Injury Discharges Regulate Calcium Channel Alpha-2-Delta-1 Subunit Upregulation in the Dorsal Horn that Contributes to Initiation of Neuropathic Pain

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Abstract

Previous studies have shown that peripheral nerve injury in rats induces increased expression of the voltage gated calcium channel (VGCC) alpha-2-delta-1 subunit ($Ca_v\alpha_2\delta_1$) in spinal dorsal horn and sensory neurons in dorsal root ganglia (DRG) that correlates to established neuropathic pain states. To determine if injury discharges trigger $Ca_v\alpha_2\delta_1$ induction that contributes to neuropathic pain initiation, we examined allodynia onset and $Ca_v\alpha_2\delta_1$ levels in DRG and spinal dorsal horn of spinal nerve ligated rats after blocking injury induced neural activity with a local brief application of lidocaine on spinal nerves before the ligation. The lidocaine pretreatment blocked ligation-induced discharges in a dose-dependent manner. Similar pretreatment with the effective concentration of lidocaine diminished injury-induced increases of the $Ca_v\alpha_2\delta_1$ in DRG and abolished that in spinal dorsal horn specifically, and resulted in a delayed onset of tactile allodynia post injury. Both dorsal horn $Ca_v\alpha_2\delta_1$ upregulation and tactile allodynia in the lidocaine pretreated rats returned to levels similar to that in saline pretreated controls two weeks post the ligation injury. In addition, preemptive intrathecal $Ca_v\alpha_2\delta_1$ antisense treatments blocked concurrently injury-induced allodynia onset and $Ca_v\alpha_2\delta_1$ upregulation in dorsal spinal cord. These findings indicate that injury induced discharges regulate $Ca_v\alpha_2\delta_1$ expression in the spinal dorsal horn that is critical for neuropathic allodynia initiation. Thus, preemptive blockade of injury-induced neural activity or $Ca_v\alpha_2\delta_1$ upregulation may be a beneficial option in neuropathic pain management.

Keywords

Nerve injury; spinal neuroplasticity; neuropathic pain; allodynia initiation

Introduction

Chronic pain derived from peripheral nerve injury (neuropathic pain) is a disorder affecting adversely the quality of patients' daily-life. Current medications for neuropathic pain have

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limited specificity and efficacy and the development of new medications relies on our better understanding of neuropathic pain mechanisms. Recent findings from our group and others have indicated that expression of the voltage-gated calcium channels (VGCC) $\alpha_2\delta_1$ subunit ($Ca_v\alpha_2\delta_1$) is highly upregulated in the spinal dorsal horn and dorsal root ganglia (DRG) after nerve injury [34,35,38,41,42], which correlates with established neuropathic pain in animal models [29,34,35]. Since a complete blockade of injury-induced $Ca_v\alpha_2\delta_1$ in dorsal spinal cord by dorsal rhizotomy or intrathecal $Ca_v\alpha_2\delta_1$ antisense oligodeoxynucleotides can reverse established neuropathic pain states in nerve injured rats [29], injury-induced spinal $Ca_v\alpha_2\delta_1$ upregulation is critical in neuropathic pain maintenance. However, it remains unclear what are the injury factors responsible for $Ca_v\alpha_2\delta_1$ induction and if this neuroplasticity also plays a critical role in neuropathic pain initiation.

The $Ca_v\alpha_2\delta_1$ is a structural subunit of the VGCC, which include low voltage-activated T-type, and high voltage-activated L-, N-, P/Q-, and R-types depending on the channel forming $\alpha 1$ subunits [17]. It contains two disulfide-linked peptides (α_2 and δ) encoded by the same gene [12,15]. The protein is highly glycosylated, contains a single transmembrane domain and five intracellular carboxyl terminal amino acids [7,19,43]. The $\alpha_2\delta$ family consists of at least four distinctive genes, $Ca_v\alpha_2\delta_1$, $Ca_v\alpha_2\delta_2$, $Ca_v\alpha_2\delta_3$ and $Ca_v\alpha_2\delta_4$ [28,39]. These $Ca_v\alpha_2\delta$ subunits show tissue-specific expression patterns [11,37], suggesting that they may process tissue-specific functions. In addition to serving as an auxiliary subunit of the VGCC in promoting and stabilizing VGCC at the plasma membrane [3,5,18–20], $Ca_v\alpha_2\delta$ may have other physiological functions, including synapse modulation as suggested recently [13,16]. So far, only $Ca_v\alpha_2\delta_1$ has been shown to be upregulated by nerve injury.

Response to nerve injury in the sensory pathways could be dynamic, involving, but not limited to, short-term changes such as injury discharges that could have immediate effects on induction of gene expression and neuropathic pain, and long-term changes such as injury-induced expression of other factors that could have slow, but prolonged effects on expression of down-stream target genes and neuropathic pain maintenance. Changes in different stages post injury may have unique contribution to neuropathic pain. In order to understand the mechanism of injury induction in $Ca_v\alpha_2\delta_1$ expression and neuropathic pain, we examined the influence of nerve injury-induced neuronal discharges on $Ca_v\alpha_2\delta_1$ regulation, and its functional role in neuropathic pain initiation.

Materials and Methods

Neuropathic lesion and lidocaine application

Adult male rats (200–250g, Sprague Dawley; Harlan Sprague-Dawley Co., Houston, TX) were housed in a room with a reverse 12/12 hr light/dark cycle (dark cycle: 8:00 A.M. – 8:00 P.M.) with free access to food and water for a week before experiments. All animal care and experiments were carried out according to protocols approved by the Institutional Animal Care Committee of the University of California, Irvine and the University of Texas Medical Branch, Galveston.

Unilateral spinal nerve ligation (SNL) was performed by the procedure described by Kim and Chung [26]. Briefly, the left L5 lumbar spinal nerve was exposed in anesthetized rats, wrapped with a small piece of Gelfoam soaked with either sterile saline or 10% lidocaine for 15 min, washed with sterile saline, and then tightly ligated with 6.0 silk suture distal to DRG. For tissue collections from the saline or lidocaine pretreated SNL rats, both left L4/5 spinal nerves were ligated to increase sampling tissue volume. For the antisense and mismatch oligodeoxynucleotide pretreated SNL rats, both left L5/6 spinal nerves were ligated to increase sampling tissue volume. DRG and spinal dorsal horn tissues were collected at day 5 and 14 post surgeries and kept at $-80\ °C$ until use.

