA administration, but not ERK1/2 inhibition by U0126 administration, significantly improved total tissue sparing, white sparing and gray matter sparing in spinal cord after contusive SCI in rats. This data supported our hypothesis that selective ERK2 reduction by ERK2 siRNA with ERK1 sparing is neuroprotective after acute SCI. The results are consistent with our previous report that long-term reduction of ERK2 by

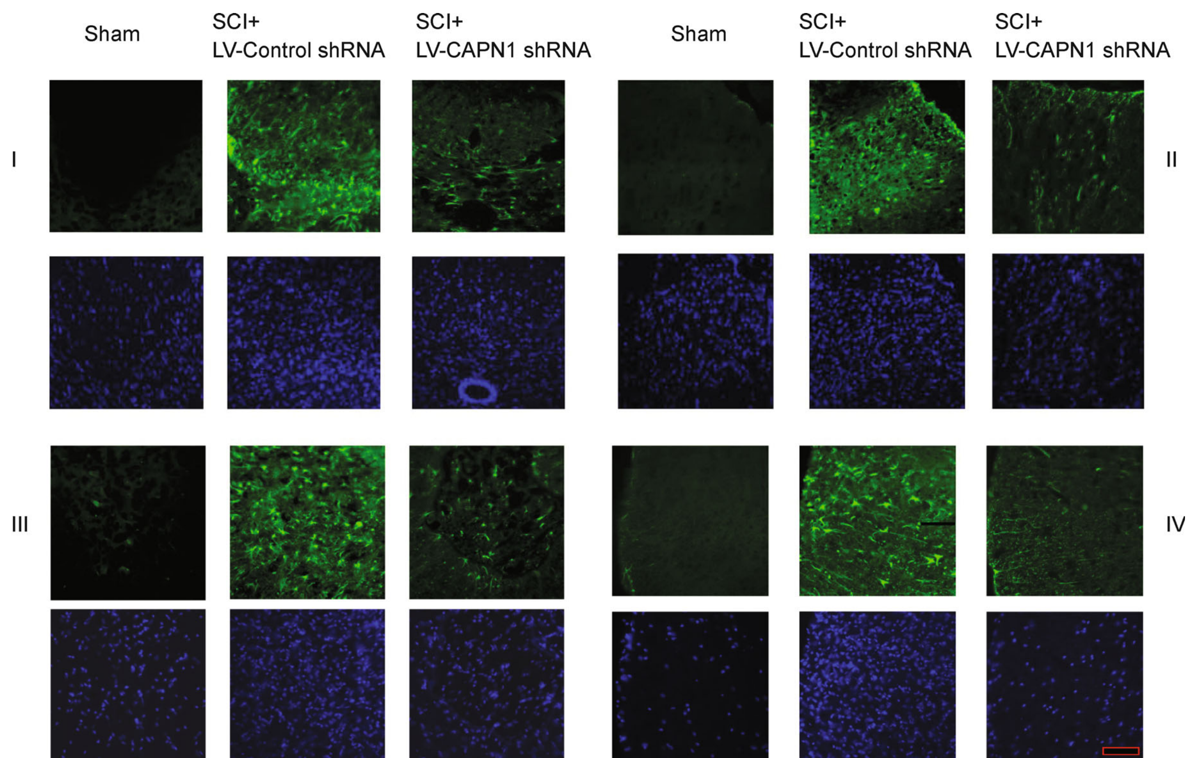

Figure 7 Photomicrographs for representatives of astroglial scar formation/astrogliosis in transverse rat spinal cord sections 42 days following contusive SCI. The cross-sections were immunofluorescence-stained with antibody against GFAP. Blue, Hoechst 33342 nuclear stain. Scale bar: 50 μm. SCI condition is described in Material and Method Section. Four designated areas (I: dorsal central area, II: dorsal horn area, III: ventral horn area, and IV: lateral area) in each section were imaged and the total fluorescent signal (histogram) from all four areas were calculated.

lentiviral ERK2 shRNA pretreatment improves long-term locomotor outcomes after contusive SCI in rats (Yu et al., 2010). The pretreatment with lentiviral shRNA vectors enabled examination of the role of chronic ERK2 activation, which is not possible using current pharmacologic inhibitors, and allowed sufficient time for genomic integration of the retroviral cDNA and the decreased gene and protein expression. However, the pretreatment with lentiviral shRNA before SCI is not viable therapeutic strategy, while post-injury administration of lentiviral shRNA is unable to target acute ERK2 activation after SCI. To be a viable therapeutic, the treatment must be administered post injury. We therefore examined post injury delivery of ERK2 siRNA to knockdown acute ERK2 activation after SCI.

Synthetic siRNA has great potential as a novel therapeutic strategy (Aigner, 2006; Lu and Woodle, 2008; Wittrup and Lieberman, 2015). Developing suitable siRNA delivery methods *in vivo* is an important issue for consideration in potential use of clinical application. Local delivery of siRNA with *in vivo* jetPEI has been shown to be a promising delivery method (Aigner, 2006; Li et al., 2012; Kim et al., 2013) and is currently being used in clinical trials (Drake et al., 2010). To develop a clinically relevant siRNA therapeutic strategy for acute SCI, we examined intrathecal delivery of siRNA into spinal cord after SCI in rats using *in vivo* jetPEI. *In vivo* jetPEI was chosen because it is widely used for siRNA delivery *in vivo*, with low cytotoxicity, and is currently being used for human clinical trials (www.polyplus-transfection.com). Importantly, the PEI complexation provides almost complete protection against enzymatic or nonenzymatic degradation of chemically unmodified siRNA molecules (Aigner, 2006). In our previous study, we validated the gene silencing efficiency of ERK2 siRNAs *in vitro* (Yu et al., 2010). In the present study, we identified the gene silencing of ERK2 in spinal cord by intrathecal administration of the ERK2 siRNA following contusive SCI. The dose of siRNA was chosen based on previous studies, which indicated that intrathecal administration of siRNA up to 400 μg/d did not cause neurotoxicity (Luo et al., 2005). The results obtained with siRNA approach supported the detrimental role for ERK2 in the secondary injury mechanisms. However, ERK2 signal transduction for the neuroprotective effects of ERK2 siRNA in functional outcomes remains to be determined.

