Published in final edited form as:

Pain. 2011 March ; 152(3): 649–655. doi:10.1016/j.pain.2010.12.014.

Calcium channel alpha-2-delta-1 protein upregulation in dorsal spinal cord mediates spinal cord injury induced neuropathic pain states

Amin Boroujerdi¹, Jun Zeng^{2,#}, Kelli Sharp³, Donghyun Kim², Oswald Steward³, and Z. David Luo^{1,2}

¹Department of Pharmacology University of California Irvine, School of Medicine, Irvine, CA 92697

²Department of Anesthesiology & Perioperative Care, University of California Irvine, School of Medicine, Irvine, CA 92697

³Reeve-Irvine Research Center, University of California Irvine, School of Medicine, Irvine, CA 92697

Abstract

Spinal cord injury (SCI) commonly results in the development of neuropathic pain, which can dramatically impair the quality of life for SCI patients. SCI induced neuropathic pain can be manifested as both tactile allodynia (a painful sensation to a non-noxious stimulus) and or hyperalgesia (an enhanced sensation to a painful stimulus). The mechanisms underlying these pain states are poorly understood. Clinical studies have shown that gabapentin, a drug that binds to the voltage gated calcium channel alpha-2-delta-1 subunit (Ca_vα₂δ-1) proteins is effective in the management of SCI induced neuropathic pain. Accordingly, we hypothesized that tactile allodynia post SCI is mediated by an upregulation of Ca_vα₂δ-1 in dorsal spinal cord (DSC). To test this hypothesis, we examined if SCI-induced dysregulation of spinal Ca_vα₂δ-1 plays a contributory role in below-level allodynia development in a rat spinal T9 contusion injury model. We found that Ca_vα₂δ-1 expression levels were significantly increased in L4-6 dorsal, but not ventral, spinal cord of SCI rats that correlated with tactile allodynia development in the hindpaw plantar surface. Furthermore, both intrathecal gabapentin treatment and blocking SCI induced Ca_vα₂δ-1 protein upregulation by intrathecal Ca_vα₂δ-1 antisense oligodeoxynucleotides could reverse tactile allodynia in SCI rats. These findings support that SCI induced Ca_vα₂δ-1 upregulation in spinal dorsal horn is a key component in mediating below-level neuropathic pain development and selectively targeting this pathway may provide effective pain relief for SCI patients.

© 2010 International Association for the Study of Pain. Published by Elsevier B.V. All rights reserved.

Corresponding author: Z. David Luo, Department of Anesthesiology & Perioperative Care, University of California Irvine Medical Center Bldg 53, Room 227, 101 The City Drive South, Orange, CA 92868, Phone: 714-456-7962, Fax: 714-456-7903.

#Current address: Department of Anesthesiology, West China Hospital, China, 610041

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

All the authors did not have financial and other relationships that might lead to a conflict of interest in the study.

Introduction

Approximately 1,275,000 individuals in the United States suffer from spinal cord injuries (SCI) with 11,000 cases occurring each year [35,42]. Although the primary effect of SCI is the loss of motor functions, the development of neuropathic pain, or pain derived from injuries to the nervous systems, is a common consequence prevalent in more than half of individuals with SCI [41]. Neuropathic pain can be manifested in some SCI patients as a painful sensation evoked by a non-noxious stimulus (tactile allodynia) or exaggerated pain sensations to mild painful stimuli (hyperalgesia). Retrospective clinical studies have shown that 65% of SCI patients suffer from pain that interferences with activities of their daily lives, and 60% of those patients use medications for pain management [9,32]. Unfortunately, current pain medications are only partially effective and associated with unwanted side effects. This lack of effective and safe pharmacological agents for alleviation of SCI pain is mainly due to our insufficient knowledge in understanding the mechanisms underlying SCI-induced neuropathic pain.

Previous findings have demonstrated that peripheral nerve injury induces upregulation of the voltage gated calcium channel (VGCC) $\alpha_2\delta$ -1 ($\text{Ca}_v\alpha_2\delta$ -1) subunit protein in dorsal spinal cord (DSC) that contributes to the development of tactile allodynia [28]. The $\text{Ca}_v\alpha_2\delta$ -1 protein is a highly glycosylated extracellular subunit associated with VGCC. The $\text{Ca}_v\alpha_2\delta$ -1 plays an important role for functional assembly of the VGCC in the cell membrane [2,11,14,23]. In addition, studies have shown that upregulated $\text{Ca}_v\alpha_2\delta$ -1 subunit modulates calcium channel current density and synaptogenesis [8,13,29]. These factors may contribute to neuropathic pain development. In fact, development of tactile allodynia was either prevented [4] or reversed [28] by blocking injury-induced DSC $\text{Ca}_v\alpha_2\delta$ -1 protein upregulation with dorsal rhizotomy or intrathecal $\text{Ca}_v\alpha_2\delta$ -1 antisense oligodeoxynucleotide treatment, supporting that spinal $\text{Ca}_v\alpha_2\delta$ -1 plays a critical role in neuropathic pain development after peripheral nerve injury.