Multi-unit dorsal root recording

Nine rats (3 rats each for saline, 2% lidocaine, and 10% lidocaine groups) were anesthetized with sodium pentobarbital. The left L5 spinal nerve was exposed following the procedure described earlier [10], and a L1 to L6 laminectomy was performed. The rat was mounted on a spinal investigation frame and a heated mineral oil pool (37 °C) was made over the exposed tissue to prevent drying. The dura mater was opened and the L5 dorsal root was cut close to the cord. The distal part of the cut dorsal root was placed on a mirror-based platform and divided into four equal-size fascicles with a pair of forceps under a dissecting microscope. One fascicle was placed on a pair of bipolar recording hook electrodes for multi-unit recording. The proximal part of the exposed L5 spinal nerve was wrapped around with a small piece of Gelfoam soaked with either saline, or 2% or 10% lidocaine solution for 15 minutes, and then washed with saline before nerve ligation. The receptive field of foot pad was tapped with a glass rod at 3 different time points including before lidocaine application, 6 and 10 minutes during the 15 minutes lidocaine or saline application period. After 15 minutes of lidocaine application, the Gelfoam piece was removed and the spinal nerve was washed with saline, then tightly ligated with a 6-0 silk suture. Multi-unit activity was recorded, amplified with an AC-coupled amplifier (WPI, DAM-80), displayed on an oscilloscope and processed with a data acquisition system (CED 1401).

Preemptive intrathecal oligodeoxynucleotide treatment

Oligodeoxynucleotides were sterilized with ethanol precipitation and dissolved in sterile saline, and directly injected through the L5/L6 spinal region of an isofluorane anesthetized rat in a total volume of 10 μL, once daily, using a microinjector connected to a 30 G needle. The preemptive treatment started one day prior to spinal nerve ligation surgery and lasted for five days.

Behavioral testing

Behavioral tests were performed blindly so that the examiner did not know the experimental manipulation. For experiments of local saline and lidocaine pre-treatment before the ligation injury, paw withdrawal thresholds to mechanical stimuli were measured at designated time points only in the planter surface of the left (L5 spinal nerve ligated) hind paw. For experiments of preemptive intrathecal antisense treatment, paw withdrawal thresholds to mechanical stimuli were measured in the planter surface of both left (L5/6 spinal nerve ligated) and right (non-injured) hind paws at designated time points. For each test, the animal was habituated for at least 15 minutes in a plastic chamber placed on top of a mesh screen so that mechanical stimuli could be applied to the plantar surface of the left hind paw. Paw withdrawal thresholds were determined by the up-down method [4,8,14] using a set of von Frey monofilaments. Specifically, a von Frey filament (starting with the 4.31 one) was applied perpendicularly to the most sensitive areas of the plantar surface at the base of the 3rd or 4th toes with sufficient force to bend the filament slightly for 2 to 3 seconds. An abrupt foot withdrawal during stimulation or immediately after stimulus removal was considered a positive response that led to the use of the next weaker filament. When no response was obtained, the next stronger filament was applied. This testing pattern continued until responses to six von Frey stimuli, starting from the one that elicited the first change in response, were measured. The responses were then converted into a 50% threshold value using the formula: 50% threshold = $10^{(X + kd)}/10^4$, where X = the value of the final von Frey hair used (in log units), k = the tabular value for the pattern of positive/negative responses, and d = the mean differences between stimuli in log units (0.22) [14]. When positive or negative responses were still observed at the end of a stimulus session, values of 3.54 or 5.27 were assigned, respectively, by assuming a value of ± 0.5 for k in these cases. In some cases, the von Frey filament values had been converted to gram force values.

Western blot analysis

To measure protein expression, frozen tissues were homogenized and extracted in 50 mM Tris buffer, pH 8.0, containing 0.5% Triton X-100, 150 mM NaCl, 1 mM EDTA, and protease inhibitors. The protein concentration was determined using the Bicinchonic Acid Protein Assay Kit (Pierce, Rockford, IL). The protein extracts were separated by NuPAGE 3–8 % Tris–acetate gel (Invitrogen, Carlsbad, CA) electrophoresis under a reducing condition (0.05 M DTT). After electrophoresis, the gel was transferred onto a nitrocellulose membrane (BioRad, Hercules, CA), which was subsequently incubated in 5 % blocking buffer (0.1% Tween 20 and 5% nonfat dry milk in PBS) for 1 hr at room temperature. The membrane was then incubated with monoclonal antibodies for $Ca_v\alpha_2\delta_1$ (1:1000 dilution, Sigma, St. Louis, MO), or GFAP (BD Pharmingen, 1:1000 dilution) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:10000 dilution, for verifying equal protein loading, Ambion, Austin, TX) overnight at 4 °C. After washing, the membranes were incubated with anti-mouse IgG secondary antibodies conjugated to horseradish peroxidase (1:10000 dilution, Cell Signaling Tech, Danvers, MA) for one hr at room temperature. The bands were then visualized using an enhanced chemiluminescent reagent (Super Signal West Pico; Pierce, Rockford, IL). Under reducing conditions the delta peptide separates from the alpha2 subunit [23], therefore the detected band by the $Ca_v\alpha_2\delta_1$ primary antibody is reflective of the alpha 2 subunit only. Rat brain extracts containing high levels of $Ca_v\alpha_2\delta_1$ proteins were used as positive controls. Densitometric analysis of the immunoreactive bands was performed using a Kodak Imaging station (2000MM) within the linear range of the CCD camera and X-ray films.

Statistical Analyses

Data were presented as the means ± S.E.M, and significant changes were defined by the two-tailed p value < 0.05 as determined with unpaired Student's t test.

Results

Blocking nerve injury-induced discharges with lidocaine delayed the onset of tactile allodynia in spinal nerve ligated rats

To investigate whether nerve injury induced discharges play a role in the genesis of neuropathic pain, we pretreated spinal nerves at the ligation site with different concentrations of lidocaine 15 min before the ligation to block ligation-induced discharges. As shown in Fig. 1, spinal nerve ligation (SNL) induced brief injury discharges. Treatment of the spinal nerve with 2% lidocaine did not completely block injury discharges, or conduction of impulses generated by tapping the receptive field. However 10% of lidocaine completely blocked both nerve conduction and injury discharges. Thus, 10% lidocaine was used to block nerve ligation induced discharges in SNL rats for the biochemical experiments.

