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Post-translational modification of cortical GluA receptors in rodents following spinal cord lesion

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Abstract

Previous studies investigating the pathophysiology of neuropathic pain caused by injury to the spinal cord suggest that pain may result, at least in part, from maladaptive plasticity in the somatosensory cortex and associated pain networks. However, little is known about the molecular and cellular mechanisms leading to maladaptive plasticity in the cortex and how they contribute to the development of neuropathic pain.

AMPA-type glutamate receptors (GluARs) mediate fast excitatory synaptic transmission in the mammalian brain and play an important role in pain processing. Here we used an electrolytic lesion model of spinal cord injury in animals to study the expression and phosphorylation of GluA1 and 2 in the primary somatosensory cortex (S1). Experiments in rats and mice revealed that maladaptive plasticity and hypersensitivity after SCL are associated with a reduction in the fraction of GluA1 subunits that are phosphorylated at serine 831 (S831) in the hindlimb representation of S1 (S1HL). Manipulations that reduce the fraction of phosphorylated S831 in S1HL of non-lesioned animals, including low frequency electrical stimulation and viral-mediated gene transfer of mutant S831, were associated with the development of hypersensitivity. Taken together, these findings suggest that phosphorylation of GluA1 at S831 play an important role in the development of hypersensitivity after SCL.

Introduction

Injury to the spinal cord is associated with neurological changes not only at the site of injury but also in supra-spinal structures, including the thalamus and cerebral cortex, that lead to the development of chronic neuropathic pain. Most of the evidence concerning spinal injury-

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related cortical changes arise from neuroimaging studies. These studies illustrate that neuropathic pain is associated with enhanced BOLD signals and anatomical and neurochemical changes in primary somatosensory cortex (S1) of humans (Cesaro et al., 1991; Peyron et al., 2000; Ducreux et al., 2006; Wrigley et al., 2009; Gustin et al., 2010; Stanwell et al., 2010; Henderson et al., 2011; Widerstrom-Noga et al., 2013) and animals (Morrow et al., 2000; Paulson et al., 2005; Endo et al., 2007; Endo et al., 2008b; Endo et al., 2008a). More recently, it has been shown that injury-induced neuropathic pain is also associated with altered electroencephalogram patterns in humans (Jensen et al., 2013b; Jensen et al., 2013a). Consistent with these findings, we found that neurons in S1 of rats with spinal cord lesions have significantly higher spontaneous firing rates, greater evoked responses to innocuous and noxious mechanical stimulation and a greater tendency to fire bursts of action potentials (Quiton et al., 2010). Using resting state functional MRI, we also found a reduction in functional connectivity between thalamus and S1, and subsequently an increased connectivity between S1 and cortical areas involved in nociceptive processing (Seminowicz et al., 2012). Thus, the excruciating pain following spinal cord injury may result, at least in part, from *hyperactivity* in S1 and associated pain networks. However, little is known about the molecular and cellular mechanisms leading to maladaptive plasticity in the cortex and how they contribute to the development of neuropathic pain.

The studies discussed above show that the capacity of S1 to compensate for injury-induced anatomical and functional disturbances is impaired, suggesting that homeostatic plasticity of cortical synapses is compromised. Homeostatic synaptic plasticity is regulated primarily via the regulation of the expression and function of AMPA-type glutamate receptors (GluARs) (Wang et al., 2012). Therefore, it is likely that changes in expression and function of GluARs in S1 may contribute to development of neuropathic pain following spinal cord injury.

Although several studies have shown that GluARs play an important role in nociceptive processing in the spinal cord (Wang et al., 2012), very little is known about their role in nociceptive processing in S1 after spinal cord injury. GluARs are tetrameric assemblies of highly homologous subunits, GluA1-4, and are assembled as two identical heterodimers with GluA1 and GluA2 being the most predominant GluAR subunits in S1 (Conti et al., 1994; Kondo et al., 1997). Alterations in synaptic strength can be achieved through regulation of GluAR expression and the composition of tetramers, and by phosphorylation of specific sites on GluAR subunits (Lee et al., 2010; Lee and Kirkwood, 2011). Phosphorylation of serine 831 (S831) and serine 845 (S845), located at the C-terminal end of the GluA1 subunit, is implicated in long-term potentiation and depression of synapses (Lee et al., 2003; Lee et al., 2007) via modulation of GluAR conductance (S831) (Derkach et al., 1999; Kristensen et al., 2011) and open channel probability (S845) (Roche et al., 1996; Banke et al., 2000).

