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Calpain 1 Knockdown Improves Tissue Sparing and Functional Outcomes after Spinal Cord Injury in Rats

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Calpain 1 Knockdown Improves Tissue Sparing and Functional Outcomes after Spinal Cord Injury in Rats

Chen Guang Yu, Yanzhang Li, Kashif Raza, Xin Xin Yu, Sarbani Ghoshal, and James W. Geddes

Abstract
To evaluate the hypothesis that calpain 1 knockdown would reduce pathological damage and functional deficits after spinal cord injury (SCI), we developed lentiviral vectors encoding calpain 1 shRNA and eGFP as a reporter (LV-CAPN1 shRNA). The ability of LV-CAPN1 shRNA to knockdown calpain 1 was confirmed in rat NRK cells using Northern and Western blot analysis. To investigate the effects on spinal cord injury, LV-CAPN1shRNA or LV-mismatch control shRNA (LV-control shRNA) were administered by convection enhanced diffusion at spinal cord level T10 in Long-Evans female rats (200–250 g) 1 week before contusion SCI, 180 kdyn force, or sham surgery at the same thoracic level. Intraspinal administration of the lentiviral particles resulted in transgene expression, visualized by eGFP, in spinal tissue at 2 weeks after infection. Calpain 1 protein levels were reduced by 54% at T10 2 weeks after shRNA-mediated knockdown (p<0.05, compared with the LV-control group, n=3 per group) while calpain 2 levels were unchanged. Intraspinal administration of LV-CAPN1shRNA 1 week before contusion SCI resulted in a significant improvement in locomotor function over 6 weeks postinjury, compared with LV-control administration (p<0.05, n=10 per group). Histological analysis of spinal cord sections indicated that pre-injury intraspinal administration of LV-CAPN1shRNA significantly reduced spinal lesion volume and improved total tissue sparing, white matter sparing, and gray matter sparing (p<0.05, n=10 per group). Together, results support the hypothesis that calpain 1 activation contributes to the tissue damage and impaired locomotor function after SCI, and that calpain1 represents a potential therapeutic target.

Key words: behavior; gene therapy; lentiviral; locomotor; pathology; RNAi; tissue sparing

Introduction

The locomotor deficits resulting from traumatic spinal cord injury (SCI) are the result of both the initial mechanical insult as well as a cascade of secondary mechanisms that exacerbate the tissue damage. Excessive calpain activation is one secondary mechanism strongly implicated in the secondary neuronal and axonal degeneration after SCI. Calpains are calcium activated neutral proteases that cleave a wide range of cellular proteins including cytoskeletal, membrane-bound, and soluble proteins. Loss of function of these calpain substrates contributes to the death of neurons and oligodendrocytes, axonal demyelination and degeneration, and locomotor deficits after SCI. In addition, calpains serve essential physiological roles including signal transduction, cell migration, membrane fusion, and cell differentiation. The challenge is to prevent the excessive calpain activity that leads to neurodegeneration, but retain the essential physiological roles of calpains.

Of the 15 mammalian calpain isoforms identified to date, the best characterized calpains in the central nervous system (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). The two calpain isoforms share many properties. Both are cytosolic and appear to have similar substrate specificities, with the major difference being that purified m-calpain requires mM Ca2+ for activation while µ-calpain requires µM Ca2+. Knockout of calpain 2 is embryonically lethal, while calpain 1 null mice are viable with minimal phenotype. In addition, CNS calpain 1 expression increases post-natally while calpain 2 expression is relatively constant during CNS development and maturation. Together, the above results suggest that knocking down or inhibiting calpain 1 might limit the damage caused by excessive calpain activity, and that by targeting only a single calpain isoform that essential physiological roles of calpains would be preserved.

In the present study, we examined the hypothesis that decreased calpain 1 will attenuate secondary tissue damage and locomotor deficits after contusive injury to the spinal cord. Calpain 1 levels were reduced using lentiviral CAPN1 short hairpin ribonucleic acid (shRNA) injected into the rat spinal cord. Because of the relatively
rapid calpain activation that occurs after SCI compared with the several days needed for knockdown of calpain protein levels using RNA interference or antisense approaches. It was necessary to administer the lentiviral CAPN1 siRNA 1 week pre-injury. Locomotor function, lesion volume, and tissue sparing were evaluated post-injury. The longer range goal is to evaluate the potential of CAPN1 inhibition as a druggable target.