After spinal nerve ligation injury, the animals developed tactile allodynia manifested as reduced paw withdrawal thresholds to mechanical stimulation. The tactile allodynia was fully developed three days post nerve ligation (Fig. 2) and lasted for at least 10 weeks [35]. Interestingly, 10% lidocaine pretreatment for 15 min at the ligation site delayed the onset of tactile allodynia to day 14 post ligation in SNL rats (Fig. 2).

Blocking nerve injury-induced discharges with lidocaine delayed calcium channel $Ca_v\alpha_2\delta_1$ subunit upregulation in the dorsal horn of spinal nerve ligated rats

It has been reported that SNL injury causes upregulation of the $Ca_v\alpha_2\delta_1$ gene in sensory neurons and spinal dorsal horn [29,35,38,42]. In addition, upregulated $Ca_v\alpha_2\delta_1$ correlates with established neuropathic allodynia [29,35,38], suggesting a role of this neuroplasticity in neuropathic pain. However, how nerve injury regulates $Ca_v\alpha_2\delta_1$ expression and whether

$Ca_v\alpha_2\delta_1$ upregulation plays a critical role in neuropathic pain induction are not clear. To test the hypothesis that injury induced neuronal discharges play a critical role in $Ca_v\alpha_2\delta_1$ upregulation, which in turn contributes to the onset of neuropathic pain in SNL rats, we examined the $Ca_v\alpha_2\alpha_1$ protein levels at designated time points post SNL in DRG and dorsal spinal cord of SNL rats pretreated with 10% lidocaine or saline. If nerve injury discharges are required for the initiation of $Ca_v\alpha_2\delta_1$ upregulation in DRG and spinal dorsal horn that is important to neuropathic pain initiation, then blocking the discharges with lidocaine should delay or prevent $Ca_v\alpha_2\delta_1$ upregulation and neuropathic pain onset. In contrast, if nerve injury discharges are not required for $Ca_v\alpha_2\delta_1$ upregulation and neuropathic pain development, then blocking neuronal discharges should not affect $Ca_v\alpha_2\delta_1$ upregulation and neuropathic pain initiation.

As shown in Fig. 3, SNL induced dramatic increases in $Ca_v\alpha_2\delta_1$ protein levels in DRG and spinal dorsal horn of saline pretreated SNL rats five days post SNL, which correlated with the full onset of tactile allodynia in the SNL rats (Fig. 2). SNL also led to a further increase in dorsal horn $Ca_v\alpha_2\delta_1$ expression at day 14 post SNL in saline pretreated rats (Fig. 3B). These changes were consistent with findings reported previously in the same model [29,35]. However, injury-induced upregulation of $Ca_v\alpha_2\delta_1$ in DRG was diminished (Fig. 3A) and that in the spinal dorsal horn was blocked (Fig. 3B) at post injury day five by a brief lidocaine pretreatment at the ligation site for 15 min. In contrast to saline pretreated SNL rats, these lidocaine pretreated SNL rats did not develop tactile allodynia at post SNL day five (Fig. 2).

The lidocaine blockade of injury-induced $Ca_v\alpha_2\delta_1$ upregulation and allodynia onset was transient. Injury-induced $Ca_v\alpha_2\delta_1$ upregulation in dorsal horn returned to the control level observed in the saline pretreated SNL rats, and that in the DRG was higher than the control level observed in the saline pretreated SNL rats 14 days post ligation injury (Fig. 3), a time point coincided with the delayed onset of tactile allodynia in the lidocaine pretreated SNL rats (Fig. 2).

To determine the specificity of lidocaine effects on spinal $Ca_v\alpha_2\delta_1$ expression, we examined GFAP expression in spinal dorsal horn of SNL rats with similar lidocaine pretreatment. SNL injury induced significant upregulation of GFAP in the injury side of dorsal horn from saline pretreated rats at both day five and 14 post SNL. Similar pretreatment with 10% lidocaine at the ligation site did not change significantly the injury-induced GFAP upregulation at both time points (Fig. 4), suggesting that the lidocaine pretreatment blocked dorsal horn $Ca_v\alpha_2\delta_1$ expression specifically. However, our data could not exclude the possibility that lidocaine might have altered the expression of other proteins that were not examined.

Injury-induced dorsal horn $Ca_v\alpha_2\delta_1$ upregulation is critical for allodynia initiation

To determine directly if injury-induced dorsal horn $Ca_v\alpha_2\delta_1$ upregulation played a critical role in allodynia initiation, we examined the onset of allodynia in SNL rats pretreated with intrathecal $Ca_v\alpha_2\delta_1$ antisense oligodeoxynucleotides that were known to be effective in blocking $Ca_v\alpha_2\delta_1$ upregulation and reverse established allodynia in SNL rats (Li et al., 2004). As shown in Fig. 5, initiation of injury-induced allodynia was blocked in all but one SNL rats treated with intrathecal $Ca_v\alpha_2\delta_1$ antisense, but not mismatch, oligodeoxynucleotides (50 µg/rat/day) started one-day before the SNL. The antisense effects in blocking allodynia onset were evident after four days of treatment and lasted for at least two days after the last injection. Neither the antisense nor mismatch oligodeoxynucleotide treatment affected the paw withdrawal thresholds in the non-injury side. As shown in Fig. 6, data from Western blot analysis indicated that the degrees of dorsal horn $Ca_v\alpha_2\delta_1$ upregulation by injury were similar among SNL rats showing tactile allodynia, including mismatch treated and antisense-insensitive SNL rats at the end of the intrathecal treatments (four days post injury) and SNL rats 10 days after the last intrathecal mismatch or antisense treatment, all of which were similar

to that seen in dorsal spinal cord of saline and mismatch treated SNL rats with established allodynia [29]. In contrast, intrathecal treatment with $Ca_v\alpha_2\delta_1$ antisense oligodeoxynucleotides, which had minimal effects on basal level dorsal horn $Ca_v\alpha_2\delta_1$ expression (in the contralateral side) [29], blocked injury-induced dorsal horn $Ca_v\alpha_2\delta_1$ upregulation only in SNL rats sensitive to antisense blockade of allodynia initiation, but not in the SNL rat insensitive to the antisense blockade of allodynia initiation (Fig. 5).

