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Chronic at-level thermal hyperalgesia following rat cervical contusion spinal cord injury is accompanied by neuronal and astrocyte activation and loss of the astrocyte glutamate transporter, GLT1, in superficial dorsal horn

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Abstract

Neuropathic pain is a form of pathological nociception that occurs in a significant portion of traumatic spinal cord injury (SCI) patients, resulting in debilitating and often long-term physical and psychological burdens. While many peripheral and central mechanisms have been implicated in neuropathic pain, central sensitization of dorsal horn spinothalamic tract (STT) neurons is a major underlying substrate. Furthermore, dysregulation of extracellular glutamate homeostasis and chronic astrocyte activation play important underlying roles in persistent hyperexcitability of these superficial dorsal horn neurons. To date, central sensitization and astrocyte changes have not been characterized in cervical SCI-induced neuropathic pain models, despite the fact that a major portion of SCI patients suffer contusion trauma to cervical spinal cord. In this study, we have characterized two rat models of unilateral cervical contusion SCI that behaviorally result in chronic persistence of thermal hyperalgesia in the ipsilateral forepaw. In addition, we find that STT neurons are chronically activated in both models when compared to laminectomy-only uninjured rats. Finally, persistent astrocyte activation and significantly reduced expression of the major CNS glutamate transporter, GLT1, in superficial dorsal horn astrocytes are associated with both excitability changes in STT neurons and the neuropathic pain behavioral phenotype. In conclusion, we have characterized clinically-relevant rodent models of cervical contusion-induced neuropathic pain that result in chronic activation of both STT neurons and astrocytes, as well as compromise in astrocyte glutamate transporter expression. These models can be used as important tools to further study mechanisms underlying neuropathic pain post-SCI and to test potential therapeutic interventions.

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Keywords

spinal cord injury; contusion; cervical; neuropathic pain; hyperalgesia; astrocyte; GLT1; glutamate transporter; hyperexcitability

1. Introduction

A significant portion of traumatic spinal cord injury (SCI) patients experience one or more forms of neuropathic pain, resulting in debilitating and often long-term physical and psychological burdens. These include enhanced responsiveness to noxious peripheral stimuli (hyperalgesia), painful sensation in response to formally innocuous peripheral stimuli (allodynia), and often spontaneous pain in the absence of peripheral stimulation (Hulsebosch et al., 2009).

Hyperexcitability of dorsal horn pain projection neurons (“central sensitization”) is a major substrate for neuropathic pain following SCI (Gwak and Hulsebosch, 2011b). This includes decreased threshold for activation (i.e. action potential generation), increased spontaneous activity, expansion of peripheral receptive field, and the occurrence of after-discharges in dorsal horn spinothalamic tract (STT) pain projection neurons (Latremoliere and Woolf, 2009). In particular, alterations in the properties and proportion of wide-dynamic range pain projection neurons (in both superficial and deeper laminae) occur following SCI. Furthermore, many of these changes in dorsal horn neuron properties are persistent, likely accounting for the chronic nature of neuropathic pain after SCI.

Dysregulation of extracellular glutamate homeostasis is thought to play a major mechanistic role in dorsal horn neuron hyperexcitability following SCI (Tao et al., 2005). Glutamate is the primary neurotransmitter released from primary afferent terminals onto 2nd order dorsal horn pain projection neurons. Following injury, elevated levels of extracellular glutamate can result from increased synaptic release by primary afferent pain fibers, release from injured neurons, axons and glial cells, and compromised glutamate clearance due to glutamate transporter dysfunction. Increased activation of glutamate receptors (particularly NMDA receptors) is mediated by elevated dorsal horn levels of extracellular glutamate detailed above, as well as by changes in the expression, localization and function (via phosphorylation, for example) of glutamate receptor subunits in these neurons (Bleakman et al., 2006). Additional mechanisms that might account for glutamate-mediated central changes in nociceptive neurotransmission in the dorsal horn after SCI include: 1) increased activation of glial cells such as astrocytes and microglia by glutamate (Cao and Zhang, 2008; Hansson, 2006; Ren, 2010), 2) decreased inhibitory tone due to glutamate-mediated excitotoxic loss of GABAergic interneurons (Gwak and Hulsebosch, 2011a), and 3) reduced recycling of glutamine from astrocytes back to inhibitory neurons for GABA synthesis because of decreased glutamate uptake and subsequent conversion of glutamate to glutamine in activated astrocytes (Ortinski et al., 2010).

In the CNS, glutamate is efficiently cleared from the synapse and from other sites by glutamate transporters located on the plasma membrane (Maragakis and Rothstein, 2004). Astrocytes are supportive glial cells that play a host of crucial roles in CNS function (Pekny

and Nilsson, 2005). In particular, astrocytes express the major CNS glutamate transporter, GLT1, which is responsible for the vast majority of functional glutamate uptake in the CNS, particularly in the spinal cord (Maragakis and Rothstein, 2006). Studies have focused on therapeutically targeting glutamate receptor over-activation in SCI models. However, regulation of extracellular glutamate homeostasis by GLT1 following SCI has not been extensively addressed, particularly with respect to modulation of the excitability of dorsal horn neurons involved in pain neurotransmission. This is despite the fact that astrocyte GLT1 plays the central role in regulating extracellular glutamate homeostasis in the spinal cord (Maragakis and Rothstein, 2006).

Following SCI, astrocyte loss/dysfunction and/or altered GLT1 physiology can result in dysregulation of extracellular glutamate homeostasis (Lepore et al., 2011a; Lepore et al., 2011b), as well as further glial activation and consequent additional compromise in GLT1 function. Vera-Portocarrero and colleagues (Vera-Portocarrero et al., 2002) reported changes in GLT1 protein levels within 24 hours following thoracic contusion, while Olsen et al. (Olsen et al., 2010) reported longer-term decreases in GLT1 expression after thoracic SCI. Our group has also demonstrated long-term decreases in GLT1 expression and functional GLT1-mediated glutamate uptake following thoracic contusion (Lepore et al., 2011a; Lepore et al., 2011b). As glutamate homeostasis dysregulation can persist after SCI, it is important to characterize and therapeutically target both early and longer-term changes in transporter expression and function, as well as to do so in the context of dorsal horn neuron hyperexcitability and neuropathic pain following cervical SCI.