Data from preclinical studies have demonstrated that neuropathic pain states, similar to that seen in SCI patients, also develop in SCI rat models [6,7] due at least partially to sensitization of dorsal horn neurons [7,18,22]. It has been shown that increased $\text{Ca}_v\alpha_2\delta$ -1 expression in a transgenic mouse line leads to dorsal horn neuron hyperexcitability [29,34]. Preclinical and clinical studies have shown that gabapentin, an anti-neuropathic pain drug that binds to the $\text{Ca}_v\alpha_2\delta$ -1 protein, is effective in alleviating neuropathic pain states in SCI animals [25] and patients [1,27,38,40]. Together, these data suggest that $\text{Ca}_v\alpha_2\delta$ -1 may play a role in SCI-induced neuropathic pain. However, it is not clear if SCI induces dysregulation of $\text{Ca}_v\alpha_2\delta$ -1 expression in spinal dorsal horn, and if so, whether this neuroplasticity contributes to SCI-induced neuropathic pain states. In this study, we examined the expression of spinal cord $\text{Ca}_v\alpha_2\delta$ -1 and its functional role in neuropathic pain states in a SCI model derived from spinal cord T9 contusion injury.

Methods

Spinal cord injury

Spinal cord contusion injury was produced in female Sprague Dawley rats (180–200 g). Intraperitoneal injection of ketamine/xylazine (100:10 mg/kg) was used to anesthetize the rats. The spinal cord thoracic region was shaved and prepped aseptically with betadine for surgery. A midline incision was made at the T8–T11 region followed by a blunt dissection to expose the T9 region of the spinal cord. The spinous process of the T9 vertebrae was then removed by laminectomy exposing the corresponding spinal cord region. A contusion injury was generated using the Infinite Horizon device that emulates a 200 kilo-dynes weight-drop onto the spinal cord from a distance of 3–4 mm [36]. Sham rats were also

generated by producing lameinectomy without the contusion injury. After the contusion injury, connective tissue and muscle were closed with 5-0 chromic gut and the skin was stapled. Immediately post surgery, animals were placed on heating pads until they recovered from anesthesia. Postoperative care involved subcutaneous injections of saline (5mL/100g), Buprenex (3mg/kg) for 3 days and prophylactic Baytril (2.5 mg/kg/d) for 7 days to prevent dehydration and urinary tract infection. SCI animals received manual bladder expression twice daily for 10–14 days until their bladder functions were fully recovered.

Locomotor function recovery test

Locomotor function recovery post SCI was monitored using the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale [3]. Rats were acclimated to their testing environment by handling and exposing them to the open field test apparatus (circular metal enclosure 111. 25 cm diameter, 30cm wall height) 30 to 60 minutes daily for 10 days prior to surgery. The locomotor recovery is based on a scale ranging from 0 (no movement) to 21 (normal functioning). The lower segment of the scale (0–7) measures the early recovery phase, which consists of isolated joint movement of hip, knee, and ankle. The middle portion of the scale (8–12) represents the intermediate recovery phase that corresponds to plantar placement of hindpaw with or without weight support and plantar stepping. The upper portion of the scale (14–21) evaluates the late recovery phase and is assessed after the animal shows consistent coordination between the front and hind limbs. This portion of the scale focuses on paw position, toe clearance, trunk stability and tail position.

Behavioral testing

After SCI rats regained their ability to support their body weight (10 days post SCI), the development of tactile allodynia was determined using the 50% withdrawal threshold (PWT) method described by Chaplan et al (1994). All behavioral testing was performed in a blinded manner, so animals from each group were mixed for the testing. SCI rats with complications such as urinary track infection, permanent loss of motor functions (dragging hindlimb), spontaneous vocalization (spontaneous pain) and severe body weight loss were excluded from the studies and euthanized. Sham and SCI rats were placed in individual clear chambers positioned on top of a mesh screen for 30 minute acclimation. A series of von Frey filaments were then used to measure the degree of mechanical sensitivity based on the up-down method described by Dixon [10]. Each filament was applied in a perpendicular fashion to the glabrous plantar surface of each hind paw (L4-6 dermatomes) with a force causing the filament to buckle. This degree of force was maintained for 6 seconds or until a positive response in the form of sharp withdrawal or paw licking was observed. No withdrawal response prompted the use of next filament with a higher buckling force. A positive response prompted the use of next filament with a lower buckling force. This paradigm continued until 6 measurements were obtained, beginning with the one before the first change in response, or until five consecutive positive (assigned score 0.25g) or negative (assigned score 15g) measurements occurred.

Intrathecal oligodeoxynucleotide injection

The sequences of the $\text{Ca}_v\alpha_2\delta\text{-1}$ (GenBank accession number: M86621) antisense (AGCCATCTTCGCGATCGAAG) and mismatch (CGATACCTCGCTGGCTAAAG) oligodeoxynucleotides with phosphothioate modification on three nucleotides at each end were synthesized commercially (GeneLink, Hawthorne, NY). The antisense, but not mismatch control, oligodeoxynucleotides were effective in reversing peripheral nerve injury-induced $\text{Ca}_v\alpha_2\delta\text{-1}$ upregulation and neuropathic allodynia [28]. The oligodeoxynucleotides were precipitated, washed in 75% ethanol and dissolved in sterile saline before injections. Direct intrathecal injections into allodynic rats (40-day post SCI) were performed using the technique described by Mestre et al., (1995). Briefly, rats were