Discussion

It is appreciated that spinal nerve injury induces upregulation of $Ca_v\alpha_2\delta_1$ in sensory neurons and spinal dorsal horn that contributes to neuropathic pain [29,30,35,38]. However, it is not clear how nerve injury regulates $Ca_v\alpha_2\delta_1$ expression, and whether this neuroplasticity affects initiation of neuropathic pain specifically. Using lidocaine pretreatment at the ligation site that blocks injury induced neuronal discharges, we have shown that blocking injury-induced neuronal discharges in SNL rats can transiently diminish or block injury-induced $Ca_v\alpha_2\delta_1$ upregulation in DRG and spinal dorsal horn, respectively, and delay the onset of tactile allodynia. Delayed tactile allodynia appeared precisely in lidocaine-pretreated SNL rats when injury-induced dorsal horn $Ca_v\alpha_2\delta_1$ upregulation returned to a level similar to that observed in saline-pretreated SNL rats. Preemptive intrathecal $Ca_v\alpha_2\delta_1$ antisense oligodeoxynucleotide treatment blocked injury-induced dorsal horn $Ca_v\alpha_2\delta_1$ upregulation only in SNL rats that are also sensitive to antisense-blockade of allodynia initiation. Together, these findings suggest that injury induced neuronal discharges play an important role in the initiation of injury-induced $Ca_v\alpha_2\delta_1$ upregulation in DRG and spinal dorsal horn, and this plasticity in spinal dorsal horn is critical for the induction of tactile allodynia.

While findings from our previous studies have indicated that injury-induced dorsal horn $Ca_v\alpha_2\delta_1$ upregulation, which is mainly from elevated DRG $Ca_v\alpha_2\delta_1$ proteins, is the driving force for established tactile allodynia [29], findings from this study indicate that increased dorsal horn $Ca_v\alpha_2\delta_1$ expression is critical for tactile allodynia initiation. Even though lidocaine pretreatment diminished significantly injury-induced DRG $Ca_v\alpha_2\delta_1$ expression at day five post ligation injury, the increased $Ca_v\alpha_2\delta_1$ levels in injured DRG were still about 10-folds over that seen in non-injured DRG from the contralateral side. However, lidocaine pretreated SNL rats failed to develop tactile allodynia while saline pretreated SNL rats fully developed tactile allodynia at this time point. This suggests that increased DRG $Ca_v\alpha_2\delta_1$ expression is not a dominant factor in tactile allodynia initiation post SNL injury. In contrast, brief lidocaine pretreatment in SNL rats led to a concurrent blockade of injury-induced dorsal horn $Ca_v\alpha_2\delta_1$ upregulation and tactile allodynia five days post injury, both returned to levels similar to that observed in saline pretreated SNL rats 14 days post SNL, supporting a critical role of injury discharges in the induction of dorsal horn $Ca_v\alpha_2\delta_1$ and tactile allodynia. Furthermore, preemptive intrathecal antisense oligodeoxynucleotide treatment blocked both injury-induced dorsal horn $Ca_v\alpha_2\delta_1$ upregulation and tactile allodynia at the same time point post injury only in SNL rats sensitive to the antisense treatment. Together, it seems that injury-induced neural activity is the driving force for dorsal horn $Ca_v\alpha_2\delta_1$ upregulation that in turn plays important roles in tactile allodynia initiation. This conclusion is consistent with findings that blocking signaling pathways that mediate injury-induced $Ca_v\alpha_2\delta_1$ upregulation at the spinal cord level is sufficient to block initiation, but not maintenance, of neuropathic pain in SNL animals [22].

Since the effective duration of lidocaine in circulation is short (< 2 hrs) [25], the impact of delayed but sustained ectopic discharges, which start 13 to 24 hrs post injury [21,32,33], on $Ca_v\alpha_2\delta_1$ expression could not be studied in our experimental settings. The temporal blockade of injury-induced $Ca_v\alpha_2\delta_1$ upregulation in DRG and spinal dorsal horn indicates that brief local application of high concentrations of lidocaine did not cause irreversible toxic effects in DRG

and spinal dorsal horn, at least for the cells expressing $Ca_v\alpha_2\delta_1$. The lack of lidocaine effects on injury-induced dorsal horn GFAP expression further supports that the lidocaine effects on dorsal horn $Ca_v\alpha_2\delta_1$ expression are specific and not due to toxicity, even though there remains possibility that lidocaine may also alter expression of other proteins that are not examined in our study. Indeed, injury-induced discharges are likely a regulatory factor that plays a critical role in the induction of $Ca_v\alpha_2\delta_1$ upregulation. The discrepancy between an over 10-fold $Ca_v\alpha_2\delta_1$ upregulation in injured DRG but the control level $Ca_v\alpha_2\delta_1$ expression in the injury side of dorsal horn in five-day SNL rats pretreated briefly with lidocaine suggests that transportation of $Ca_v\alpha_2\delta_1$ proteins from DRG to dorsal horn post SNL injury was not regulated passively by concentration gradients, but by active axonal transport that may be modulated by initial injury-induced discharges. This conclusion is consistent with in vitro findings that lidocaine can inhibit axonal transport in isolated DRG neurons [24]. However, our data do not allow us to exclude the possibility that injury-induced discharges also regulate post-synaptic dorsal horn $Ca_v\alpha_2\delta_1$ expression through a trans-synaptic regulatory mechanism.

Enhanced expression of the $Ca_v\alpha_2\delta_1$ gene in spinal dorsal horn and DRG has been found to correlate with the expression of neuropathic hypersensitivity in nerve injured animals [29,35, 38], and preventing such an increase, especially that at the presynaptic terminals of central sensory afferents, leads to a reversal of established tactile allodynia in nerve injured animals [29]. Overexpression of the $Ca_v\alpha_2\delta_1$ gene in neuronal tissues of transgenic mice results in exaggerated dorsal horn neuron excitability and behavioral hypersensitivity to mechanical and thermal stimuli. These hypersensitivities occur without concurrent changes in dorsal horn neurons' responses to windup stimulation and receptive fields, implying normal dorsal horn neuron intrinsic excitability post $Ca_v\alpha_2\delta_1$ upregulation [30]. All these findings suggest that overexpression of the $Ca_v\alpha_2\delta_1$ gene contributes to, at least partially, the development of abnormal sensation through enhanced excitability at the presynaptic terminals. Our current findings indicate that this neuroplasticity may represent one of the possible molecular bases for the pharmacological efficacy of lidocaine in alleviating neuropathic pain sensations derived from peripheral nerve injuries in preclinical and clinical experiments [1,2,6,9,36,40].