**Methods**

**Calpain 1 shRNA**

Calpain 1 (CAPN1) small interfering RNA (siRNA) sequences (Genebank accession number: NM_019152) were selected and chemically modified based on accepted criteria for the rational design of siRNA with off-target control using two web-based programs (Dharmacon and Invitrogen). To minimize the potential off-target effects of siRNA, the CAPN1 siRNA sequence 1 (Sense, start 5¢-GCACAAUCAGAGCACUUUAUC-3¢), the CAPN1 siRNA sequence 2 (Sense, start 5¢-GGGACUUCAUGGAGUCA-3¢), and mismatch control siRNA (5¢-GGAGAAUCACAGGACUUUAUC-3¢) were subjected to BLAST analysis and synthesized in the modification option with off-target control by Thermo Scientific-Dharmacon Products (Lafayette, CO). To determine the efficacy of CAPN1 siRNA sequences, normal rat kidney (NRK) cells (3×10⁵/well in 6-well plates) were transfected with 100 nM calpain 1 siRNAs complexed with Lipofectamine 2000, based on the manufacturer’s instructions (Invitrogen, Carlsbad, CA). NRK cells were assigned to the following groups: (A) mismatch control siRNA transfection, (B) negative control siRNA transfection, (C) calpain 1 siRNA transfection, and (D) calpain 1 siRNA 2 transfection. Western blot analysis was used to evaluate the relative levels of calpain 1 or calpain 2 protein at 48 h post-transfection (*n*=4/group).

NRK cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco modified eagle medium supplemented with 5% fetal bovine serum, 10% heat-inactivated donor horse serum, 50 μg/mL streptomycin, and 50 IU/mL penicillin in a humidified atmosphere at 37°C and 5% CO₂ as described in the protocol provided by ATCC.

**Lentiviral vectors**

Lentiviral vectors were chosen because they induce little or no immune response within the host, they provide long-lasting transgene expression, and they infect dividing and non-dividing cells including neurons and glia. LentiLox 3.7 vector was purchased from ATCC (pLL3.7, Manassas, VA). It contains a cytomegalovirus (CMV) promoter driven enhanced green fluorescent protein (eGFP) reporter and a U6 promoter with downstream of cloning sites (HpaI and XhoI) to allow the introduction of oligonucleotides encoding CAPN1 shRNA or mismatch control shRNA to produce LV-CAPN1shRNA or LV-control shRNA. The LV-CAPN1shRNA or LV-control shRNA was cotransfected with packaging plasmid PAX2 and envelope plasmid pMD2G (obtained from Dr. Didier Trono, University of Geneva, Switzerland) into 293FT cells (Invitrogen) using calcium-phosphate precipitate. Viral supernatants were harvested and concentrated after 72 h. The titer of the lentiviral stocks was assessed by using 10-fold serial dilutions to transduce HT 1080 human fibrosarcoma cells (ATCC) using the eGFP reporter of the pLL3.7 vector to identify infected cells. The viral titer was 5×10⁸ to 1×10⁹ TU/mL for LV-CAPN1shRNA and LV-control shRNA.

Knockdown of calpain 1 mRNA and protein in the NRK cells treatment was determined using Northern blot and Western blot analysis, respectively. The NRK cells were grown to 80% confluence, then infected with 10⁵ TU/well in six-well plates. Five days after lentiviral infection, the cells were harvested for Northern or Western blotting. NRK cells were chosen because they express relatively high levels of both calpain 1 and calpain 2. NRK cells were assigned to the following groups: (A) Lentiviral CAPN1 shRNA and (B) Lentiviral control shRNA. Northern blotting and Western blotting were used to evaluate the relative levels of calpain 1 mRNA and protein at 5 days post-lentiviral infection, respectively (*n*=3/group).