In this study, we propose that spinal cord lesion is associated with maladaptive homeostatic plasticity and post-translational modification of GluA1 subunits. We test this hypothesis in rodents and predict that spinal cord lesion is associated with alterations in GluA1 phosphorylation at specific serine residues heavily involved in synaptic plasticity. We also

predict that changes in GluA1 serine residue phosphorylation correlate with changes in hypersensitivity observed after spinal lesions.

Materials and Methods

Animals

All experimental procedures were carried out in strict accordance with the Animal Welfare Act regulations, Public Health Service guidelines, the International Association for the Study of Pain guidelines, and a protocol approved by the University of Maryland Baltimore Institutional Animal Care and Use Committee.

Male and female Sprague Dawley rats weighting 250–300g were purchased from Envigo (n = 84). Transgenic mice in which inhibitory neurons are labeled with green fluorescent protein (GAD65-GFP mice; n = 16) obtained from Dr. Adam Puche. Transgenic B6.129-Gria1^{tm5Rlh/J} (S831 mutant) mice (n = 6) and strain-matched wild-type mice (C57BL/6; n = 6) were obtained from the Jackson Laboratory (Bar Harbor, Maine). All animals were housed in climate-controlled facilities with a 12h light/dark cycle and free access to food and water.

Spinal cord lesion (SCL)

We used an animal model of SCL, in which an electrolytic lesion was generated in the anterolateral quadrant of the spinal cord at the level of C6 or T10. Under aseptic conditions, the animals were anesthetized with ketamine/xylazine (rat: 80mg/kg / 10mg/kg, mouse: 150mg/kg / 16mg/kg) and placed on a thermo-regulated heating pad to maintain the body temperature. A laminectomy was performed to expose the spinal cord, and the dura was removed. In rats (n=18), a metal electrode (5 μ m tip) was advanced to the anterolateral quadrant of the spinal cord at C6 level and direct current of 10 μ A was passed through the electrode for 10 sec for 4 times to produce an electrolytic lesion, as we described previously (Masri et al., 2009). In mice (GAD65-GFP mice, n=16), a metal electrode with same tip size was advanced to the anterolateral quadrant of spinal cord at T10 level and direct current of 10 μ A was passed through the electrode for 10 sec twice to produce an electrolytic lesion. After surgery, the muscles and skin were sutured in layers to approximate incision sites.

Low frequency electrical stimulation (LFS)

Rats were anesthetized with isoflurane (1–3%) and placed into a stereotaxic frame (Narishige). The bone overlying the primary motor cortex was removed and the custom-made epidural bipolar platinum electrodes (diameter: 70 μ m, exposed tip: 50 μ m, distance between the electrodes: 500 μ m) were placed above the right primary motor cortex (M1) and stabilized using dental resin, as we described previously (Davoody et al., 2011; Lucas et al., 2011; Cha et al., 2013; Jiang et al., 2014).

A stimulator (A-M Systems) was used to deliver LFS (50 μ A, 1 Hz, and 300 μ s, for 30 minutes/session) to the right M1. One session of LFS was performed daily and repeated over 5 consecutive days. In control experiments, animals implanted with electrodes received

sham stimulation in which the electrodes were connected to the stimulator but no current was passed.

In non-lesioned rats, changes in mechanical withdrawal thresholds were assessed daily (see below for details) before and immediately after LFS or sham LFS ($n = 6/\text{group}$). In a separate group of animals, Western blot analysis (described below) was performed on tissue samples extracted from the hindlimb representation of S1 (S1HL) immediately after one stimulation session (1X), five stimulation sessions (5X), or five sham stimulation sessions ($n = 14/\text{group}$). We focused on S1HL because of the below-level hypersensitivity in lesioned animals following injury (Wang and Thompson, 2008; Davoody et al., 2011) and because we have shown that functional and metabolic activity in S1HL is abnormally enhanced in neuropathic pain (Seminowicz et al., 2012; Hubbard et al., 2014).