anesthetized with 2–3% isoflurane. The hair over the lumbar region was shaved and prepped aseptically with betadine. The rats were held securely in one hand at the pelvic rim while a 25-gauge (1/2 inch) needle was inserted between the lumbar 5 and 6 spinous process. The needle entered the subarachnoid region in a perpendicular fashion producing a sudden involuntary tail movement indicative of proper insertion. No other signs of distress or pain were observed. The needle was placed on a needle holder that was connected through tubing to a Hamilton syringe attached to the microinjector (MINJ-PD microINJECTOR All-Digital Positive Displacement System, Tritech Research, Inc., Los Angeles) and the system was filled up with mineral oil to ensure proper small volume injection. The speed of drug uptake and injection was programmed through the microinjector to 0.3S/ μ L. Gabapentin, antisense or mismatch (control) $\text{Ca}_v\alpha_2\delta$ -1 oligodeoxynucleotides in 10 μ L sterile saline or saline only were up-taken into the needle last after the sterile saline cushion (at least 10 μ L) by reversing, then injected by forwarding motion of the microinjector. After each injection, the needle was held in the same position for 15–20 seconds and slowly withdrawn to avoid any outflow of drugs. The daily treatment lasted for 4 to 6 days, and von Frey test was performed daily before the injection.

Western blotting

Frozen lumbar 4–6 dorsal or ventral spinal cord (VSC) sections from sham or SCI rats were pulverized and extracted in 50 mM Tris buffer, pH 7.5, containing 0.5% Triton X-100, 150 mM NaCl, 1 mM EDTA and protease inhibitors. Protein concentrations of extracts were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce; Rockford, IL). Equal amounts of total proteins were then loaded onto a NuPAGE 3–8% Tris-acetate gel (Invitrogen; Carlsbad, CA) under reducing conditions (0.05 M Dichloro-Diphenyl-Trichlorethane) and separated by electrophoresis. The protein bands were transferred onto a nitrocellulose membrane (Bio-Rad; Hercules, CA). The membrane was then incubated in 5% blocking buffer for 1 hour at room temperature followed by overnight incubation at 4°C with monoclonal antibodies for $\alpha_2\delta$ -1 (1:1000; Sigma), GAPDH (1:10000; Ambion) or β -actin (1:10000; Novus) in PBS-T (PBS with 0.1% Tween 20). The membrane was washed 3 times in PBS-T and incubated with secondary antibody conjugated to horseradish peroxidase for 1 hour at room temperature. The membrane was washed again for 3 times, and protein bands were detected using a chemiluminescence reagent (Pierce; Rockford IL) followed by luminescence detection (Kodak 2000MM) or x-ray film exposure within the linear range of the CCD camera or X-ray films. The band densities were quantified using Kodak Image Analysis version 4.0.

Statistic analysis

Significance was indicated by a two-tailed p value < 0.05 performed by ANOVA analyses for multi-group comparisons and by unpaired Student's t tests for pair-wise comparisons as indicated. Summarized data are presented as the means $\pm$ SEM from independent determinations as indicated in each figure legend.

Results

SCI results in the development of allodynia

To determine if SCI led to the development of below-level neuropathic pain states, we tested the behavioral sensitivity to von Frey filament stimulation of sham and T9 spinal contusion rats at the glabrous plantar surface of the hindpaw. Our data indicated that SCI led to a gradual reduction in the hindpaw withdrawal thresholds to the mechanical stimulation to a level considered allodynic (around and below 5 g) in roughly half of the SCI rats (Fig. 1). The hindpaw withdrawal thresholds to mechanical stimulation in another half of SCI rats showed an initial reduction (up to 16 days post SCI) to around 10 g, which is similar to that

seen in sham operated rats in a peripheral nerve injury model [30], then remained at a level similar to that seen in sham operated rats for the rest of the experiment (Fig. 1). These indicate that allodynia is developed below the lesion site in this SCI model (Fig. 1). The slight drop in PWT in the sham group post SCI may be due to the fact that sham and SCI rats were tested together blindly in the same room, so discomfort and ultrasonic vocalization from rats with allodynia may have created additional stress for the sham rats. The behavioral hypersensitivity was not due to a difference in locomotor functional recovery after SCI since locomotor functional recovery scores (BBB) were similar between allodynic and non-allodynic SCI rats at designated time points up to 5 weeks post SCI (Fig. 2). These behavioral data support that spinal cord injury leads to the development of below-level tactile allodynia, a neuropathic pain symptom seen in SCI patients. Thus, this SCI model is suitable for our study.

Upregulated levels of $\text{Ca}_v\alpha_2\delta\text{-1}$ subunit correlates with SCI induced tactile allodynia

Data from our previous experiments have shown that peripheral nerve injury induced upregulation of $\text{Ca}_v\alpha_2\delta\text{-1}$ protein in lumbar DSC mediates tactile allodynia [28]. To determine if SCI also caused $\text{Ca}_v\alpha_2\delta\text{-1}$ upregulation that may lead to allodynia development, we measured $\text{Ca}_v\alpha_2\delta\text{-1}$ protein levels at 14, 30–40 days post injury. These time points correlated respectively with the pre-onset, and well established allodynia post SCI. Our results indicated that there was no significant elevation of $\text{Ca}_v\alpha_2\delta\text{-1}$ protein levels in the DSC of SCI rats 14 day post injury compared with that in the sham rats (Fig. 3A). At 30–40 days post SCI, the $\text{Ca}_v\alpha_2\delta\text{-1}$ protein levels were significantly upregulated in the DSC (Fig. 3B), but not VSC (Fig. 3C), of SCI rats with allodynia compared to that in SCI rats without allodynia or sham rats.