**Animals and surgical procedures**

Thirty-two female Long-Evans adult rats (Charles River, Indianapolis, IN) weighing 200–250 g were used for the in vivo experimental studies. All experimental procedures were approved and performed in accordance with the Guidelines of the US National Institutes of Health and Institutional Animal Care and Use Committee of the University of Kentucky.

Animals were kept under standard housing conditions for at least 1 week prior to arrival. Lentiviral particles containing LV-CAPN1-shRNA and LV-control shRNA were administered by convection enhanced delivery at spinal segment T10. Intraspinal injections were made bilaterally at 0.5 mm lateral to the midline and 1 mm ventral. For each injection site, a volume of 3.0 μL was slowly administered over 30 min.

Contusive SCI was produced using a T10 laminectomy using an Infinite Horizons (IH) spinal cord injury device (Precision Systems & Instrumentation, Fairfax Station, VA) as described previously. Briefly, adult female Long-Evans rats, weighing 200–250 g, were anesthetized with ketamine (80 mg/kg, intraperitoneal [ip]) and xylazine (10 mg/kg, ip), and a laminectomy was made to expose spinal segment T10. The exposed vertebral column was stabilized by clamping the rostral T9 and caudal T11 vertebral bodies with two spinal forceps. SCI was then applied with the IH device using a 180 kdyn force setting, which resulted in moderately severe conduction injury. Impact analyses, including actual force applied to the spinal cord, displacement of spinal cord, and velocity, were recorded. The impact tip was automatically retracted immediately, the wound irrigated with saline, and the muscle and skin openings were closed with sutures. The surgical procedure and postoperative care were similar to that described previously.

**Northern blot analysis**

Lentivirus-infected NRK cells were lysed with Trizol reagent (Invitrogen) and prepared for total RNA isolation according to the manufacturer’s instructions. 1.0 μg of total RNA was fractioned on a 10% denaturing polyacrylamide gel and transferred to a nitrocellulose membrane. Blots were hybridized to a probe consisting of a 5¢-digoxin-labeled 21-nt calpain 1 siRNA sense strand (DIG RNA Labelling Kit, Roche Applied Science, Mannheim, Germany). A 5¢-digoxin-labeled actin probe was used to determine equal loading of RNA. The hybridization and the detection of the hybridized probe were performed according to DIG Application Manual (Roche Applied Science, Mannheim, Germany).

**Western blot analysis**

NRK cells were washed with 1× phosphate buffered saline (PBS), lysed by adding buffer, and sonicated for 15 sec to shear deoxyribonucleic acid and reduce sample viscosity. For spinal cord samples, animals were euthanatized by pentobarbital (100 mg/kg, ip injection) and decapitated 2 weeks post-injury. A 5-mm block of spinal cord centered on T10 was removed and snap-frozen on dry ice, then stored at ~80°C. The spinal cord samples were homogenized in a lyses buffer and sonicated. The protein samples were obtained by microcentrifugation at 14,000 rpm for 10 min. Protein quantities were determined using the bicinchoninic method.
Western blotting was performed as described previously.21,22 Briefly, NRK cell protein samples or spinal cord protein samples were loaded on SDS-PAGE gels and electrotransferred to nitrocellulose membranes. Blots from cell samples or spinal cord samples were probed with a polyclonal anti-rabbit antibody against calpain 1 (1:1000, Chemicon, Temecula, CA). Blots were then incubated with infrared-labeled anti-rabbit secondary antibody (1:5000). The blots were reprobed with a monoclonal anti-mouse antibody against actin (1:1000, Chemicon, Temecula, CA) and then incubated with infrared-labeled anti-mouse secondary antibody (1:5000). All blots were visualized and analyzed on the LI-COR Odyssey infrared imaging system (Lincoln, NE).

Immunofluorescence imaging

At 2 weeks post-injection with LV-CAPN1shRNA and LV-control shRNA, rats were transcardially perfused with cold saline followed by buffered 4% paraformaldehyde. The spinal cord was removed, cut into blocks according to the segments, and postfixed with the same fixative overnight. The fixed spinal cords were serially and transversely cryosectioned at a thickness of 20 μm. eGFP expression was viewed using a fluorescence microscopy system (Olympus DSU) (n = 3/group).