Virus injection

Replication-deficient Herpes Simplex Viruses 1 (HSV1) that over-express wild-type GluA1 subunits [HSV-GluA1-mCherry], or mutant GluA1 subunits, where S831 is replaced by alanine [HSV-GluA1-S831A-mCherry] were obtained from Dr. Rachael Neve at the MIT Viral Core. These viral vectors have been shown previously to be effective in the expression of wild-type GluA1 or mutant GluA1 subunits starting 2–3 hours after viral injection and reaching maximal expression 2–5 days post-injection, before diminishing to undetectable levels 8–10 days after injection (Carlezon et al., 2000b; Carlezon et al., 2000a; Choi et al., 2003; Neve et al., 2005; Todtenkopf et al., 2006; Bachtell et al., 2008; Hobson et al., 2013).

To deliver the virus, Sprague Dawley rats ($n=12$) were anesthetized with isoflurane (1–3%) and placed in a stereotaxic frame. The bone overlying S1 was exposed and stereotaxic coordinates were used to guide a Hamilton syringe loaded with the virus to S1HL. The viral vector was microinjected (2 μL , titer: $3\text{--}5 \times 10^8$ infectious units/mL) in S1HL over a 10-minute period. The Hamilton syringe was left in place for 20 minutes after viral injection before withdrawal.

Rats injected with virus expressing mutant or wild-type GluA1 ($n = 6/\text{group}$) were tested daily for changes in mechanical withdrawal thresholds at both the left and right hindpaws.

Behavioral testing

Mechanical hypersensitivity was assessed using the up-down method (Dixon, 1965; Chaplan et al., 1994), and measured as hindpaw withdrawal thresholds with calibrated von Frey filaments (Stoelting). Animals were habituated for two weeks before behavioral testing in order to minimize the animals' anxiety. On the day of the behavioral test, animals were placed in Plexiglas boxes on a mesh surface. Von Frey filaments were applied in sequence to the plantar surface of the hindpaw. Hindpaw withdrawal upon application of the filament constituted a positive response. A positive response prompted the use of the next lightest filament, while absence of response prompted increasing filament weight. This paradigm was continued for 4 measurements after the initial change in behavior, or when 5 consecutive negative responses were obtained. The sequence of positive and negative scores was used to interpolate the 50% response threshold.

Baseline behavioral tests were conducted on three consecutive days before SCL, LFS, or viral injection. SCL animals underwent further behavioral testing on days 3, 7, 14, and 21 post surgery. Animals receiving LFS and animals receiving virus injection (see below) also underwent behavioral testing daily for 4–5 consecutive days. One way ANOVA on ranks followed by Dunnett's test was used to assess changes in mechanical withdrawal thresholds relative to baseline. A $p < 0.05$ was considered as significant. Data is reported as mean $\pm$ standard deviation.

Western blot

Flash frozen brains of animals were sectioned (300 μ m for rats and 250 μ m for mice) using a cryostat (Leica CM1950), and S1HL was visually identified using an anatomical atlas as a reference ((Paxinos and Watson, 2007); (Paxinos and Franklin, 2012)). A tissue sample corer (AgnTho's, SE) with a sample diameter of 2 mm was used to punch and collect tissues from S1HL separately from other parts of S1 (S1o). The punched tissues were then stored at -80°C until preparation of protein extracts.

S1HL tissue samples from 1 or 2 animals were pooled together and homogenized in ice-cold buffer comprised of 0.32 M sucrose, 10 mM HEPES pH7.4, 2 mM EDTA, freshly added protease inhibitor cocktail (Complete Mini, Roche Applied Science), and phosphatase inhibitors (5 mM NaF, 1 mM NaVO_4) using a Potter-Elvehjem homogenizer. The homogenate was first centrifuged 15 minutes at 1000g to pellet nuclei and cellular debris, and the supernatant was then centrifuged 30 minutes at 12,500g to sediment synaptosomes. The pellet was washed in a resuspension buffer (25 mM HEPES pH7.4, 2 mM EDTA, with freshly added protease and phosphatase inhibitors) and centrifuged 45 minutes at 100,000g (4°C) to obtain synaptosomal membranes. Protein concentration was determined using Pierce BCA protein assay kit (Thermo Fisher).