Chronic glial activation contributes to the development and persistence of neuropathic pain following SCI. The anatomical mechanisms of hyperalgesia, allodynia and spontaneous pain differ; nevertheless, significant astrocyte activation occurs in all of these pain states, regardless of whether there is central SCI or peripheral nerve damage (Hansson, 2006). Activated astrocytes (and microglia) play key roles in neuropathic pain (Scholz and Woolf, 2007). Following SCI, elevation in the levels of factors such as glutamate, CGRP and substance P can activate astrocytes, resulting in astrocytic release of ATP, NO and pro-inflammatory cytokines that facilitate nociceptive neurotransmission by increasing excitability of dorsal horn pain neurons. These signaling molecules can also activate neighboring astrocytes to maintain and possibly anatomically spread pain to uninjured areas (Hulsebosch, 2008). Astrocyte activation can also compromise key physiological functions such as glutamate uptake (Lepore et al., 2011a), resulting in further dysregulation of extracellular glutamate homeostasis. As described above, dysregulation of glutamate signaling can itself promote dorsal horn neuron hyperexcitability and can also lead to additional astrocyte stimulation, both locally and in more distant locations. Furthermore, this overall gliopathic response can persist, possibly maintaining a chronic neuropathic state (Cao and Zhang, 2008).

Glutamate has been shown to play a central role in this astrocyte response and in neuropathic pain in general (Hulsebosch, 2008; Hulsebosch et al., 2009). Glutamate uptake inhibition or administration of glutamate receptor agonists to intact spinal cord results in spontaneous pain behaviors similar to those seen after SCI (Liaw et al., 2005). There is high level expression of AMPARs, NMDARs, mGluRs (and GLT1) in superficial dorsal horn

(Bleakman et al., 2006), and a role for these glutamatergic receptor types has been documented in neuropathic pain using pharmacological agents targeting both ionotropic and metabotropic receptors (Bleakman et al., 2006; Mills et al., 2000; Mills et al., 2001; Mills and Hulsebosch, 2002; Mills et al., 2002a; Mills et al., 2002b). In models of chronic neuropathic pain induced by peripheral nerve injury, there are reduced dorsal horn GLT1 levels, and maintenance of GLT1 expression via drugs such as ceftriaxone and propentofylline can block neuronal hyperexcitability and the development of pathological pain (Hobo et al., 2011; Hu et al., 2010; Inquimbert et al., 2012; Nie and Weng, 2010; Tawfik et al., 2008). It is therefore crucial to address the underlying role of glutamate - and specifically the crucial role played by astrocytes - in breaking this “loop” of glial activation-neuronal hyperexcitability-neuropathic pain following SCI.

Importantly, studies on neuropathic pain need to model clinically-relevant aspects of human SCI. Injury to cervical spinal cord represents greater than half of all human SCI cases, in addition to often resulting in the most severe physical and psychological debilitation (Lane et al., 2008). Furthermore, contusion-type SCI predominates in humans (McDonald and Becker, 2003). Despite this make-up of the clinical population, the vast majority of animal studies have not employed cervical contusion models, and therapies targeting neuropathic pain have not been tested with cervical contusion. Although use of thoracic SCI animal models has predominated, cervical contusion SCI models have recently been developed (Aguilar and Steward; Gensel et al., 2006; Lee et al.; Sandrow-Feinberg et al.; Sandrow-Feinberg et al., 2009; Sandrow et al., 2008; Stamegna et al.), including our own (Nicaise et al., 2012a; Nicaise et al., 2012b; Nicaise et al., 2013).

In this study, we have characterized two clinically-relevant rat models of unilateral cervical contusion SCI. Given that astrocyte activation, loss of astrocyte GLT1 function and consequent dysregulation of extracellular glutamate homeostasis can contribute to the hyperexcitability of second order dorsal horn STT neurons following SCI, we have spatiotemporally examined (in the superficial dorsal horn) astrocyte and STT neuron activation, as well as alterations in GLT1 expression, in these models. We report that both SCI models result in the development and chronic persistence of at-level thermal hyperalgesia, as well as in chronic activation of STT neurons in the cervical spinal cord. Furthermore, persistent astrocyte activation and significantly reduced astrocyte GLT1 expression in superficial dorsal horn are associated both with changes in STT neurons and the neuropathic pain behavioral phenotype. Going forward, these models can be used as important tools to further study mechanisms underlying the development of neuropathic pain post-SCI and to test potential therapeutic interventions.

2. Results

2.1 Unilateral cervical contusion SCI resulted in persistent thermal hyperalgesia

In this study, we have characterized two rat models of unilateral cervical contusion SCI for the development and persistence of one form of at-level neuropathic pain, thermal hyperalgesia. Specifically, rats received either a unilateral 200 kilodyne contusion injury (with a 2 second dwell time) at level C5 or C6 using the IH Impactor (Fig. 1A-B), while

uninjured control animals received laminectomy only (Nicaise et al., 2012a; Nicaise et al., 2012b; Nicaise et al., 2013).

To assess changes in thermal sensitivity, laminectomy-only control ($n = 9$) and injured rats ($n = 9$ per injury type) were tested using the Hargreaves apparatus (Fig. 1C-D). Pre-surgery baseline data for both injury groups and the uninjured control group were similar, indicating the consistency across animals of the Hargreaves test for assessing thermal sensitivity. Rats almost always displayed supraspinal behaviors associated with perception of the thermal stimulus, including licking of the plantar surface of the stimulated paw and sometimes sharp vocalization. The laminectomy control animals showed no significant changes in thermal sensitivity in either the right (Fig. 1C) or left (Fig. 1D) forepaws for up to six weeks following surgery. In contrast, both C5 and C6 injury groups showed a statistically significant and robust increase in thermal sensitivity in the right forepaw following SCI (Fig. 1C). One week after surgery, both the C5 and C6 injured rats exhibited an almost 50% reduction in latency to right forepaw withdrawal from the noxious thermal stimulus, which persisted to at least the chronic time point of 6 weeks post-injury. However, left forepaw withdraw latency showed no significant changes following either C5 or C6 contusion (Fig. 1D). These findings demonstrate that both cervical contusion SCI models result in the development and chronic persistence of ipsilateral at-level thermal hyperalgesia, while no changes were observed in the contralateral forepaw.