Gabapentin treatment in SCI rats blocked allodynia

To confirm pharmacologically that dysregulation of $\text{Ca}_v\alpha_2\delta\text{-1}$ expression at the spinal level of SCI rats contributes to development of allodynia, we injected gabapentin (300 $\mu\text{g}/\text{rat}$) intrathecally into the L5/6 region of SCI rats with allodynia followed by behavioral sensitivity assessment. Bolus gabapentin treatment reversed SCI-induced allodynia (Fig. 4). The gabapentin effects started 30 min after the injection, peaked in 1 hr and diminished in 4 hrs.

Intrathecal treatment with $\text{Ca}_v\alpha_2\delta\text{-1}$ antisense in SCI rats resulted in reversals of allodynia and upregulated $\text{Ca}_v\alpha_2\delta\text{-1}$ protein in the dorsal spinal cord

To verify whether enhanced expression of $\text{Ca}_v\alpha_2\delta\text{-1}$ subunit in the DSC of SCI rats contributes to allodynia, we treated allodynic SCI rats with intrathecal antisense oligodeoxynucleotides against $\text{Ca}_v\alpha_2\delta\text{-1}$ mRNA to see if this treatment could reverse allodynia by diminishing SCI-induced $\text{Ca}_v\alpha_2\delta\text{-1}$ upregulation in DSC. Our data showed that $\text{Ca}_v\alpha_2\delta\text{-1}$ antisense oligodeoxynucleotides significantly reversed SCI induced allodynia compared to that in allodynic rats treated with mismatch control oligodeoxynucleotides (Fig. 5A). For the same dose of treatment (50 $\mu\text{g}/\text{rat}/\text{day}$), longer treatment duration (6 days vs 4 days) resulted in a longer duration of anti-allodynic effects after the last injection (Fig. 5A). To determine if the antisense effects were due to selective blockade of SCI induced $\text{Ca}_v\alpha_2\delta\text{-1}$ upregulation, we examined $\text{Ca}_v\alpha_2\delta\text{-1}$ protein levels in L4-6 DSC samples from SCI rats treated with $\text{Ca}_v\alpha_2\delta\text{-1}$ antisense or mismatch oligodeoxynucleotides for 6 days. Our data supported that $\text{Ca}_v\alpha_2\delta\text{-1}$ protein levels were significantly lowered in the SCI group treated with antisense oligodeoxynucleotides compared with that in the SCI group treated with mismatch oligodeoxynucleotides (Fig. 5B), supporting that the effects of $\text{Ca}_v\alpha_2\delta\text{-1}$ oligodeoxynucleotides are mediated through the antisense mechanism. Interestingly, the peak effects of allodynia reversal by intrathecal antisense oligodeoxynucleotides were dose-

dependent in a bell shape, indicating that 5 $\mu\text{g}/\text{rat}/\text{day}$ is the optimal dose in allodynia reversal (Fig. 6).

Discussion

Findings from this study support that elevated $\text{Ca}_v\alpha_2\delta-1$ subunit in dorsal spinal cord plays a causal role in mediating SCI induced neuropathic pain. Our major findings supporting this conclusion include that enhanced $\text{Ca}_v\alpha_2\delta-1$ expression in the DSC post SCI correlates with the development of tactile allodynia. Treatment with intrathecal gabapentin reverses SCI-induced tactile allodynia, consistent with findings from previous studies of systemically administered gabapentin [25], and antisense oligodeoxynucleotides against $\text{Ca}_v\alpha_2\delta-1$ block injury-induced $\text{Ca}_v\alpha_2\delta-1$ expression and tactile allodynia.

Data from our behavioral testing showed that roughly half of the SCI rats developed below injury level allodynia, with no significant difference in locomotor function recovery between the allodynic and non-allodynic groups. These results are similar to clinical data showing that approximately half of SCI patients develop neuropathic pain and most of the chronic pain is below the level of SCI [32]. Since SCI-induced allodynia takes weeks to develop, it is likely that SCI at the thoracic level leads to pathological changes in the lumbar region that mediates chronic neuropathic allodynia.

Our data suggest that enhanced $\text{Ca}_v\alpha_2\delta-1$ expression in lumbar spinal cord post SCI is highly likely an important mediator at least for the maintenance of below injury level neuropathic pain states. $\text{Ca}_v\alpha_2\delta-1$ protein levels in dorsal spinal cord were not increased significantly compared with that in sham SCI group during the initiation stage of allodynia. It is not practical to determine at this time point if $\text{Ca}_v\alpha_2\delta-1$ protein levels are increased specifically in allodynic rats because collected dorsal spinal cord samples are from both non-allodynic and allodynic rats, which could not be determined at this time point (14 days post SCI). However, our findings in the later time point (30–40 days post SCI) have shown that $\text{Ca}_v\alpha_2\delta-1$ is significantly upregulated in allodynic rats compared to sham and non-allodynia SCI rats (Fig. 3B). This indicates a correlation between SCI induced $\text{Ca}_v\alpha_2\delta-1$ upregulation in the DSC and allodynia maintenance, and suggests a role of SCI-induced $\text{Ca}_v\alpha_2\delta-1$ dysregulation in maintaining centrally induced allodynia. This is similar to findings from previous studies showing that peripheral nerve injury induces dysregulation of $\text{Ca}_v\alpha_2\delta-1$ in the spinal dorsal horn that plays a critical role in the initiation and maintenance of neuropathic pain [4,28].