It is worthy to point out that systemic lidocaine administration provides biphasic anti-hyperalgesic effects in spinal nerve injured rats. An acute phase followed by a sustained phase that lasts for several weeks [2]. Since the prolonged lidocaine effects over last the effective duration of the drug substantially, the sustained anti-hyperalgesic effects of lidocaine must be mediated by mechanisms independent of its direct actions on nerve blockade initially. Since it has been shown that nerve injury-induced discharges regulate gene expression in DRG cells [27,31], it is possible that nerve injury-induced discharges propagate to sensory neurons to activate transcription factors that enhance transcription of genes encoding $Ca_v\alpha_2\delta_1$ and possibly other molecules required for enhancing transportation of elevated DRG $Ca_v\alpha_2\delta_1$ proteins to the spinal dorsal horn. These regulatory mechanisms in injury discharge-induced gene regulation in DRG remain to be explored. Nevertheless, blocking injury-induced upregulation of dorsal horn $Ca_v\alpha_2\delta_1$ by lidocaine may underlie the sustained anti-hyperalgesic effects of lidocaine in some neuropathic pain states, and the preemptive dosing strategy in preventing nerve injury-induced neuropathic pain may open the door for further investigation in preventing the occurrence of neuropathic pain states from other conditions, such as diabetic- and chemotherapy-induced neuropathies [34,44], which could also lead to dorsal spinal cord $Ca_v\alpha_2\delta_1$ upregulation.