2.2 Unilateral cervical contusion SCI produced focal cystic cavitation

Eriochrome cyanine/Cresyl violet stained transverse sections of the cervical spinal cord were processed to characterize the extent of the lesion following SCI at 2 and 6 weeks ($n = 7-8$ per group) post-injury (Nicaise et al., 2012a; Nicaise et al., 2012b; Nicaise et al., 2013). Analysis at 2 weeks was chosen because secondary degeneration has been reported to occur for several weeks post-injury; therefore, a time point towards the end of this period was selected. Tissue was analyzed at 6 weeks because of interest in the evolution of the lesion out to a chronic state following SCI, particular in the context of persistent neuropathic pain. Uninjured laminectomy-only rats showed intact spinal cord anatomy when stained with Eriochrome cyanine/Cresyl violet (Fig. 2A). As both injury paradigms were unilateral in nature, only the ipsilateral hemi-cord was affected at both time points, at least when assessed for overt tissue compromise using standard Eriochrome/Cresyl violet histology. Both the C5 (Fig. 2B) and C6 (Fig. 2C) lesions were characterized by a cystic cavitation of the spinal cord surrounded by a border of fibrous tissue, which resembles the human contusive SCI condition (Norenberg et al., 2004). This cystic cavitation resulted in the partial destruction of the dorsal horn and the lateral funiculus, areas important to nociceptive neurotransmission. The gross structure of the spinal cord appeared mostly normal at 2.0mm caudal to the epicenter (Fig. 2D), a location that was used in immunohistochemical analysis in this study. The maximum extent of the lesion area at the injury epicenter (defined as the section with the largest percent lesioned tissue relative to total tissue area in the same section (Nicaise et al., 2012a; Nicaise et al., 2012b; Nicaise et al., 2013)) following C5 injury was $55 \pm 4\%$ of the total ipsilateral hemi-cord at 6 weeks. Similarly, the maximum extent of the C6 lesion encompassed $47 \pm 5\%$ of the total ipsilateral hemi-cord at the epicenter at 6 weeks (Fig. 2E). The lesion extended approximately 2mm in the rostral-caudal axis from the

impact epicenter, with no statistical difference in the rostral-caudal spread of the injury between the two paradigms at either time point (Fig. 2E).

2.3 Dorsal horn neuron activation was chronically increased following unilateral cervical contusion

Central sensitization of second order spinothalamic tract (STT) neurons in the superficial laminae of the dorsal horn is one major mechanism implicated in the pathogenesis of neuropathic pain (Gwak and Hulsebosch, 2011b). These changes can be chronic, resulting in the persistence of neuropathic pain. An established biomarker of chronic neuronal activation is FosB (McClung et al., 2004; Nestler, 2008). FosB is a truncated splice variant of the transcription factor FosB, and is thought to transcriptionally mediate some of the long-term changes that result from chronic activation of pain transmission neurons (Luis-Delgado et al., 2006; Robison and Nestler, 2011).

To assess for the chronic activation of neurons in the superficial dorsal horn, FosB immunohistochemistry (IHC) was employed at 2 and 6 weeks post-injury (n = 7-8 per group). Counting of FosB-positive nuclei was performed in the ipsilateral dorsal horn in superficial laminae I-II (Fig. 2F-G) at the most caudal extent of the lesion (i.e. 2.0mm caudal to epicenter: approximately 1 spinal segment away) (Fig. 1A-B). Analysis was conducted at this location to determine whether chronic dorsal horn neuronal activation occurs at locations removed from the injury. In addition, the dermatomes corresponding to the plantar surface of the forepaw in rats primarily innervate dorsal horn of the most caudal segments of the cervical spinal cord (Takahashi and Nakajima, 1996). We focused all of our IHC analyses to the ipsilateral dorsal horn because we only observed thermal hyperalgesia in the ipsilateral forepaw.

FosB-expressing neurons exhibited a defined pattern of nuclear staining (Fig. 3A), which allows for quantification of positive nuclei. As compared to laminectomy-only controls (Fig. 3B), there was a significant increase in the numbers of FosB-expressing neurons at both 2 (Fig. 3C) and 6 weeks (Fig. 3D) post-injury in both C5 and C6 (Fig. 3C-D) contusion paradigms. Importantly, these FosB changes occurred only in the superficial dorsal horn (Fig. 3C-D), demonstrating that increased FosB expression and by extension neuronal hyperexcitability were not generally observed in neurons throughout the spinal cord, but were specific to superficial dorsal horn populations. Both injury paradigms showed statistically significant increases at areas caudal to the injury in the ipsilateral dorsal horn (Fig. 3F). The ipsilateral dorsal horn at the lesion epicenter was not used for FosB quantification because the structural integrity of superficial laminae was mostly damaged following injury. In summary, dorsal horn neurons are chronically activated in both cervical contusion SCI models when compared to laminectomy-only uninjured rats.

2.4 Astrocyte activation and cell proliferation were chronically increased following unilateral cervical contusion

Astrocyte activation is a key molecular mechanism that has been implicated in the central sensitization of STT neurons following PNS and CNS injury (Cao and Zhang, 2008). To spatiotemporally assess astrocyte activation, GFAP and Ki67 (to assess for proliferating

cells) IHC was employed at 2 and 6 weeks post-injury ($n = 7-8$ per group). Astrocyte activation results in robustly increased GFAP expression. In the intact spinal cord, gray matter astrocytes express levels of GFAP protein that are very low using IHC; therefore, GFAP analysis is an established approach for histologically quantifying astrocyte activation at specific anatomical locations (Lepore et al., 2008a). The intensity of GFAP immunostaining, the total numbers of Ki67-expressing cells, and the phenotypic lineage of proliferation cells were quantified in the ipsilateral dorsal horn (in superficial laminae I-II: Fig. 2F-G) at the lesion epicenter and at the most caudal extent of the lesion (Fig. 1A-B).