The involvement of dorsal horn $\text{Ca}_v\alpha_2\delta-1$ proteins in SCI neuropathic pain was further supported by our behavioral data showing that intrathecal treatment with gabapentin, an anti-neuropathic pain drug that binds to the $\text{Ca}_v\alpha_2\delta-1$ protein with high affinity [15], could reverse established neuropathic pain states in the SCI rats. It has been shown that systemic gabapentin treatment in a similar SCI model also attenuates behavioral hypersensitivities [25]. Findings from this study confirm that the anti-hyperalgesia properties of gabapentin in SCI animals are mediated at least at the spinal level through normalization of central hyperexcitability, presumably induced by $\text{Ca}_v\alpha_2\delta-1$ dysregulation. This is supported by our findings that intrathecal $\text{Ca}_v\alpha_2\delta-1$ antisense, but not mismatch, treatments also caused allodynia reversal in SCI rats. The anti-allodynic effects of intrathecal gabapentin and $\text{Ca}_v\alpha_2\delta-1$ antisense oligodeoxynucleotides in SCI rats were reversible (Fig. 5A), indicating that they were not derived from toxicity. In addition, the anti-allodynic effects of intrathecal $\text{Ca}_v\alpha_2\delta-1$ antisense oligodeoxynucleotides were dose-dependent (Fig. 6). Furthermore, a longer duration of treatment resulted in a longer duration of allodynia reversal after the last injection (Fig. 5A). Western blot results validated that antisense, but not mismatch, oligodeoxynucleotide treatments had lowered the expression levels of $\text{Ca}_v\alpha_2\delta-1$ in DSC

compared to the pre-treatment level (Fig. 5B). Together, these findings suggest that upregulated $\text{Ca}_v\alpha_2\delta-1$ in the DSC post SCI is a mediator for below-level tactile allodynia. Detail information regarding the cell-type and regional specific regulation of this gene at the spinal cord level before and after SCI and $\text{Ca}_v\alpha_2\delta-1$ antisense treatments would provide critical information related to the mechanisms of $\text{Ca}_v\alpha_2\delta-1$ mediated SCI neuropathic pain.

The mechanism underlying SCI induced chronic pain remains elusive. Data from previous studies have shown that $\text{Ca}_v\alpha_2\delta-1$ overexpression in transgenic mice causes enhanced voltage gated calcium channel activity in isolated dorsal root ganglion neurons and dorsal horn neuron hyperexcitability to peripheral stimuli in vivo [29]. Findings from extracellular recordings in spinal dorsal horn of various SCI models have also shown neuronal hyperexcitability [12,19,22,26,43]. The $\text{Ca}_v\alpha_2\delta-1$ mediated dorsal horn neuron hyperexcitability is likely derived from enhanced release of excitatory neurotransmitters from pre-synaptic terminals in the spinal dorsal horn [34]. This is supported by the findings that SCI leads to a 50-fold increase in excitatory amino acids glutamate at the spinal dorsal horn level [31]. Even though the exact mechanism of this enhanced neurotransmitter release post injury is not clear, findings from a recent study have shown that protein factors secreted from activated astrocytes interact with the $\text{Ca}_v\alpha_2\delta-1$ subunit in promoting excitatory synaptogenesis [13]. In addition, there is a dramatic shift in microglial status from a resting to an activated state in the lumbar spinal cord after SCI at a time when dorsal horn nociceptive neurons exhibit hyperexcitability [20]. Activated astrocytes and microglia have been implicated in the development of SCI neuropathic pain [5,33,37,39], including below-level neuropathic pain [16,17,24]. It is highly possible that increased $\text{Ca}_v\alpha_2\delta-1$ in the DSC post SCI interacts with other factors from glial cells that results in abnormal synaptogenesis and enhanced glutamate release, which in turn cause dorsal horn neuron hyperexcitability and neuropathic pain states. However, we cannot rule out potential interactions of SCI-induced $\text{Ca}_v\alpha_2\delta-1$ with other factors and pathways that also contribute to SCI-induced below-level neuropathic pain development [18,21].

In summary, we provided evidence to support that the $\text{Ca}_v\alpha_2\delta-1$ protein plays a critical role in the maintenance of neuropathic pain with a central origin. While the underlying mechanisms are unknown and still under investigation, findings from this study suggest that SCI induced $\text{Ca}_v\alpha_2\delta-1$ or its interacting proteins could serve as new therapeutic targets for the development of more specific medications for SCI pain management.

Acknowledgments

This study is supported in part by grants from the Christopher & Dana Reeve Foundation and the Roman Reed Spinal Cord Injury Research Fund of California (ZD Luo). A. Boroujerdi was the recipient of a Roman Reed Predoctoral Fellowship.