Acknowledgements

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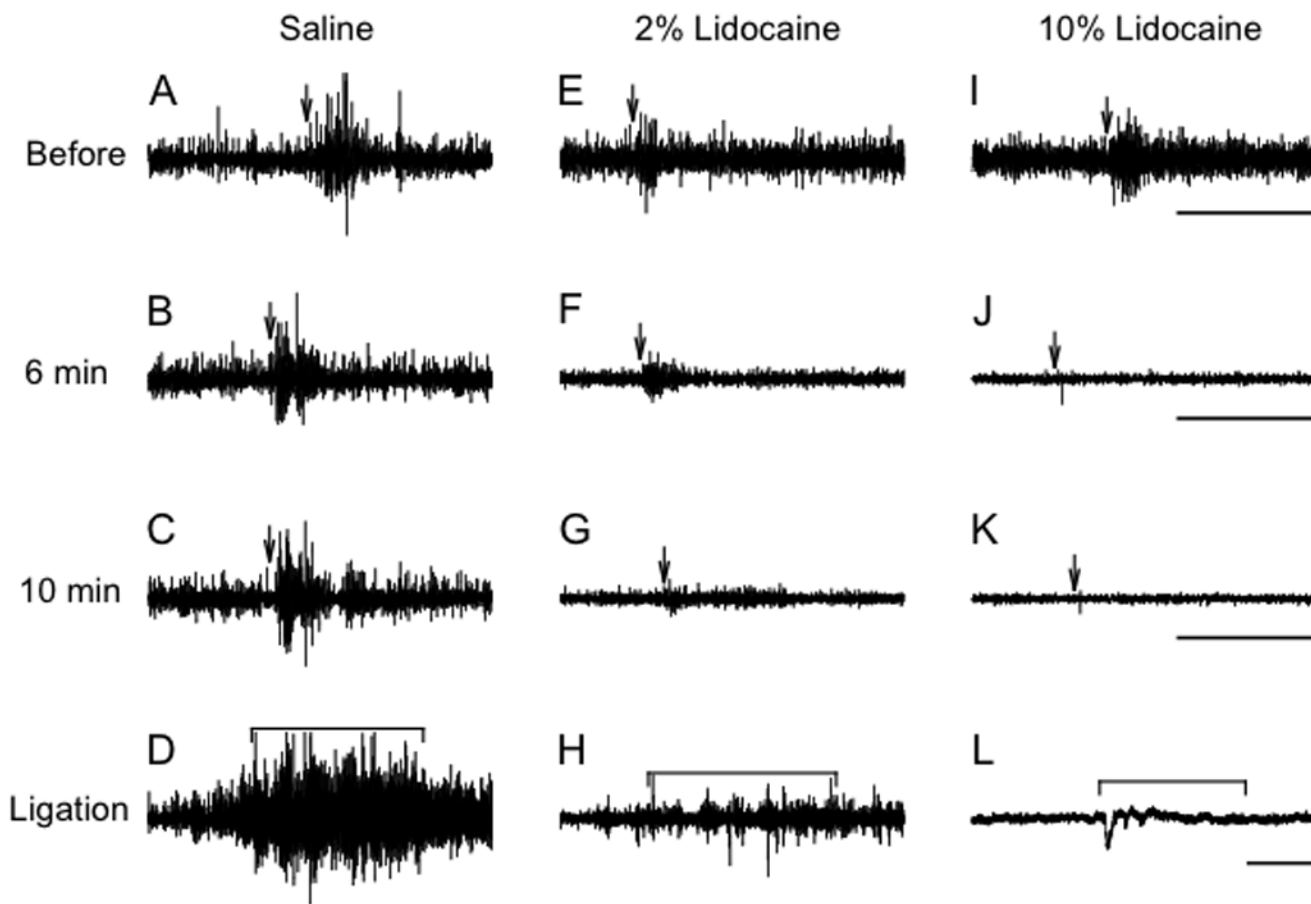
Fig. 1. Blockage of injury discharges by local lidocaine application
In three different experiments (A–D, E–H, and I–L), multi-unit activity was recorded from a fascicle of the L5 dorsal root before brief application of saline or one of the two concentrations (2% or 10%) of lidocaine to the left L5 spinal nerve for 15 min. During this period, the footpad (receptive field) was tapped with a glass rod at the times indicated by arrows - before lidocaine application (top row), and at 6 (2nd row) and 10 min (3rd row) after lidocaine (or saline) application. After 15 min, the spinal nerve was washed with saline, and then tightly ligated with a 6-0 silk suture (during the periods indicated by brackets in the bottom row). Calibration bars on the right column indicate 0.2 sec for each row. Note that nerve conduction (response to tapping) or injury discharges were completely eliminated with 10%, but not 2%, lidocaine. Signals shown in L represent an artifact associated with spinal nerve movement during the ligation.
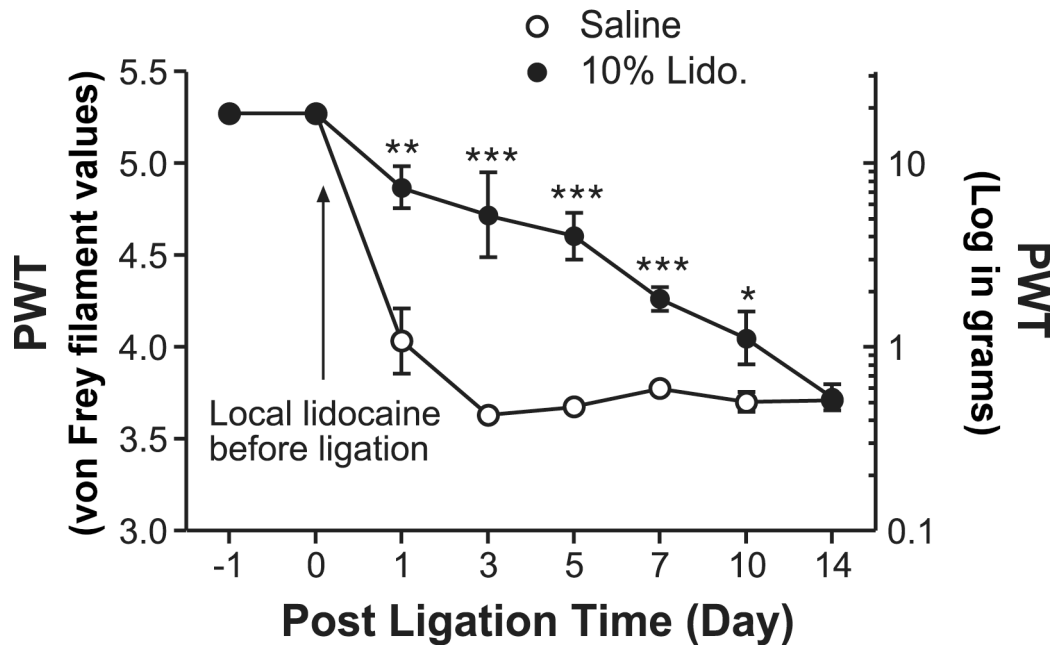
Fig. 2. Brief local-application of lidocaine delayed injury-induced neuropathic allodynia
Paw withdrawal thresholds (PWT) to von Frey filament stimulations were tested in rats before and at designated times after L5 spinal nerve ligation following pretreatment with saline or 10% lidocaine at the ligation site for 15 min. The arrow indicates the time of local lidocaine pretreatment before the ligation. Data presented are the means ± SEM from seven rats in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with saline pretreated group.
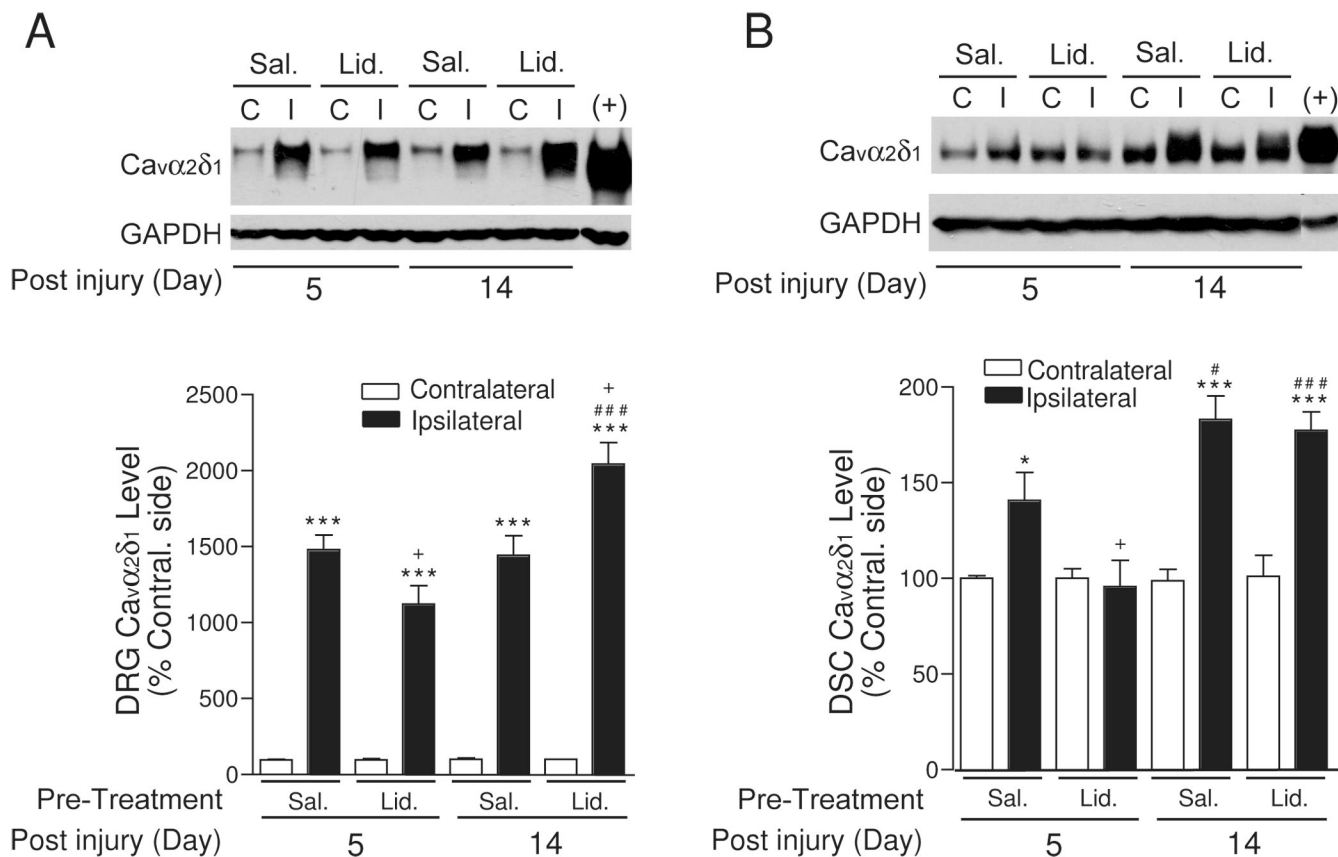
Fig. 3. Influence of brief local-application of lidocaine on nerve injury-induced $Ca_v\alpha_2\delta_1$ upregulation in spinal dorsal horn and DRG