Following cervical contusion SCI, there was a pronounced activation of astrocytes in the dorsal horn (both at the lesion epicenter and in intact caudal spinal cord) at both 2 and 6 weeks in both injury paradigms, which was characterized by significantly increased expression of GFAP in individual astrocytes and an increase in the total numbers of astrocytes (Fig. 4A-B). Compared to laminectomy-only controls (Fig. 4C), there was an increase in GFAP expression at 2 weeks and 6 weeks post-injury in both C5 and C6 contusion paradigms. These statistically significant changes in GFAP expression for both the C5 and C6 injuries were observed in the ipsilateral superficial dorsal horn both at the level of the epicenter (Fig. 4D) and at areas caudal to the injury (Fig. 4E) (quantification of all analyses shown in Fig. 4F).

Furthermore, this astrocyte activation response was associated with marked increases in the numbers of proliferating Ki67-positive cells, including large numbers of proliferating astrocytes (Fig. 5A). Astrocyte proliferation represents a major phenotype along the spectrum of astrocyte activation in response to CNS disease/perturbation (Faulkner et al., 2004). At both 2 and 6 weeks post-injury in both the C5 and C6 contusion paradigms, there was a significant increase in the number of total proliferating cells in the superficial dorsal horn compared to uninjured animals, as assessed with Ki67 (Fig. 5E). This was observed both at the ipsilateral lesion epicenter (Fig. 5C) and in ipsilateral caudal spinal cord (Fig. 5D). In the dorsal horn of the intact spinal cord of laminectomy-only control rats, almost no proliferation was observed (Fig. 5B). Similar to our previous observations (Lepore et al., 2011a), a large percentage of these Ki67⁺ cells in the superficial dorsal horn were GFAP⁺ astrocytes both at the lesion epicenter and caudal spinal cord at 2 and 6 weeks post-injury in both contusion paradigms (Fig 5A; quantification in Fig 5F), suggesting that astrocyte proliferation at least partially accounts for the increase in number of astrocytes post-injury and that a significant portion of astrocytes following injury are in a highly activated state.

2.5 GLT1 expression was reduced in the superficial dorsal horn following cervical contusion SCI

Confocal analysis shows that in superficial dorsal horn laminae of uninjured rats virtually all GFAP⁺ astrocytes expressed GLT1 (Fig. 6A). This is in line with the established almost-exclusive expression of GLT1 by astrocytes in the intact spinal cord (Regan et al., 2007). Following cervical contusion SCI, there was a pronounced activation of astrocytes in the superficial dorsal horn (both at the lesion epicenter and in intact caudal spinal cord) at all time points characterized by significantly increased expression of GFAP in individual astrocytes, as well as an increase in the total numbers of astrocytes (Fig. 4, Fig. 6B).

However, a significant portion of activated astrocytes following injury (proliferating astrocytes and/or non-dividing astrocytes that have increased GFAP expression) did not express GLT1 (Fig. 6B), especially with closer proximity to the injury site. These data suggest that activated astrocytes are compromised in their capacity for glutamate uptake.

GLT1 expression was spatiotemporally quantified following injury using IHC. The intensity of GLT1 immunostaining was quantified in the ipsilateral dorsal horn (in superficial laminae I-II: Fig. 2F-G) at the same epicenter and caudal locations (Fig. 1A-B) as described for

FosB, GFAP and Ki67 ($n = 3$ per group). Laminectomy-only uninjured rats showed greater expression levels of GLT1 in the superficial dorsal horn compared to white matter and more ventral gray matter locations (Fig. 6C). However, following C6 injury (the C5 injury paradigm was not assessed by GLT1 IHC), GLT1 expression decreased in superficial dorsal horn, both at the lesion epicenter (Fig. 6D) and at intact caudal locations (Fig. 6E). This decrease in total GLT1 protein expression in the superficial dorsal horn was statistically significant at both the injury epicenter and caudal to the injury (Fig. 6F).

To complement these IHC studies, GLT1 immunoblotting analysis (Lepore et al., 2011b) was conducted on whole-tissue spinal cord homogenate that was sub-dissected from ipsilateral spinal cord segments at the level of the lesion epicenter and one segment caudal to the epicenter (Fig. 1A-B). Western blotting results illustrate that total spinal cord GLT1 protein levels were reduced at both 2 and 6 weeks post-C6 injury, compared to uninjured laminectomy-only control (Fig. 6G). Specifically, decreases were significant at both 2 and 6 weeks at the lesion epicenter, but were only significant at 6 weeks at the caudal location (Fig. 6H).

3. Discussion

We sought to characterize clinically-relevant animal models of cervical contusion SCI-induced neuropathic pain and to examine associated changes in populations of astrocytes and neurons of the superficial dorsal horn. When designing the injury paradigms, care was taken to develop behaviorally robust models of evoked neuropathic pain without the occurrence of animal death or severe disability, which is a danger associated with cervical-targeted trauma. Despite the rostral location of the injury, no rats required ventilatory assistance following either contusion paradigm in the present study, likely due to the unilateral nature of the trauma. This lack of respiratory impairment corresponds to our previous work with similar models of unilateral cervical contusion that we developed to study diaphragmatic compromise in both rats and mice (Nicaise et al., 2012a; Nicaise et al., 2012b; Nicaise et al., 2013). To target forepaw sensory function, we employed a relatively moderate force of impact at either the C5 or C6 level. These levels were chosen because a significant proportion of human SCI cases occur at the mid-cervical region (Lane et al., 2008), which lends clinical relevance to these models. In addition, caudal segments of the cervical spinal cord correspond to sensory input from primary nociceptive afferents innervating the plantar surface of the forepaws in rats (Takahashi and Nakajima, 1996).

We behaviorally examined one type of evoked neuropathic pain, thermal hyperalgesia, and consistently observed increased sensitivity in only the ipsilateral forepaw following both

injuries, while no sensory changes were noted in the contralateral forepaw at any time following surgery. Thermal hyperalgesia manifested itself one week post-contusion and continued out to chronic time points, providing a model that can be used to study both initial developmental of the neuropathic pain phenotype and its chronic persistence. We also observed supraspinal behaviors associated with the decreased latency to withdraw such as vocalization and paw licking, suggesting that these animals are likely exhibiting an enhanced pain response as opposed to just local increases in spinal reflexes. In addition, forelimb motor dysfunction was minimal following injury (data not shown), likely due to the moderate severity of these injuries compared to our previous rat SCI models that produced chronic forelimb motor compromise (Nicaise et al., 2012a; Nicaise et al., 2012b; Nicaise et al., 2013). In conclusion, we have successfully characterized clinically relevant models of cervical contusion SCI that result in robust and persistent at-level thermal hyperalgesia.