Involvement of ERK1/2-calpain 1-NF-κB signal transduction in astrogliosis and astroglial scar formation after SCI

One important finding observed in the present study for the first time is the ERK1/2-calpain 1 signal transduction after SCI. In the present study, observations in U0126 intervention following contusive SCI showed that ERK1/2 inhibition by

U0126 reduced calpain 1 expression in spinal cord. The results demonstrated the involvement of calpain 1 in the downstream signal transduction of ERK1/2 in the secondary tissue damage after SCI.

Calpain activation is widely considered as a pathogenic target in the secondary injury cascade in CNS pathological conditions (Ray et al., 2003, Saatman et al., 2010; Sribnick et al., 2010). Current evidence is mounting to suggest that calpain activation also serve essential physiologic roles in cell signaling and neuronal plasticity (Kuchay and Chishti, 2007; Liu et al., 2008; Doshi and Lynch, 2009). However, the contribution of individual calpain isoform signal transduction to secondary damage and functional deficits after SCI in rodents remain unknown. In a recent study (Yu et al., 2013), we demonstrated that lentiviral calpain 1 shRNA intraspinal administration pre-injury resulted in a significant reduction in calpain 1 expression in spinal cord, attenuation in lesion volume, and improvement in tissue sparing and locomotor function after contusive SCI in rats. The signal transduction mechanisms underlying the neuroprotection with the calpain 1 reduction by lentiviral calpain 1 shRNA pretreatment in SCI are not yet known. Secondary tissue damage following traumatic SCI is associated with astroglial NF-κB activation (a key neuroinflammatory factor), astrogliosis/astroglial scar formation, and inflammation (Brambilla et al., 2009; Wu et al., 2012). We previously demonstrated that ERK1/2 activation mediated NF-κB pathway in spinal cord after SCI (Yu and Yezierski, 2005). To further evaluate if calpain 1 reduction by lentiviral-calpain 1 shRNA pretreatment protects against SCI through inhibiting delayed astroglial NF-κB activation, and astrogliosis/astroglial scar formation following traumatic SCI, we examined nuclear pNF-κBp65 activity in astrocytes and astrogliosis/astroglial scar formation in contusion injured rats with treatment of LV-calpain 1 shRNA or LV-control shRNA. Calpain and NF-κB signaling pathways are major contributors to proliferation of astrocytes at delayed/chronic phases post-injury, leading to inflammatory responses and astroglial scar formation around the injury site after SCI (Tian et al., 2006; Yiu and He, 2006; Bramlett and Dietrich, 2007; Brambilla et al., 2009). The astrogliosis and scarring process play a key role in the regulation of axonal regeneration and the cascades of neuro-inflammation and secondary tissue damage. Transgenic inhibition of astroglial NF-κB leads to increased axonal sparing and sprouting, reduced expression of CSPGs and inflammation, and improved functional recovery after chronic contusion SCI (Brambilla et al., 2009). Astroglial NF-κB signaling as a new target for SCI therapy has been proposed (Brambilla et al., 2009). Calpain cleaves IkB, the endogenous inhibitor of NF-κB, which activates NF-κB and facilitates translocation of NF-κB from cytosol to nucleus (Sribnick et al., 2010). In the present study, we demonstrated that reduced calpain 1, using the lentiviral vector-mediated shRNA delivery approach, resulted in significant attenuation in nuclear NF-κB activation and astrogliosis that may contribute to the secondary tissue damage including progressive tissue loss, inflammation, and glial scar formation after traumatic SCI.

Our data suggest that calpain 1 is the downstream target of ERK1/2 signaling, while astroglial NF-κB is the downstream molecular and cellular targets of calpain 1 activity after chronic SCI. Therefore, the inhibition of astroglial NF-κB and astrogliosis/astroglial scar formation are major mechanisms by which reduced ERK-calpain 1 signal transduction results in neuroprotective effects after SCI. However, astroglial ERK2 reduction and its downstream calpain 1-NF-κB pathway activation, as well as the therapeutic window for the neuroprotective effects of ERK2 siRNA in functional outcomes remains to be determined.

Identifying novel signal transduction targets and developing novel therapeutic strategies for SCI are urgently needed. Our present studies provide evidence that selective inhibition of ERK2 and ERK-calpain 1 pathway reduce secondary tissue damage and astrogliosis/astroglial scar formation. pERK1/2-calpain 1-NF-κB signal transduction is an attractive candidate for joint targeting for neuroprotection after SCI. Therefore, in the future study, targeting individual ERK2 and calpain 1 isoforms and ERK1/2-calpain-NF-κB signal transduction, while sparing ERK1 signaling, may provide greater potential as novel therapeutics for minimizing secondary damage and resultant functional deficits following SCI.

Acknowledgements

This research was support by grants from KSCHIRT #7-6A and 11-19A, and NIH p30 NS051220 and NIH CTSA UL1TR000117. We thank Dr. James Geddes for reviewing the paper and his kind comments. We also thank Linda Simmerman for expert assistance with microscopy and imaging.

Compliance with ethics guidelines

All authors (Xin Xin Yu, Vimala Bondada, Colin Rogers, Carolyn A. Meyer, and Chen Guang Yu) declare that they have no conflict of interest.

The article does not contain any studies with human subjects performed by any of the authors.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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