Western blot analysis was performed in L4/5 DRG (A) and spinal dorsal horn (B) samples taken at indicated time points post injury from injury (I) and contralateral (C) sides of spinal nerve ligated rats, pretreated with either saline or 10% lidocaine at the ligation site for 15 min. Representative Western blot images from each group were presented at the top of each panel of summarized Western blot data. Band density ratios of the $Ca_v\alpha_2\delta_1$ band over the GAPDH band within each sample were calculated for loading normalization before data comparison between injury and non-injury sides. Data presented are the means ± SEM from at least six independent determinations. DSC – dorsal spinal cord. Sal. – Saline pretreated. Lid. – 10% lidocaine pretreated. * $p < 0.05$, *** $p < 0.001$ compared with saline pretreatment within each group. # $p < 0.05$, ### $p < 0.001$ compared with the group of post-injury day five received the same pretreatment at the ligation site. + $p < 0.05$ compared with values from the injury (ipsilateral) side of saline pretreated rats within the same time point.
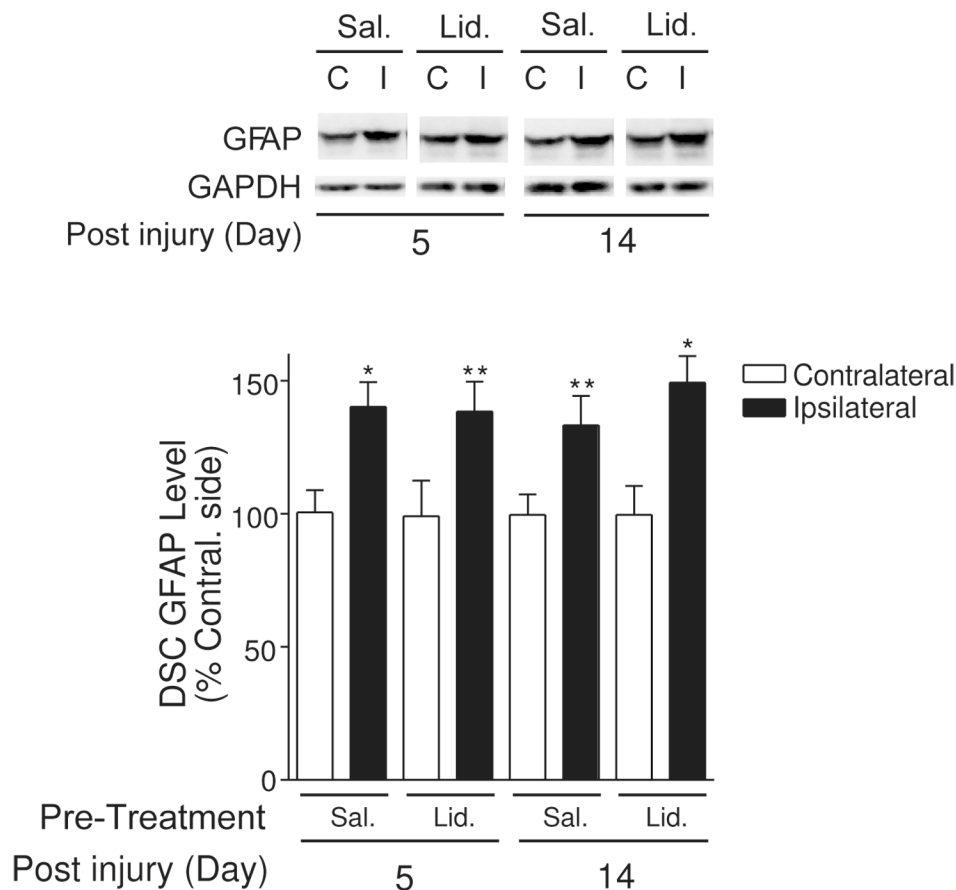
Fig. 4. Influence of brief local-application of lidocaine on nerve injury-induced GFAP upregulation in dorsal spinal cord

Western blot analysis was performed in L4/5 spinal dorsal horn samples taken at indicated time points post injury from injury, or ipsilateral, (I) side and contralateral (C) side of SNL rats, pretreated with either saline or 10% lidocaine at the ligation site for 15 min before the nerve ligation. Representative Western blot images from each group were presented at the top of each panel of summarized Western blot data. Band density ratios of the GFAP band over the GAPDH band within each sample were calculated for loading normalization before data comparison between injury and non-injury sides. Data presented are the means ± SEM from at least six independent determinations. DSC – dorsal spinal cord. Sal. – Saline pretreated. Lid. – 10% lidocaine pretreated. * $p < 0.05$, ** $p < 0.01$ compared with saline pretreatment within each group.
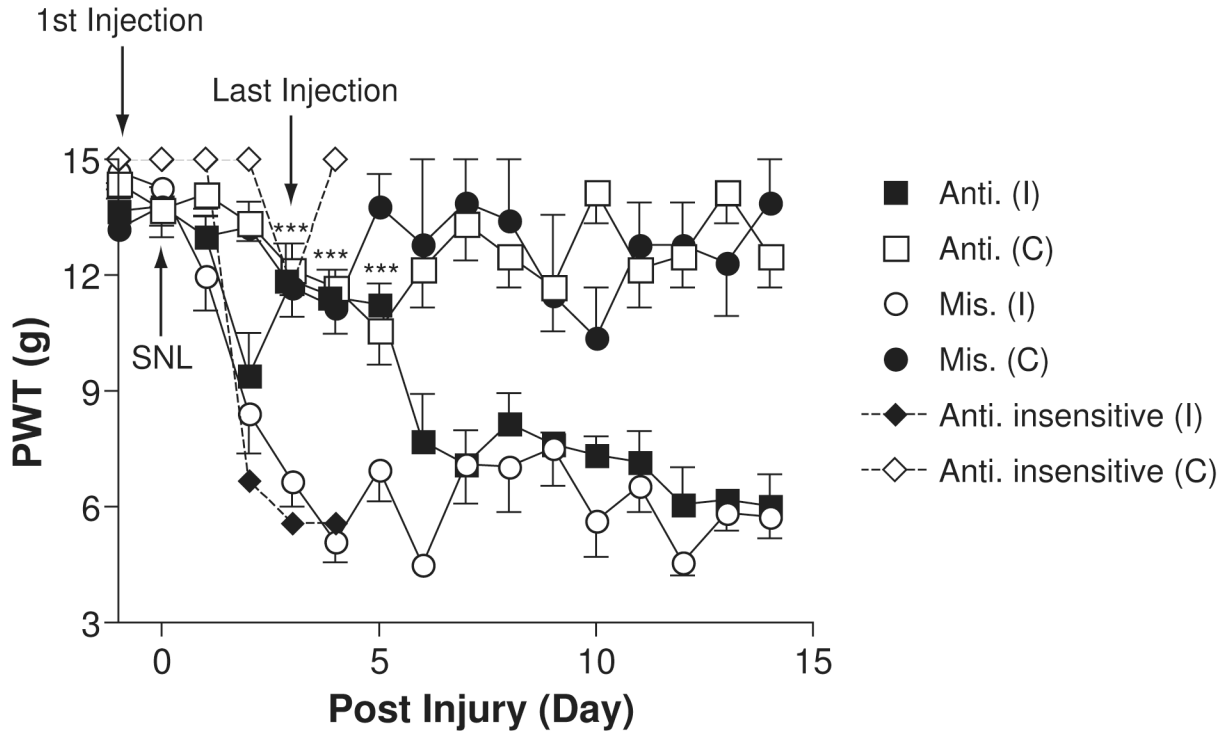
Fig. 5. Effects of preemptive intrathecal $Ca_v\alpha_2\delta_1$ antisense treatment on spinal nerve ligation induced tactile allodynia