However, we did not assess tactile allodynia or spontaneous pain, two additional forms of neuropathic pain commonly observed post-injury (Hulsebosch et al., 2009). Like thermal hyperalgesia, tactile allodynia has been an outcome measure often examined in models of SCI, particularly in above-, at-, below-level dermatomes following thoracic trauma. Spontaneous pain is particularly relevant to the clinical situation (Mogil, 2009; Wang et al., 2004) and needs to be assessed in SCI models, despite greater technical difficulty than evoked behavioral paradigms of pathological pain. In ongoing work, we are attempting to characterize spontaneous pain behaviors using, for example, a conditioned place preference approach that can unveil spontaneous neuropathic pain in rats (De Felice et al., 2011; King et al., 2012). We are also working to optimize this assay for use in a mouse model of cervical contusion-induced neuropathic pain that we have developed (Renninger et al., in review), as other investigators have shown that such an approach can be used in mouse models of chronic pain (He et al., 2012).

We also did not assess the below-level phenotype. A number of previous studies have shown that various SCI paradigms can produce above- and/or below-level neuropathic pain using Hargreaves and von Frey filament testing (Detloff et al., 2010). Nevertheless, we focused on the at-level phenotype because we sought to also assess in detail the histological and biochemical changes occurring at various locations of the cervical spinal cord and to correlate these changes with forepaw pain behavior. In the future, we could expand our behavioral, histological and biochemical analyses to other spinal cord regions in these cervical contusion SCI models.

Interestingly, we only observed ipsilateral hyperalgesia, with the contralateral forepaw being unaffected by the contusion. Almost no work has been published on neuropathic pain following cervical contusion SCI. An important recent finding by Detloff and colleagues demonstrated that unilateral mid-cervical contusion in the adult rat produces contralateral thermal hyperalgesia (Detloff et al., 2010). A number of possibilities could explain this discrepancy with our results, including differences in the specifics of the injury paradigms such as impactor tip size, location of the injury, and use of dwell time. In fact, the cumulative effect of many subtle experimental differences such as surgical and behavior testing techniques and animal strain and sex have been shown to result in varied functional outcomes with sensory testing (Callahan et al., 2008; Hoschouer et al., 2010; Mogil, 2009).

Importantly, our hyperalgesia phenotype is quite robust, reproducible and observed in all injured animals, even with contusion targeting two different spinal cord levels. These results suggest that injury to cervical spinal locations in general can result in at-level neuropathic pain, but that differences in injury can alter the specifics of the neuropathic pain outcome, similar to variability in the human SCI population.

We attempted to more fully characterize these injury paradigms using various histological and biochemical approaches and to correlate these changes with the behavioral phenotype. Our lesion analysis corresponds to previous work in which we characterized cervical contusion models in both the rat and mouse to target the respiratory phrenic motor neuron pool (Nicaise et al., 2012a; Nicaise et al., 2012b; Nicaise et al., 2013). At least based on standard Eriochrome cyanine and Cresyl violet staining, the lesion appeared relatively focal in the rostral-caudal axis and was restricted to only the side ipsilateral to the trauma site. Histological assessment of both the C5 and C6 injury types revealed a similar anatomical profile. Dorsal horn locations in the caudal cervical spinal cord that correspond to sensory afferent input from the plantar surface of the forepaw were undamaged, while changes in neuronal and astrocyte activation and glutamate transporter down-regulation were both pronounced and persistent at these same locations removed from the lesion that appeared grossly intact. We did not observe any expansion of the lesion between 2 and 6 weeks post-injury, suggesting that the process of secondary neurodegeneration that characterizes SCI occurred mostly prior to 2 weeks. This finding coincides with our previous observations in which we showed expansion of the lesion only during the first week following cervical contusion (Nicaise et al., 2013).

We focused our immunohistochemical analyses to the very superficial laminae of the dorsal horn because of the involvement of neurons in these regions in nociceptive transmission. However, deeper laminae also play important roles in pain signaling (Kuner, 2010). In addition, more rostral locations of the CNS neuraxis such as the thalamus and somatosensory cortex are also major sites of pain neurotransmission. Future work could be aimed at characterizing changes in these locations in our SCI models.

Using analysis of FosB expression in individual cells, we demonstrated persistent activation of superficial dorsal horn neurons following injury, both at the lesion epicenter and at intact caudal locations. Furthermore, we observed changes at an early time point following injury (i.e. 2 weeks) that coincided with the initial development of hyperalgesia, as well as changes at chronic time points (i.e. 6 weeks) that coincided with persistence of the behavioral phenotype. However, we did not assess very early events (i.e. prior to two weeks post-SCI) such as neuronal and astrocyte activation and inflammatory signaling that likely play a role in neuropathic pain development. Such analysis will be important in future work. Previous studies have shown that FosB is a reliable and better-suited histological marker of persistent activation of neuronal populations than other activity-induced factors such as c-Fos, Fra1, or other FosB analogs (Nestler, 2008). FosB is a truncated splice variant of the transcription factor FosB. Importantly, the truncated region of the protein contains ubiquitination sites that target the protein for proteasomal degradation (McClung et al., 2004). As a result, FosB expression persists for longer periods of time following neuronal activation than other FosB analogs and its levels are further elevated with persistent

activation. Furthermore, FosB is thought to transcriptionally mediate some of the long-term changes that result from chronic activation of pain transmission neurons (Luis-Delgado et al., 2006; Robison and Nestler, 2011). In particular, FosB regulates expression of key genes involved in plasticity associated with persistent neuropathic pain, including CDK5 (which is involved in regulating increased dendritic spine density) and the GluA2 and GluN1 subunits of AMPARs and NMDARs, respectively (Nestler, 2008). We did not employ electrophysiological approaches to further document changes in the excitability of dorsal horn neurons. However, such work has been previously conducted in models of thoracic contusion SCI, correlating increased responsiveness of wide-dynamic range neurons following peripheral sensory stimulation with neuropathic pain behavioral phenotypes (Crown et al., 2008).