Assessment of locomotor function

Open-field locomotor function was assessed preinjury and 0, 3, 7, 14, 21, 28, 35, and 42 days post-injury using the Basso, Beattie, Bresnahan (BBB) locomotor rating scale.19,23 The two examiners participating in the BBB evaluation were blinded to the experimental treatment received by each animal.

Assessment of tissue sparing

At 6 weeks post-injury, animals were euthanatized and transcardially perfused with cold saline followed by buffered 4% paraformaldehyde. The spinal cord was removed and prepared for histological assessment as described previously.19,24 Spinal cords were serially and transversely cryosectioned at a thickness of 20 μm. Every fifth section was mounted onto gelatin-coated slides and stored at −20°C. A modified eriochrome cyanine 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 lesion volume, total tissue sparing, white matter sparing, and gray matter sparing in transverse sections of the injured cords were performed as described previously.19,24

FIG. 1. Knockdown of calpain 1 with siRNA in vitro. Two calpain 1 siRNA sequences were evaluated in transient transfection assays. Normal rat kidney cells (3x10^5/well in six-well plates) were transfected with 100 nM calpain 1 siRNA sequence 1 or sequence 2 using lipofectamine 2000 (Invitrogen). Relative protein levels of calpain 1 (80 kDa) were measured 48 h after transfection using Western blot and compared with levels of actin. Each siRNA sequence decreased calpain 1 levels by approximately 60%, ***p < 0.001, compared with mismatch control siRNA and to negative control siRNA, repeated measures analysis of variance, and Bonferroni post hoc test, n = 4 per group.

FIG. 2. Knockdown of calpain 1 with LV-CAPN1 shRNA in vitro. Calpain 1 siRNA (sense sequence: 5'GCACAATCAGAGCATTTATC3', 1462-1482 of NM_019152) or mismatch control siRNA were inserted into the Lentilox 3.7 shRNA vector. Infection of normal rat kidney cells with LV-CAPN1 shRNA or LV-Control shRNA was performed. The cells were grown to 80% confluency, then infected with 10^5 TU/well in six well plates. A reduction in Capn1 in mRNA was evident at 5 days after lentiviral infection using Northern blotting (A). Calpain 1 protein levels were significantly reduced in the cells infected with LV-CAPN1 shRNA or LV-control shRNA and were measured at 5 days after infection using Western blot analysis (B, C). **p < 0.001, compared with control group, t test, n = 3/group.
**Statistical analysis**

Analysis was performed using StatView (SAS Institute, Cary, NC). Data are presented as mean ± standard error of the mean. Group differences were evaluated by t test or repeated measures analysis of variance and Bonferroni post hoc test as described previously. Null hypotheses were rejected at the p < 0.05 level. Although the BBB scale is an ordinal scale, differences between the treatments were compared using parametric statistical methods recommended by Scheff and colleagues.

**Results**

**Calpain 1 siRNA reduces calpain 1 levels in vitro**

Initially, we evaluated two calpain 1 (100 nM) siRNAs (n = 4/group) in NRK cells (3 × 10^5/well in six-well plates), measuring calpain 1 or calpain 2 levels by Western blotting 48 h after transfection (Fig. 1). Both siRNAs significantly reduced calpain 1 levels while a negative control and mismatch control that differed from the calpain 1 siRNA sequence by a single nucleotide (see Methods) did not significantly alter calpain 1 levels. Calpain1 siRNA 1 did not decrease calpain 2 levels (Fig. 1).

**LV-CAPN1 shRNA reduces calpain 1 in vitro**

At 5 days after transient transfection of NRK cells with LV-CAPN1 shRNA or LV-control shRNA (n = 3/group), LV-CAPN1 shRNA significantly decreased levels of calpain 1 mRNA (Fig. 2A) and protein (Fig. 2B, 2C) by 80% at 5 days after viral infection in NRK cells compared with LV-control shRNA infection.