$Ca_v\alpha_2\delta_1$ antisense or mismatch oligodeoxynucleotides (50 µg/rat, once daily) were injected directly into the L5/6 lumbar spinal regions for five consecutive days, starting one-day before SNL. Paw withdrawal thresholds (PWT) to von Frey filament stimulations were tested blindly in the rats before the daily intrathecal injection, then daily after the last intrathecal injection for a total of 14 days after the ligation injury. Data presented are the means ± SEM from 14 rats in the antisense group (not including one rat in the antisense-insensitive group shown in dashed lines) and 15 rats in the mismatch group up to post SNL day 4 (one-day after the last intrathecal injection), and from four (mismatch) to five (antisense) rats from post SNL day 5 to 14. C – Contralateral (non-injury) side. I – Ipsilateral (injury) side. *** $p < 0.001$ between data from the antisense (filled square) and mismatch (open circle) oligodeoxynucleotide treatments at the injury side.
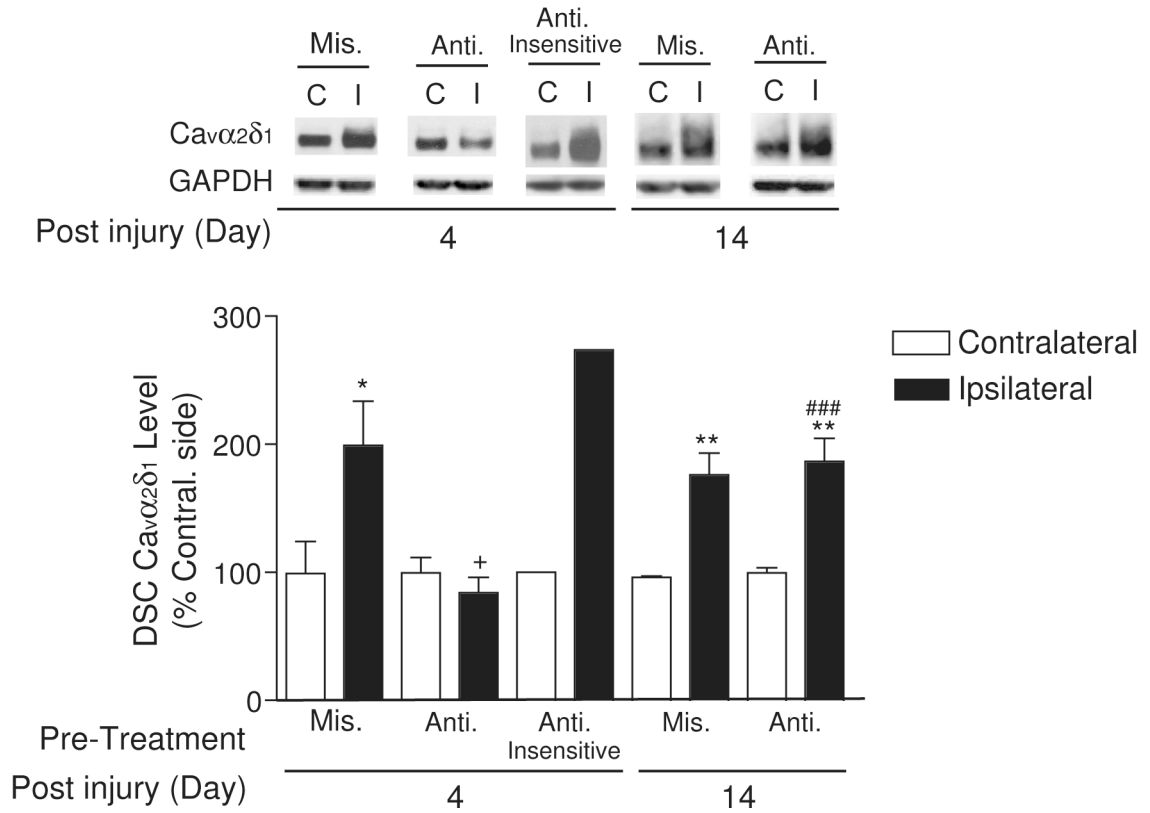
Fig. 6. Influence of intrathecal preemptive Ca$_V$α$_2$δ$_1$ antisense treatment on nerve injury-induced Ca$_V$α$_2$δ$_1$ upregulation in spinal dorsal horn

Western blot analysis was performed in L5/6 spinal dorsal horn samples taken from injury, or ipsilateral, (I) side and contralateral (C) side of four-day or 14-day SNL rats that were pretreated with either Ca$_V$α$_2$δ$_1$ antisense or mismatch oligodeoxynucleotides for five days, starting one-day before the SNL as described for Fig. 5. Representative Western blot images from each group were presented at the top of each panel of summarized Western blot data. Band density ratios of the Ca$_V$α$_2$δ$_1$ band over the GAPDH band within each sample were calculated for loading normalization before data comparison between injury and non-injury sides. Data presented are the means ± SEM from five (antisense) to six (mismatch) rats, and the mean of two independent Western blots from one antisense-insensitive rat, in the four-day post SNL groups; and the means ± SEM from four (mismatch) to five (antisense) rats in the 14-day post SNL groups. DSC – dorsal spinal cord. Anti. – antisense oligodeoxynucleotide pretreated. Mis. – mismatch oligodeoxynucleotide pretreated. * p < 0.05, ** p < 0.01 compared with contralateral side within each group. ### p < 0.001 compared with the ligation site of the four-day post-SNL group that was sensitive to the antisense pretreatment. + p < 0.05 compared with values from the injury (ipsilateral) side of mismatch oligodeoxynucleotide pretreated rats within the same time point.