We sought to examine potential mechanisms underlying long-term changes in dorsal horn neurons in our SCI models. We observed chronic astrocyte activation in the superficial dorsal horn, including even proliferation of some of these astrocytes. Spinal trauma can result in the elevation of signaling factors such as glutamate, CGRP and substance P that can activate astrocytes (Hulsebosch, 2008). Release of molecules such as ATP, NO and pro-inflammatory cytokines by activated astrocytes can then directly facilitate nociceptive neurotransmission by increasing excitability of dorsal horn pain neurons (Gwak and Hulsebosch, 2011b). In addition, astrocyte activation can also compromise key homeostatic glial functions such as synaptic glutamate uptake (Lepore et al., 2011a), thereby indirectly affecting pain transmission neurons via dysregulation of extracellular glutamate levels. Astrocyte activation has also been noted in other animal models of SCI-induced neuropathic pain, particularly following thoracic contusion (Gwak et al., 2012). Furthermore, manipulation of signaling mechanisms in astrocytes has been shown to alter the neuropathic pain phenotype in some of these SCI models (Hulsebosch, 2005).

The response of astrocytes to CNS disease/trauma is a complex phenomenon. In the context of SCI, astrocyte activation has traditionally been viewed negatively in association with the astroglial scar and its inhibitory effects on axon regeneration/plasticity. However, astrocytes normally play an integral role in the CNS via a number of important functions, including control of extracellular ion and neurotransmitter homeostasis, control of cerebral blood flow, neurotrophic factor support, metabolism, and regulation of synaptic transmission, to name but a few (Pekny and Nilsson, 2005). The response of astrocytes following SCI should not be approached only as a dichotomy of quiescent versus reactive, but should be viewed as occurring along a spectrum of phenotypes, depending on the type, location, timing and severity of the insult (Sofroniew, 2005). Importantly, it is crucial to study specific astrocyte functions in the diseased nervous system, not just to characterize a generalized response of astrocytes. Along these lines, we characterized changes in the expression of the major CNS glutamate transporter, GLT1, in our two contusion paradigms. Our findings of compromised GLT1 expression in the superficial dorsal horn are in line with previously published work from our lab (Lepore et al., 2011a; Lepore et al., 2011b) and others (Olsen et al., 2010) in models of SCI. Given the potential role of extracellular glutamate dysregulation in neuronal hyperexcitability (Tao et al., 2005), as well as the central role played by GLT1 in controlling glutamate signaling (Maragakis and Rothstein, 2004; Maragakis and Rothstein, 2006), these astrocyte changes likely play an important part in contributing to neuropathic pain. Future

studies could also quantify functional glutamate uptake and/or intraspinal glutamate levels in our cervical SCI models to verify the effects of GLT1 expression loss on actual levels of uptake and extracellular glutamate. Our previous work showed that decreased GLT1 protein expression correlates with decreased functional uptake in the spinal cord following thoracic SCI (Lepore et al., 2011b), suggesting that similar functional changes are likely occurring with GLT1 down-regulation in the current models.

Studies have also shown that GLT1 expression is compromised in animal models of neuropathic pain induced by peripheral nerve injury (Hobo et al., 2011; Hu et al., 2010; Inquimbert et al., 2012; Nie and Weng, 2010; Tawfik et al., 2008; Wang et al., 2008). Furthermore, increasing expression is able to partially reverse the pathological pain phenotype in these injury models (Hobo et al., 2011; Hu et al., 2010; Inquimbert et al., 2012; Tawfik et al., 2008). We and others have previously demonstrated in various models of CNS disease that targeting GLT1 expression via approaches such as pharmacological agents, cell transplantation and viral vector delivery can increase intraspinal GLT1 expression and can result in therapeutic efficacy (Lepore et al., 2008b; Rothstein et al., 2005). Given both that neuropathic pain is a major debilitating complication of human SCI and that a significant portion of the human SCI population suffers from mid-cervical contusion, it will be important to test the efficacy of manipulating GLT1 using such approaches for preventing the development and/or reversing chronic persistence of neuropathic pain in our new cervical contusion SCI models.

In addition, peripherally-administered agents for treating various forms of pathological pain such as glutamate receptor antagonists are associated with unwanted side effects (Bleakman et al., 2006). Our findings of pronounced GLT1 dysfunction specifically in the superficial dorsal horn of the cervical spinal cord suggests that anatomically targeting therapies to these locations represents an improved strategy for efficiently and focally targeting astrocyte dysfunction following SCI, particularly in the context of neuropathic pain.

In conclusion, we have characterized two cervical contusion SCI models in rats that result in robust at-level neuropathic pain and that are associated with astrocyte activation and loss of astrocyte GLT1 expression. These two mechanisms have also been histologically associated with chronic activation of neurons of the superficial dorsal horn. Using these models, future work will be aimed at testing relevant therapies to attenuate, prevent or reverse neuropathic pain post-SCI. These SCI-induced neuropathic pain models can also be used to study other underlying mechanisms of STT neuronal hyperexcitability both in the periphery and centrally at various levels of the CNS neuroaxis, including changes in glial populations.

4. Experimental Procedures

4.1 Animal Studies

4.1.1 Animals—In total, 54 adult female Sprague-Dawley rats (250-300 grams at the time of surgery; Charles River Laboratories, USA) were housed 3 per cage in a controlled light-dark environment, and the rats were given *ad libitum* access to food and water in the Thomas Jefferson University Animal Facility. All animal care and treatment were conducted in compliance with the *European Communities Council Directive* (2010/63/EU, 86/609/EEC

and 87-848/EEC), and the *NIH Guide for the care and use of laboratory animals*. All experimental procedures performed were previously approved by the Thomas Jefferson University IACUC.