**LV-CAPN1 shRNA reduces calpain 1 expression in vivo**

After intraspinal administration via convection enhanced delivery, expression of LV-CAPN1 shRNA was examined by direct visualization of eGFP with fluorescent microscopy (Fig. 3A). Intraspinal delivery of LV-CAPN1 shRNA (n = 3/group) resulted in reduced levels of calpain 1 (B, C), but not calpain 2 (B, D), at two weeks post-injury, *p < 0.05.

**Lentiviral-CAPN1 shRNA improves locomotor function 6 weeks after contusive SCI in rats**

No significant differences in actual force, displacement, or velocity were found between LV-CAPN1 shRNA and LV-control groups, indicating similar injuries to all animals (Table 1).

Immediately after SCI, all animals exhibited complete bilateral hindlimb paralysis. Behavioral assessment demonstrated that rats receiving LV-CAPN1 shRNA exhibited improved locomotor performance as early as 3 days post-injury, compared with rats receiving the LV-control shRNA, and the improvement persisted until the last observation at 6 weeks post-injury, (Fig. 4, n = 10/group). At 6 weeks post-injury, the rats treated with LV-CAPN1 shRNA had a mean BBB score of 12.4, indicative of frequent to consistent weight-supported plantar steps and occasional to frequent front limb-hind limb coordination. Rats administered LV-control shRNA had a mean BBB score of 9 at 6 weeks post-injury, indicative of plantar placement of the paw with weight support in stance only, but without weight-supported plantar stepping.

**Table 1. Injury Parameters**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Actual force (kdyne)</th>
<th>Displacement (microns)</th>
<th>Velocity (mm/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV-Control shRNA</td>
<td>182 ± 2</td>
<td>832 ± 79</td>
<td>122 ± 0.4</td>
</tr>
<tr>
<td>LV-CAPN1 shRNA</td>
<td>180 ± 2</td>
<td>995 ± 89</td>
<td>122 ± 0.3</td>
</tr>
</tbody>
</table>

Values are mean ± standard error of the mean. No significant differences in impact force, displacement, and velocity were found between the treatment groups (n = 10/group).
Lentiviral-CAPN1 shRNA attenuates lesion volume and improves tissue sparing at 6 weeks after contusive SCI

Histological assessment of lesion volume and tissue sparing showed that intraspinal administration of LV-CAPN1 shRNA resulted in a decrease in lesion volume (Fig. 5) and significant increase in total tissue sparing, white matter sparing, and gray matter sparing (Fig. 6). The tissue sparing was evident up to 5 mm both rostral and caudal to the lesion epicenter at 6 weeks post-injury compared with animals receiving LV-control shRNA ($n = 10$/group).

Discussion

Knockdown of calpain1 in the rat spinal cord by lentiviral shRNA resulted in a significant attenuation in lesion volume and improvement in tissue sparing and locomotor function after contusive SCI. Although pre-injury administration of lentiviral particles is not feasible from a therapeutic standpoint, this approach enabled examination of the role of the calpain 1 isoform, which is not possible using current pharmacologic inhibitors. The results support the hypothesis that calpain 1 activation contributes to the tissue damage and impaired locomotor function after SCI, and therefore represents a potential druggable target.

The improvement in locomotor function and tissue sparing observed with calpain 1 knockdown is comparable to that observed previously with broad-spectrum calpain inhibitors. This suggests that of the several calpain isoforms present in the CNS, excessive activation of calpain 1 is a major contributor to the neurodegeneration and resultant loss of motor function. Calpain 1 activation is relatively rapid after SCI, and the pre-injury knockdown would be optimal for neuroprotection. This may account for the improvement in BBB scores observed as early as 3 days after injury. The early improvement in locomotor function is consistent with pre-injury or immediate post-injury administration of broad-spectrum small molecule calpain inhibitors.

**FIG. 4.** LV-CAPN1 shRNA improves locomotor function after spinal cord injury (SCI). Pre-injury administration of LV-CAPN1 shRNA (triangles) resulted in improved locomotor performance compared with LV-control–pretreated animals (circles). Contusive SCI was produced using the Infinite Horizons Impactor, 180 kdyn setting, at T10. Data were presented as mean±standard error of the mean and analyzed with repeated measures analysis of variance followed by Bonferroni post hoc analysis, *$p < 0.05$.