Equal amounts of protein from each synaptosomal membrane sample were loaded and separated on NuPage® 4 – 12% Bis-Tris gels with MES running buffer (Invitrogen). Gels were transferred to nitrocellulose membranes at 30 V for 16 h at 4°C . Nitrocellulose membranes were incubated in blocking buffer consisting of 10% dry milk/TBST (10 mM Tris-buffered saline, pH 7.4, 150 mM NaCl, and 1% Tween 20) and probed with primary antibody in 5% milk/TBST. Primary antibodies used here included: GluA1 (mouse monoclonal, Sigma-Aldrich), GluA1-phospho S831 (rabbit polyclonal, Sigma-Aldrich), GluA1-phospho S845 (rabbit polyclonal, Sigma-Aldrich), and β -actin (mouse monoclonal, Sigma-Aldrich). Secondary antibodies used were horseradish peroxidase (HRP)-conjugated goat anti-rabbit (Cell Signaling) and goat anti-mouse (KPL). Pierce West Pico and/or SuperSignal West Femto Chemiluminescent substrates were used to visualize immunoreactivity. Chemiluminescence images were obtained with Kodak Blue XB-1 film, and subsequently scanned and quantified by densitometry using Image Studio Lite (ver 3.1, LI-COR Biosciences). One-way ANOVA followed by Dunnett's test was used for statistical analysis of the Western blotting data. A $p < 0.05$ was considered as significant.

Results

GluA1 subunits are dephosphorylated in SCL animals

We and others have shown previously that SCL in rats results in mechanical and thermal hypersensitivity below the level of the spinal injury (below-level) that emerge after 7 days, and reach a plateau 14–21 days after SCL (Wang and Thompson, 2008; Masri et al., 2009). We also demonstrated that these animals develop signs of ongoing pain, using the conditioned place preference test (Davoody et al., 2011). In these animals (day 21 post SCL), we used Western blots to assess changes in phosphorylation of GluARs at S831 and S845 in S1HL (see Methods). Western blot analysis revealed a significant reduction in the fraction of phosphorylated S831 in S1HL of SCL rats compared to sham-operated controls (sham: $100 \pm 13\%$, $n = 5$; SCL: $64 \pm 8\%$, $n = 4$; $p = 0.04$; Fig. 1A, B). There was no significant change in the fraction of phosphorylated S845 (sham: $100 \pm 12\%$, $n = 5$; SCL: $97.76 \pm 5\%$, $n = 4$; $p = 0.9$; Fig. 1C, D). There was also no significant change in total protein level of GluA1 relative to β -actin between SCL animals and sham-operated controls ($p = 0.3$; Fig. 1A).

We tested if similar post-translational modifications occur in SCL mice. In these mice, we performed SCL (or sham) surgery at the level of T10, to injure spinothalamic projections of lower extremities, while sparing ascending inputs from rostral parts of the body to S1.

Similar to what we observed in rats, the fraction of phosphorylated S831 was significantly lower in S1HL of SCL mice than sham-operated controls (sham: $100 \pm 8\%$, $n = 7$; SCL: $57 \pm 7\%$, $n = 8$; $p = 0.004$; Fig. 2A, B). There was no significant change in total GluA1 levels or the fraction of phosphorylated S831 in other parts of S1 (S1o) that receive inputs from body parts above the lesion level (sham: $100 \pm 8\%$, $n = 7$; SCL: $88 \pm 14\%$, $n = 8$; $p = 0.5$; Fig. 2A, B). The total level of GluA1 relative to β -actin was also not significantly changed ($p = 0.7$). We also found no changes in the fraction of phosphorylated S845 in S1HL of SCL mice (sham: $100 \pm 5\%$, $n = 5$; SCL: $112 \pm 5\%$, $n = 5$; $p = 0.1$; Fig. 2C, D).

GluA2 subunits of GluARs are also abundant in S1 (Conti et al., 1994; Kondo et al., 1997). Therefore, we also tested if total protein levels of GluA2/3 in S1HL change. We found no changes in GluA2/3 levels, relative to β -actin, after SCL (sham: $100 \pm 9\%$, $n = 5$; SCL: $85 \pm 11\%$, $n = 5$; $p = 0.3$; Fig. 2E, F).

These findings are consistent with what we found in rats (Fig. 1), and describe a maladaptive plastic mechanism that may be involved in the pathophysiology of below-level hypersensitivity observed following spinal lesions.