4.1.2 Contusion—SCI Models: Rats were anesthetized with a cocktail of ketamine (100 mg/kg), xylazine (5 mg/kg), and acepromazine (2 mg/kg) via intraperitoneal injection. Once anesthetized, the skin and muscle layers between the base of the skull and the top of the shoulder blades (i.e. between the spinous processes of C2 and T1) were incised to expose the cervical spinal column. The dorsal muscle layers and skin were pulled back with retractors to expose the paraspinal muscles. The paraspinal muscles over C3-C8 were incised to expose the underlying vertebrae. A unilateral right-side laminectomy at level C5 or C6 was then performed to expose the spinal cord, extending from the midline blood vessel to the lateral edge of the bone. The dorsal spinous processes of C3 and T1 were clamped with Adson toothed forceps to align and stabilize the spinal column. Before the C5 or C6 contusion took place, the entire area was bathed in 0.9% sterile saline. Using the Infinite Horizon Impactor (Precision Systems and Instrumentation; Lexington, KY), rats were injured at either level C5 or C6 (Nicaise et al., 2012a; Nicaise et al., 2012b; Nicaise et al., 2013). Impact parameters included 200 kilodynes of force using a 1.0mm impactor tip with a 2 second dwell time. To avoid dorsal rootlet damage, the location of the impactor tip contact with the spinal cord was positioned between the midline blood vessel and the entry location of the dorsal rootlets. Laminectomy control animals underwent the same surgical procedure, but did not receive contusion injury. Upon completion of the injury procedure, overlying muscles were closed in layers with sterile 4–0 silk suture, and the skin incision was closed using sterile wound clips. Following surgical procedures, the animals were subcutaneously administered 5mL of lactated Ringers solution and 0.1 mg/kg of Buprenex (buprenorphine-HCl) and were allowed to recover on a circulating warm-water jacket. Once awake and moving, animals were removed from the water jacket and returned to their home cages. Daily post-operative monitoring continued for the duration of the study.

4.1.3 Behavioral testing—Hargreaves thermal testing was conducted to assess thermal hyperalgesia in each forepaw (Detloff et al., 2010). Pre-injury baselines for each individual forepaw of each animal were acquired once per week for two weeks. Following contusion or laminectomy-only surgery, behavioral testing continued once per week until sacrifice at 6 weeks post-injury. Hargreaves testing was always conducted at the same time of day. The order of animal testing was also randomly altered both within a testing session and across sessions. Following surgery, each rat forepaw was tested only if the rat was capable of full weight-support with that particular forelimb to ensure that the rat possessed the motor function to fully withdraw from the stimulus. Prior to even the first pre-operative testing session, rats were acclimated to the testing environment for seven days (20 minutes per day). During testing, rats were enclosed in a clear Plexiglass box with a thin glass bottom (UgoBasile, Comerio, VA). After acclimation, a noxious infrared stimulus with an intensity of 40 Watts was applied to the plantar surface of the forepaw, and the withdrawal response latency was recorded in seconds. Five readings were obtained per session for each forepaw and averaged to generate one mean score. For Hargreaves testing, care was taken to note supraspinal behaviors associated with limb withdraw. This included quick withdrawal from

the noxious stimulus, licking of the forepaw after the stimulus was applied, and/or sharp vocalizations following the application of the noxious stimulus.

4.2 Histological Analyses

4.2.1 Tissue Processing—All 54 rats subjected to either contusion injury or laminectomy-only were sacrificed at 2 or 6 weeks post-injury. Following anesthetic overdose, rats were transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde only in rats used for histological analysis. Brains and spinal cords were immediately harvested, washed briefly in 0.1M phosphate buffer, post-fixed in 4% paraformaldehyde overnight, washed again briefly in 0.1M phosphate buffer, and then cryoprotected in 30% sucrose / 0.1 M phosphate buffer at 4°C for 3 days. The cervical spinal cord was isolated, embedded in OCT freezing medium, and flash-frozen with dry ice. Cervical spinal cords were cut serially in the transverse orientation on a cryostat in 30µm thick sections. Slides were then stored at -20°C until processed for histology.

4.2.2 Lesion Size Analysis—Using every 5th slide, spinal cord sections were thawed, allowed to dry for 1 hour at room temperature, stained with 0.5% Cresyl-violet acetate/ Eriochrome cyanine, and imaged using a Zeiss Imager M2 upright microscope. Using ImageJ software, lesion area was outlined and quantified (Nicaise et al., 2012a; Nicaise et al., 2012b; Nicaise et al., 2013). Specifically, lesion area was determined every 150 µm on these sections by tracing both the total area of the hemi-spinal cord ipsilateral to the lesion and the actual injury area to obtain two-dimensional values. Injury was defined as areas including both lost tissue and surrounding damaged tissue in which the normal anatomical structure of the spinal cord was compromised. The lesion epicenter was defined as the section with the largest percent lesioned tissue (relative to total hemi-cord area in the same section). Lesion area was then plotted at specific rostral-caudal distances relative to the epicenter. For all histological quantification, the experimenters were always blinded to the group identity of the samples being analyzed.

4.2.3 Immunohistochemistry—For all IHC, analysis was conducted at specific locations relative to the lesion epicenter of each animal. Specifically, analysis was conducted at the level of epicenter and at a site 2.0mm caudal to the epicenter (Fig. 1A-B). Spinal cord sections were rehydrated with TBS after drying for one hour at room temperature. After rehydration, sections were washed in TBS and then blocked with 10% normal goat serum / 0.2% Triton / TBS for 1 hour at room temperature. After blocking, sections were incubated with primary antibodies overnight at 4°C. Rabbit polyclonal FosB (Luis-Delgado et al., 2006) (IHC 1:100; Santa Cruz, USA), rabbit polyclonal Ki67 (Lepore et al., 2008a) (IHC 1:200; Abcam, USA), mouse monoclonal GFAP (Lepore and Fischer, 2005) (IHC 1:400, Sigma Aldrich, USA), and rabbit polyclonal GLT1 (Lepore et al., 2008b) (IHC 1:800, kindly provided by Jeffrey Rothstein's lab at Johns Hopkins University) were incubated in 2% goat serum / 0.2% Triton / TBS. After overnight primary antibody incubation, sections were probed with rhodamine-conjugated goat-anti-rabbit IgG (1:100; Jackson Immuno, USA) or FITC-conjugated goat-anti-mouse IgG (1:100; Jackson Immuno, USA). Sections were cover-slipped with fluorescence-compatible antifade reagent (ProLong Gold, Life Technologies, Grand Island, NY). From immunostained sections at the distances described

above, images were acquired at 10X magnification with a Zeiss Imager M2 upright fluorescence microscope. High-resolution confocal images were also obtained using a FluoView FV1000 confocal microscope (Olympus, Center Valley, PA).