**FIG. 5.** Effects of the LV-CAPN1 shRNA pretreatment on lesion volume 6 weeks after severe contusive spinal cord injury (SCI). (A) Photomicrographs of representative transverse spinal cord sections from rats at 42 days after contusive SCI (180 kdyn). The sections are from the lesion epicenter and 1, 2, 4 mm rostral and caudal to the epicenter, obtained from a LV-control shRNA-treated rat (top panel) and a LV-CAPN1-shRNA treated rat (bottom panel). The sections were stained with eriochrome cyanine for myelin. Scale bar: 100 μm. (B) Pre-injury administration of LV-CAPN1 shRNA resulted in a significant decrease in lesion volume after contusion injury to the spinal cord. Injury conditions and treatment groups are as described in Figure 4. Data were presented as mean±standard error of the mean and analyzed by $t$ test. *$p < 0.05$, $n = 10$/group.
Although the results support a role for calpain 1 in the secondary injury cascade, the therapeutic window for the beneficial effects of calpain 1 knockdown or inhibition remain to be determined. Administration of LV-CAPN1-shRNA into the spinal cord would be expected to knock down calpain 1 levels in the surrounding gray matter, but not in the ascending and descending axons in the white matter whose cell bodies are distal to the areas exposed to the lentiviral particles. Thus, the sparing observed in white matter is unlikely to be the result of calpain 1 knockdown within the affected axons. Excitotoxic mechanisms are implicated in the degeneration of oligodendrocytes after SCI, and calpains are implicated in the demyelination associated with multiple sclerosis, and calpain inhibitors protect the white matter in animal models of this disorder. Thus, the white matter sparing observed with LV-Capn1-shRNA is thought to reflect protection of oligodendrocytes after SCI.

It is also of interest that the approximately 2 mm diffusion of the LV-Capn1 shRNA in the rostral and caudal direction resulted in improved tissue sparing of up to 6 mm in both directions after SCI. This is consistent with calpain-mediated cell death contributing to the increase in lesion size with time after SCI, and that early intervention minimizes lesion size and maintains axon-myelin integrity, resulting in maintained motor function.

Additional support for the hypothesis that reduced calpain 1 protects against neurodegeneration was recently provided by Bevers and colleagues, who found that calpain 1 knockdown with RNAi attenuated proteolysis of the calpain substrate spectrin after transient forebrain ischemia minimized the loss of hippocampal CA1 neurons and preserved electrophysiologic function. In addition, calpain 1 null mice exhibited less cortical degeneration after traumatic brain injury. The neuroprotective effect of decreased calpain 1 against neurodegeneration after SCI had not been examined previously.

The results also demonstrate that convection enhanced delivery is an effective delivery method for lentiviral particles in the rat spinal cord. Convection enhanced delivery is the use of a slight pressure gradient during interstitial infusion to augment diffusion and increase the distribution of both small and large molecular weight molecules, including proteins. Intraspinal administration of LV-CAPN1shRNA via convection enhanced delivery provided rostral and caudal spread away from the injection site, leading to a significant reduction in protein expression in the spinal cord.

FIG. 6. Effects of the LV-CAPN1 shRNA pre-treatment on tissue sparing 6 weeks after severe contusive spinal cord injury. Pre-injury administration of LV-CAPN1 shRNA resulted in a significant increase in total tissue sparing (A, B), white matter sparing (C, D), and gray matter sparing (E, F) after contusion injury to the spinal cord. Data were analyzed by t test (A, C, E) or repeated measures analysis of variance followed by Bonferroni post-hoc analysis (B, D, E) *p < 0.05, **p < 0.01.
Conclusion

The results demonstrated efficient lentiviral gene transfer of a calpain1 shRNA through convection enhanced delivery in the spinal cord of rats. Lentiviral calpain1 shRNA administration provided effective calpain1 knockdown and resulted in improvement in both tissue sparing and locomotor function, suggesting that reduced calpain1 activity is neuroprotective against SCI and represents a promising therapeutic target for SCI.

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Author Disclosure Statement

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References


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