Reference

1. Ahn SHPH, Lee BS, Moon HW, Jang SH, Sakong J, Bae JH. Gabapentin effect on neuropathic pain compared among patients with spinal cord injury and different durations of symptoms. *Spine* 2003;28:341–346. [PubMed: 12590206]
2. Arikath J, Campbell KP. Auxiliary subunits: essential components of the voltage-gated calcium channel complex. *Curr Opin Neurobiol* 2003;13:298–307. [PubMed: 12850214]
3. Basso DM, Beattie MS, Bresnahan JC. A sensitive and reliable locomotor rating scale for open field testing in rats. *J Neurotrauma* 1995;12:1–21. [PubMed: 7783230]
4. Boroujerdi AKH, Lyu YS, Kim DS, Figueroa KW, Chung JM, Luo ZD. Injury discharge regulates calcium channel $\alpha_2\delta-1$ subunit upregulation in the dorsal horn that contributes to initiation of neuropathic pain. *Pain* 2008;139:358–366. [PubMed: 18571852]

5. Carlton SM, Du J, Tan HY, Nesic O, Hargett GL, Bopp AC, Yamani A, Lin Q, Willis WD, Hulsebosch CE. Peripheral and central sensitization in remote spinal cord regions contribute to central neuropathic pain after spinal cord injury. *Pain* 2009;147:265–276. [PubMed: 19853381]
6. Christensen MD, Everhart AW, Pickelman JT, Hulsebosch CE. Mechanical and thermal allodynia in chronic central pain following spinal cord injury. *Pain* 1996;68:97–107. [PubMed: 9252004]
7. Christensen MD, Hulsebosch CE. Chronic central pain after spinal cord injury. *J Neurotrauma* 1997;14:517–537. [PubMed: 9300563]
8. Chu PJBP. Molecular cloning of calcium channel alpha(2)delta-subunits from rat atria and the differential regulation of their expression by IGF-1. *J Mol Cell Cardiol* 2003;35:207–215. [PubMed: 12606261]
9. de Miguel M, Kraychete DC. Pain in patients with spinal cord injury: a review. *Rev Bras Anesthesiol* 2009;59:350–357. [PubMed: 19488549]
10. Dixon WJ. Efficient analysis of experimental observations. *Annu Rev Pharmacol Toxicol* 1980;20:441–462. [PubMed: 7387124]
11. Dolphin ACWC, Richards J, Beattie RE, Craig P, Lee JH. The effect of alpha2-delta and other accessory subunits on expression and properties of the calcium channel alpha1. *J Physiol* 1999;519:35–45. [PubMed: 10432337]
12. Drew GM, Siddall PJ, Duggan AW. Responses of spinal neurones to cutaneous and dorsal root stimuli in rats with mechanical allodynia after contusive spinal cord injury. *Brain Res* 2001;893:59–69. [PubMed: 11222993]
13. Eroglu CAN, Susman MW, O'Rourke NA, Park CY, Ozkan E, et al. Gabapentin receptor alpha2delta-1 is a neuronal thrombospondin receptor responsible for excitatory CNS synaptogenesis. *Cell* 2009;139:380–392. [PubMed: 19818485]
14. Felix R. Voltage-dependent Ca²⁺ channel alpha2delta auxiliary subunit: structure, function and regulation. *Receptors Channels* 1999;6:351–362. [PubMed: 10551267]
15. Gee NS, Brown JP, Dissanayake VU, Offord J, Thurlow R, Woodruff GN. The novel anticonvulsant drug, gabapentin (Neurontin), binds to the alpha2delta subunit of a calcium channel. *J Biol Chem* 1996;271:5768–5776. [PubMed: 8621444]
16. Gwak YS, Crown ED, Unabia GC, Hulsebosch CE. Propentofylline attenuates allodynia, glial activation and modulates GABAergic tone after spinal cord injury in the rat. *Pain* 2008;138:410–422. [PubMed: 18353556]
17. Gwak YS, Hulsebosch CE. Remote astrocytic and microglial activation modulates neuronal hyperexcitability and below-level neuropathic pain after spinal injury in rat. *Neuroscience* 2009;161:895–903. [PubMed: 19332108]
18. Hains BC, Johnson KM, Eaton MJ, Willis WD, Hulsebosch CE. Serotonergic neural precursor cell grafts attenuate bilateral hyperexcitability of dorsal horn neurons after spinal hemisection in rat. *Neuroscience* 2003;116:1097–1110. [PubMed: 12617951]
19. Hains BC, Klein JP, Saab CY, Craner MJ, Black JA, Waxman SG. Upregulation of sodium channel Nav1.3 and functional involvement in neuronal hyperexcitability associated with central neuropathic pain after spinal cord injury. *J Neurosci* 2003;23:8881–8892. [PubMed: 14523090]
20. Hains BC, Waxman SG. Activated microglia contribute to the maintenance of chronic pain after spinal cord injury. *J Neurosci* 2006;26:4308–4317. [PubMed: 16624951]
21. Hains BC, Willis WD, Hulsebosch CE. Differential electrophysiological effects of brain-derived neurotrophic factor on dorsal horn neurons following chronic spinal cord hemisection injury in the rat. *Neurosci Lett* 2002;320:125–128. [PubMed: 11852178]
22. Hains BC, Willis WD, Hulsebosch CE. Temporal plasticity of dorsal horn somatosensory neurons after acute and chronic spinal cord hemisection in rat. *Brain Res* 2003;970:238–241. [PubMed: 12706267]
23. Hendrich J, Van Minh AT, Heblich F, Nieto-Rostro M, Watschinger K, Striessnig J, Wratten J, Davies A, Dolphin AC. Pharmacological disruption of calcium channel trafficking by the alpha2delta ligand gabapentin. *Proc Natl Acad Sci U S A* 2008;105:3628–3633. [PubMed: 18299583]
24. Hulsebosch CE. Gliopathy ensures persistent inflammation and chronic pain after spinal cord injury. *Exp Neurol* 2008;214:6–9. [PubMed: 18708053]