Dephosphorylation of S831 in non-lesioned rats results in hypersensitivity

Previous studies have shown that LFS can depress synaptic transmission and dephosphorylate GluA1 subunits at the S831 site (Lee et al., 2010). Therefore, if S831 dephosphorylation is involved in the hypersensitivity we observe, then repeated sessions of LFS may result in the emergence of pain-like behaviors in non-lesioned animals. We performed daily LFS for 5 consecutive sessions and assessed the mechanical withdrawal thresholds daily before and after each stimulation session in non-lesioned rats ($n = 6$). In

Figure 3, we demonstrate that non-lesioned animals developed hypersensitivity that was significant after 3–5 sessions of LFS ($p < 0.001$), and persisted for up to 5 days after the last stimulation session (Fig. 3A). Sham LFS had no effect on animals' mechanical withdrawal thresholds ($p = 0.4$, Fig. 3A).

We used Western blot analysis to assess changes in S831 phosphorylation in S1HL tissue obtained from non-lesioned animals that received 1 or 5 sessions of LFS and compared them to non-lesioned animals that received sham stimulation (controls). The fraction of phosphorylated S831 was significantly lower in LFS animals compared to controls (sham: $100 \pm 8\%$; 1x LFS: $71 \pm 7\%$, $p = 0.02$; 5x LFS: $50 \pm 9\%$, $p = 0.002$; $n=7/\text{group}$), and the reduction in phosphorylation was positively related to the number of stimulation sessions (Fig. 3B, C). These findings suggest that dephosphorylation at S831 correlates with the development of below-level hypersensitivity in SCL animals.

LFS may have activity-related effects that are not limited to changes in S831 phosphorylation in S1. These may include activity related changes in other phosphorylation sites on GluARs, changes in phosphorylation of NMDA type receptors (GluNRs), and effects on adjacent brain regions due to current spread. Therefore, to further test if changes in S831 phosphorylation are necessary for the development of hypersensitivity in SCL animals, we took advantage of knock-in mice (S831A mutant mice), where S831 is mutated to alanine; therefore, GluA1 cannot be phosphorylated/dephosphorylated at this site (Crombag et al., 2008; Lee et al., 2010). In S831A mutant mice, we predicted that baseline mechanical withdrawal thresholds would be lower than those in strain-matched wild-type controls. However, we found that baseline mechanical withdrawal thresholds of S831A mice were not significantly different from those of wild-type mice (wild-type: 5.83 ± 0.19 g, $n = 6$; S831A: 6.31 ± 0.12 g, $n = 6$; $p = 0.1$, Fig. 4A). This suggests that baseline nociceptive processing is not affected in these mice.

Overexpressing mutant GluA1 subunits in S1HL results in transient hypersensitivity

In S831A mice, the mutation is not specific to S1, and may be associated with compensatory changes that offset the loss of S831 and alter behavior. Therefore, if we propose that dephosphorylation of S831 in S1HL is related to the development of hypersensitivity, the overexpression of phosphorylation-resistant GluA1 specifically in S1HL should result in hypersensitivity in non-lesioned animals. We tested this hypothesis using localized viral-mediated gene transfer to over-express mutant GluA1 subunits in which S831 is mutated to alanine (see methods). We monitored changes in hindlimb mechanical withdrawal thresholds daily after virus injection, for a period of 4 days. Representative coronal sections through S1HL injected with wild-type or S831A mutant viral constructs are shown in Figures 4B and 4C, respectively. In rats over-expressing GluA1-S831A, mechanical withdrawal thresholds of the contralateral hindpaw (relative to viral injection site) significantly decreased two days after virus injection (baseline: 113.98 ± 2.17 g, $n = 6$; 2d-post: 66.40 ± 6.66 g, $p < 0.001$, Fig. 4D). The reduction was transient and mechanical withdrawal thresholds returned to baseline values at day 3. Rats over-expressing wild-type GluA1 showed no changes in mechanical withdrawal thresholds ($n=6$; $p = 0.8$; Fig. 4D).

Over-expressing mutant or wild type GluA1 had no effect on withdrawal thresholds of the ipsilateral hindpaw ($p = 0.1$; Fig. 4E).