25. Hulsebosch CE, Xu GY, Perez-Polo JR, Westlund KN, Taylor CP, McAdoo DJ. Rodent model of chronic central pain after spinal cord contusion injury and effects of gabapentin. *J Neurotrauma* 2000;17:1205–1217. [PubMed: 11186233]
26. Lampert A, Hains BC, Waxman SG. Upregulation of persistent and ramp sodium current in dorsal horn neurons after spinal cord injury. *Exp Brain Res* 2006;174:660–666. [PubMed: 16718433]
27. Levendoglu FOC, Ozerbil O, Ogun TC, Ugurlu H. Gabapentin is a first line drug for the treatment of neuropathic pain in spinal cord injury. *Spine* 2004;29:743–751. [PubMed: 15087796]
28. Li CY, Song YH, Higuera ES, Luo ZD. Spinal dorsal horn calcium channel $\alpha 2\delta$ -1 subunit upregulation contributes to peripheral nerve injury-induced tactile allodynia. *J Neurosci* 2004;24:8494–8499. [PubMed: 15456823]
29. Li CY, Zhang XL, Matthews EA, Li KW, Kurwa A, Boroujerdi A, Gross J, Gold MS, Dickenson AH, Feng G, Luo ZD. Calcium channel $\alpha 2\delta$ 1 subunit mediates spinal hyperexcitability in pain modulation. *Pain* 2006;125:20–34. [PubMed: 16764990]
30. Luo ZD, Chaplan SR, Higuera ES, Sorkin LS, Stauderman KA, Williams ME, Yaksh TL. Upregulation of dorsal root ganglion (α)2(δ) calcium channel subunit and its correlation with allodynia in spinal nerve-injured rats. *J Neurosci* 2001;21:1868–1875. [PubMed: 11245671]
31. McAdoo DJXG, Robak G, Hughes MG. Changes in amino acid concentrations over time and space around an impact injury and their diffusion through the rat spinal cord. *Exp Neurol* 1999;159:538–544. [PubMed: 10506525]
32. Miladinovic K. [Spinal cord injury and chronic pain]. *Med Arh* 2009;63:106–107. [PubMed: 19537669]
33. Nestic O, Lee J, Johnson KM, Ye Z, Xu GY, Unabia GC, Wood TG, McAdoo DJ, Westlund KN, Hulsebosch CE, Regino Perez-Polo J. Transcriptional profiling of spinal cord injury-induced central neuropathic pain. *J Neurochem* 2005;95:998–1014. [PubMed: 16219025]
34. Nguyen D, Deng P, Matthews EA, Kim DS, Feng G, Dickenson AH, Xu ZC, Luo ZD. Enhanced pre-synaptic glutamate release in deep-dorsal horn contributes to calcium channel α -2- δ -1 protein-mediated spinal sensitization and behavioral hypersensitivity. *Mol Pain* 2009;5:6. [PubMed: 19216737]
35. Paralysis Resource Center CDRF.
http://www.christopherreeve.org/site/c.mtKZKgMWKwG/b.5184255/k.6D74/Prevalence_of_Paralysis.htm.
36. Scheff SW, Rabchevsky AG, Fugaccia I, Main JA, Lumpp JE Jr. Experimental modeling of spinal cord injury: characterization of a force-defined injury device. *J Neurotrauma* 2003;20:179–193. [PubMed: 12675971]
37. Schmitt ABBA, Breuer S, Brook GA, Pech K, Martin D, Schoenen J, Noth J, Love S, Schroder JM, Kreutzberg GW, Nacimiento W. Major histocompatibility complex class II expression by activated microglia caudal to lesions of descending tracts in the human spinal cord is not associated with a T cell response. *Acta Neuropathol* 2000;100:528–536. [PubMed: 11045675]
38. Siddall PJCM, Otte A, Griesing T, Chambers R, Murphy TK. Pregabalin in central neuropathic pain associated with spinal cord injury: A placebo-control trial. *Neurology* 2006;67:1792–1800. [PubMed: 17130411]
39. Sroga JM, Jones TB, Kigerl KA, McGaughy VM, Popovich PG. Rats and mice exhibit distinct inflammatory reactions after spinal cord injury. *J Comp Neurol* 2003;462:223–240. [PubMed: 12794745]
40. Tai Q, Kirshblum S, Chen B, Millis S, Johnston M, DeLisa JA. Gabapentin in the treatment of neuropathic pain after spinal cord injury: a prospective, randomized, double-blind, crossover trial. *J Spinal Cord Med* 2002;25:100–105. [PubMed: 12137213]
41. Yeziarski RP. Pain following spinal cord injury: the clinical problem and experimental study. *Pain* 1996;185–194. [PubMed: 9121805]
42. Yeziarski RP. Spinal cord injury: a model of central neuropathic pain. *Neurosignals* 2005;14:182–193. [PubMed: 16215301]
43. Yeziarski RP, Park SH. The mechanosensitivity of spinal sensory neurons following intraspinal injections of quisqualic acid in the rat. *Neurosci Lett* 1993;157:115–119. [PubMed: 8233021]

Fig. 1. Behavioral sensitivity to von Frey filament stimulation post SCI

The 50% withdrawal thresholds to von Frey filament stimulation were tested at the glabrous plantar surface of hind paws from sham or SCI rats at designated time points. In naive rats, the normal thresholds are between 10–15 grams. After SCI, approximately half of the SCI rats developed tactile allodynia at the hind paw level as indicated by reduced paw withdrawal thresholds to von Frey filament stimulation to about 5 g. Data presented are the means $\pm$ SEM from 10 sham rats, and 30 each in the allodynic or non-allodynic groups. ** $p > 0.01$, *** $p < 0.001$ compared with the sham group; & $p < 0.05$ compared with the sham group; ### $p < 0.001$ compared with the non-allodynic group determined by two-way ANOVA analyses.

Fig. 2. Scores of BBB locomotor assessment test on SCI groups with and without tactile allodynia Locomotor functional recovery scores were obtained weekly from SCI rats with or without allodynia using the BBB locomotor assessment as described. Data presented are the means $\pm$ SEM from 7 to 15 animals in each group.

Fig. 3. $\text{Ca}_v\alpha_2\delta-1$ protein levels in L4-6 spinal cord of rats at designated time points post SCI
Total proteins were extracted from L4-6 dorsal (A and B) or ventral (C) spinal cord at 14 days (A) or 30 to 40 days (B and C) post SCI, and $\text{Ca}_v\alpha_2\delta-1$ protein levels were examined with Western blot analysis. Representative Western blots were shown on top of each bar graph in A and B. The ratio of $\text{Ca}_v\alpha_2\delta-1$ band density to that of GAPDH within each sample was calculated before cross-sample comparisons for normalization of sample loading. Data presented are the means $\pm$ SEM from 4 – 9 independent determinations. * $p < 0.05$; *** $p < 0.001$ determined by Students' t test.

Fig. 4. Effects of intrathecal gabapentin on SCI-induced allodynia

SCI rats with tactile allodynia were treated intrathecally with 300 μ g/rat of gabapentin or sterile saline via direct intrathecal injection into the L5-6 region followed by test for paw withdrawal thresholds (PWT) to von Frey filament stimulation as indicated. Data presented are the means $\pm$ SEM from 5 rats in each group. ** $p < 0.005$; *** $p < 0.001$ compared with pretreatment levels as determined by two-way ANOVA analyses.

Fig. 5. Effects of intrathecal treatment with $\text{Ca}_v\alpha_2\delta-1$ antisense or mismatch oligodeoxynucleotides on SCI induced allodynia and $\text{Ca}_v\alpha_2\delta-1$ dysregulation

SCI rats with tactile allodynia were treated daily for 4 or 6 days with 50 $\mu\text{g}/\text{rat}/\text{day}$ of $\text{Ca}_v\alpha_2\delta-1$ antisense or mismatch oligodeoxynucleotides via direct intrathecal injection into the L5-6 region. A. Paw withdrawal thresholds (PWT) to von Frey filament stimulation were measured daily before the injection, and continued after the last injection as indicated. Data presented are the means $\pm$ SEM from the rat numbers from each group as indicated below: Data from 3 and 11 rats treated with antisense oligodeoxynucleotides for four or six days, respectively, were combined, then 3 rats in each group for the recovery period post treatment; 11 rats in the mismatch treated group. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$

compared with pre-treatment levels as determined by two-way ANOVA analyses. B. $\text{Ca}_v\alpha_2\delta-1$ protein levels in L4-6 dorsal spinal cord of allodynic SCI rats after intrathecal treatment with $\text{Ca}_v\alpha_2\delta-1$ antisense or mismatch ligodeoxynucleotides. Total proteins were extracted from L4-6 dorsal spinal cord of allodynic SCI rats approximately 12 hrs after the last intrathecal injection of the six-day treatment, and $\text{Ca}_v\alpha_2\delta-1$ protein levels were examined with Western blot analysis. Representative western blots were shown on top of each bar graph, which summarized Western blot data for each group. The ratio of $\text{Ca}_v\alpha_2\delta-1$ band density to that of β -actin within each sample was calculated before cross-sample comparisons for normalization of sample loading. The dot line represents the level of $\text{Ca}_v\alpha_2\delta-1$ protein in dorsal spinal cord of allodynic SCI rats without any treatment as shown in Fig. 3B. Data presented are the means $\pm$ SEM from 7 to 8 independent determinations in each group. * $p < 0.05$ compared with the mismatch treatment data as determined by Students' t test.

Fig. 6. Dose dependent effects of intrathecal $\text{Ca}_v\alpha_2\delta-1$ antisense oligodeoxynucleotides in blocking allodynia in SCI rats

SCI rats with tactile allodynia were treated daily for 6 days with different doses of $\text{Ca}_v\alpha_2\delta-1$ antisense oligodeoxynucleotides via direct intrathecal injection into the L5-6 region. PWT were measured daily before the injection, and continued after the last injection for a total of at least eight days. The peak effects of allodynia reversal by different doses of $\text{Ca}_v\alpha_2\delta-1$ antisense oligodeoxynucleotides as defined by maximum allodynia reversal in each dose group during the treatment period. Data presented are the means $\pm$ SEM from 7 to 14 SCI rats in each group. *** $p < 0.001$ compared with the pre-treatment level, # $p < 0.05$ compared with the 5 $\mu\text{g/rat/day}$ group as determined by one-way ANOVA analyses.