Discussion

In S1, GluARs are major contributors to synaptic stability and homeostatic plasticity, and alterations in GluAR levels, composition, or phosphorylation at GluA1 subunit S831 and S845 sites are prime mechanisms by which maladaptive plasticity can occur. Twenty-one days after SCL, we found no changes in total protein levels of GluA1 and GluA2 in S1HL, or in the fraction of phosphorylated S845 (Fig. 1C and D; Fig. 2C and D). However, we observed a significant reduction in the fraction of phosphorylated S831 in S1HL of animals 21 days after SCL, when compared to sham-operated animals (Fig. 1A and B; Fig. 2A and B). These findings suggest that lesion-induced hypersensitivity is associated with maladaptive homeostatic plasticity that manifests as post-translational modification of GluARs.

Dephosphorylation of GluA1 subunits at S831 may reduce the conductance of GluARs and depress excitatory synaptic responses (Kristensen et al., 2011; Lee and Kirkwood, 2011). Thus, our findings are puzzling because they appear to contradict the long held belief that SCI-Pain results from *hyperactivity* in cortical pain networks (see Introduction). However, GluA1 subunits are expressed in a larger percentage of inhibitory neurons than excitatory neurons in S1 (Kondo et al., 1997), and unlike excitatory neurons, GluA1 subunits are homomeric in inhibitory neurons (Bochet et al., 1994; Otis et al., 1995; Isa et al., 1996; Washburn et al., 1997). Thus, it is possible that dephosphorylation of S831 has a greater impact on inhibitory neurons rather than excitatory neurons. whole cell voltage clamp recordings from visually identified inhibitory and excitatory neurons in brain slices will be used to test this hypothesis. Because of GluA1 dephosphorylation, we predict that the amplitude of evoked GluAR excitatory synaptic currents (EPSCs) relative to NMDA-type glutamate receptor (GluNR) EPSCs, or GluAR/GluNR ratio (Kauer and Malenka 2007), is reduced in animals with SCL relative to controls. Such a finding would suggest that dephosphorylation results in reduced cortical inhibition, and ultimately, *hyperactivity*.

Electrical stimulation at low frequencies (LFS) resulted in the emergence of hypersensitivity in non-lesioned animals. This is in contrast to high frequency stimulation, which we reported previously to ameliorate hypersensitivity in SCL animals (Lucas et al., 2011). This frequency-dependent effect of electrical stimulation on hypersensitivity could be due to the bidirectional effects of electrical stimulation on GluA1 subunit phosphorylation. Indeed, our results suggest a link between LFS, hypersensitivity, and dephosphorylation of GluA1 subunits. However, it remains to be seen if interventions that utilize high frequency electrical stimulation to reduce hypersensitivity in SCL and neuropathic pain animals (e.g.: stimulation of the motor cortex (Lucas et al., 2011), or deep brain stimulation (Iwata et al., 2011)) are associated with an increase in the fraction of phosphorylated S831.

S831A mutant mice did not present with reduced baseline mechanical withdrawal thresholds (Fig. 4A). This finding was not consistent with our prediction, and suggests that baseline nociceptive processing is not affected in these mutant mice. Indeed, it has been shown that

S831 mutant mice exhibit normal basal synaptic transmission (Crombag et al., 2008) and therefore, cortical processing of discrete, interrupted mechanical stimuli (von Frey) in normal conditions is unlikely to depend on receptor phosphorylation. However, it is also possible that the S831A mutation yields compensatory changes that offset the loss of S831. One mechanism could be through compensatory changes in the phosphorylation of GluA1 at the S845 site (Lee et al., 2010).

Viral-mediated over-expression of mutant GluA1 subunits in S1HL resulted in transient hypersensitivity in non-lesioned animals. Mutant GluA1 expression is driven by the CMV promoter and therefore, it is likely to influence both excitatory and inhibitory neurons in S1HL. Thus, it may not alter the excitation/inhibition balance in the cortex, which may explain the lack of persistent changes in behavior following mutant virus injection. Alternatively, it is possible that changes in S831 phosphorylation alone are not sufficient to produce persistent hypersensitivity. Dephosphorylation of S831 could be due to up-regulation of protein phosphatases 1 and 2 (PP1 and PP2) (Lee et al., 1998; Lee et al., 2000) down-regulation of specific protein kinases (CaMKII and PKC) (Roche et al., 1996; Derkach et al., 1999; Kristensen et al., 2011), or a combination of both. Such changes are likely to affect other cellular and synaptic targets in addition to GluA1. Another prediction stemming from our hypothesis is that phosphomimetic manipulations, which enhance the phosphorylation of GluA1 subunits at S831, may also ameliorate SCL-induced hypersensitivity. Therefore, additional studies assessing the molecular mechanisms underlying changes in GluAR phosphorylation, and how they contribute to the development of hypersensitivity is necessary.

We use electrolytic lesions to injure the anterolateral quadrant of the spinal cord. We specifically target the spino-thalamo-cortical tract because clinical studies suggest that deafferentation of this pathway is necessary for the development of chronic pain (Siddall et al., 2003; Bryce et al., 2012; Widerstrom-Noga et al., 2015). A limitation of this model is that it does not produce extensive damage like that observed in other spinal injury models (e.g.: contusion). However, the controlled and relatively small size of the lesions, the reduced morbidity, and the lack of motor involvement and paralysis has allowed us to specifically investigate mechanisms that lead to the development of below-level hypersensitivity.

Taken together, our results suggest that the dephosphorylation of S831 in GluA1 subunits may contribute to the development of below-level hypersensitivity in SCL animals.

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Highlights

- Electrolytic lesions in the anterolateral quadrant of the spinal cord are associated with de-phosphorylation of GluARs.
- This de-phosphorylation occurs in the somatosensory cortex of both spinal cord lesioned rats and mice.
- De-phosphorylation may play an important role in the hypersensitivity observed after spinal cord lesions.

Figure 1.

Changes in phosphorylation at of GluA1 subunits at S831 and S845 of in S1HL of SCL rats (SCL at C6). A, Representative Western blots of phosphorylated S831 relative to GluA1 subunit and β-actin in sham operated rats (control) and SCL rats (day 21). B, Quantitation of Western blot data. The fraction of phosphorylated S831 significantly decreased in SCL rats when compared to sham-operated controls. In C-D, the same animals were used to assess changes in the fraction of phosphorylated S845. No changes in phosphorylated S845 level were observed in SCL rats when compared to sham-operated controls. Error bars=standard error of the mean (SEM). n = 4 for sham group and n = 5 for SCL group. S1HL: hindlimb representation in the primary somatosensory cortex. SCL: spinal cord lesion.

Figure 2.

Changes in phosphorylation of GluA1 subunits at S831 and S845, and total GluA2 levels relative to β -actin in SCL mice (SCL: T10). A, Representative Western blots of phosphorylated S831 relative to GluA1 subunit in sham-operated mice, and SCL mice (day 21). B, Quantitation of Western blot data. The fraction of phosphorylated S831 significantly decreased in S1HL but not in S1(o) of SCL mice compared to sham-operated controls. In C–D, the same animals were used to assess changes in the fraction of phosphorylated S845. No changes in phosphorylated S845 were observed compared to sham-operated controls. In E–F, the same animals were also used to assess total GluA2 level relative to β -actin. No changes were observed in SCL mice when compared to sham control. $n = 7$ for sham group and $n = 8$ for SCL group. Error bars= SEM. S1(o): primary somatosensory cortex lateral to hindlimb representation.

Figure 3.

LFS effects on mechanical withdrawal thresholds and phosphorylation of GluA1 at S831 site. A, Daily LFS caused mechanical withdrawal thresholds to gradually decrease and reached a maximum reduction after 5 sessions of LFS (5 days) ($p < 0.001$). Mechanical withdrawal thresholds returned to baseline values 5 days after the last LFS session. No changes in mechanical withdrawal thresholds were found in non-lesioned rats receiving sham LFS ($p = 0.44$). B, Representative Western blots of tissues obtained from S1HL after 1X, 5X or sham LFS. The fraction of phosphorylated S831 was less in 1X and 5X LFS animals relative to shams. C, Quantitation of Western blot data. The fraction of phosphorylated S831 significantly decreased after 1X and 5XLFS when compared to shams. $n = 7$ for each group. Error bars=SEM. LFS: low frequency stimulation.

Figure 4.

A. Baseline mechanical withdrawal thresholds are not significantly different between S831A mutant mice and strain-matched wild-type controls. B and C, show representative examples of coronal sections obtained from rats brains injected with either HSV-GluA1-mCherry (n = 6), or HSV-GluA1-S831-mCherry (n = 6) in S1HL. D, Mechanical withdrawal thresholds of the contralateral hindpaw (relative to virus injection site) are transiently reduced 2 days after virus injection. E, Mechanical withdrawal thresholds of the ipsilateral hindpaw are not changed after virus injection. Error bars = SEM.