Quantification was specifically conducted in the superficial laminae (I-II) of the spinal cord dorsal horn (Fig. 2F-G). For FosB and Ki67 quantification, individual cell nuclei were counted in a blinded manner at all four histological locations described above. GFAP and GLT1 were quantified differently. Because these two antibodies have relatively diffuse staining throughout the dorsal horn, quantification of cell number is challenging. Instead, images of the dorsal horns were acquired at constant exposure settings for all animals and areas. GFAP and GLT1 quantification was calculated as an integrated intensity using Metamorph software (Molecular Devices, Sunnyvale, CA) (Lepore et al., 2008b). For Ki67-GFAP double-labeling, the percentage of Ki67-positive cells that co-expressed GFAP was quantified.

4.3 Immunoblotting

4.3.1 Tissue Harvest—When harvesting fresh tissue for immunoblotting, rats were perfused with only 0.9% saline (Lepore et al., 2011b). Spinal cord was quickly dissected, and whole tissue pieces were sub-dissected from two defined locations: 1) ipsilateral to the lesion epicenter at the segment of the contusion, 2) ipsilateral to the lesion epicenter at a location one segment caudal to the contusion (Fig. 1A-B). Spinal cord tissue was fast-frozen and then stored at -80°C until further processing.

4.3.2 Western Blotting—Spinal cord samples were then homogenized on ice in 0.5ml of RIPA buffer containing (in mM): 50 Tris-HCl pH 7.6, 150 NaCl, 2 EDTA, 0.1% SDS, 0.01% NP-40, Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN). Equal amounts of protein (determined by Bradford assay) were resolved by SDS-PAGE on 4-12% gels and transferred to nitrocellulose membranes. The membranes were blocked in Odyssey blocking buffer (Li-Cor, Lincoln, NE) for 1 hour at room temperature and probed with anti-GLT1 antibody (1:5,000) or anti-actin antibody (1:2,000; Abcam) overnight followed by incubation with IRDye-conjugated goat anti-rabbit or goat anti-mouse IgG (1:10,000; Li-Cor) as secondary antibody for detection. Acquisition was done on a Li-Cor Odyssey infrared imaging system. Quantification of GLT1 bands was conducted using ImageJ software by integrating band intensity, followed by normalization to actin (Lepore et al., 2011b).

4.4 Statistical Analyses

Results were expressed as means $\pm$ standard error of the mean (SEM). Statistical significance was assessed by analysis of variance (One-way ANOVA) for Hargreaves thermal testing. All histological analyses, including comparison of lesion size, FosB, GFAP, Ki67, and GLT1 IHC, were analyzed via one sample t-tests and the Shapiro-Wilk test for normality. Statistics were computed with Sigma Plot 12 (Systat Software Inc., San Jose, CA) software. $P < 0.05$ was considered as statistically significant for all analyses.

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References

- Aguilar RM, Steward O. A bilateral cervical contusion injury model in mice: assessment of gripping strength as a measure of forelimb motor function. *Exp Neurol*. 2011; 221:38–53. [PubMed: 19815010]
- Bleakman D, Alt A, Nisenbaum ES. Glutamate receptors and pain. *Semin Cell Dev Biol*. 2006; 17:592–604. [PubMed: 17110139]
- Callahan BL, Gil AS, Levesque A, Mogil JS. Modulation of mechanical and thermal nociceptive sensitivity in the laboratory mouse by behavioral state. *J Pain*. 2008; 9:174–84. [PubMed: 18088557]
- Cao H, Zhang YQ. Spinal glial activation contributes to pathological pain states. *Neurosci Biobehav Rev*. 2008; 32:972–83. [PubMed: 18471878]
- Crown ED, Gwak YS, Ye Z, Johnson KM, Hulsebosch CE. Activation of p38 MAP kinase is involved in central neuropathic pain following spinal cord injury. *Exp Neurol*. 2008; 213:257–67. [PubMed: 18590729]
- De Felice M, Sanoja R, Wang R, Vera-Portocarrero L, Oyarzo J, King T, Ossipov MH, Vanderah TW, Lai J, Dussor GO, Fields HL, Price TJ, Porreca F. Engagement of descending inhibition from the rostral ventromedial medulla protects against chronic neuropathic pain. *Pain*. 2011; 152:2701–9. [PubMed: 21745713]
- Detloff MR, Clark LM, Hutchinson KJ, Kloos AD, Fisher LC, Basso DM. Validity of acute and chronic tactile sensory testing after spinal cord injury in rats. *Exp Neurol*. 2010; 225:366–76. [PubMed: 20643128]
- Faulkner JR, Herrmann JE, Woo MJ, Tansey KE, Doan NB, Sofroniew MV. Reactive astrocytes protect tissue and preserve function after spinal cord injury. *J Neurosci*. 2004; 24:2143–55. [PubMed: 14999065]
- Gensel JC, Tovar CA, Hamers FP, Deibert RJ, Beattie MS, Bresnahan JC. Behavioral and histological characterization of unilateral cervical spinal cord contusion injury in rats. *J Neurotrauma*. 2006; 23:36–54. [PubMed: 16430371]
- Gwak YS, Hulsebosch CE. GABA and central neuropathic pain following spinal cord injury. *Neuropharmacology*. 2011a; 60:799–808. [PubMed: 21216257]
- Gwak YS, Hulsebosch CE. Neuronal hyperexcitability: a substrate for central neuropathic pain after spinal cord injury. *Curr Pain Headache Rep*. 2011b; 15:215–22. [PubMed: 21387163]
- Gwak YS, Kang J, Unabia GC, Hulsebosch CE. Spatial and temporal activation of spinal glial cells: Role of gliopathy in central neuropathic pain following spinal cord injury in rats. *Exp Neurol*. 2012; 234:362–72. [PubMed: 22036747]
- Hansson E. Could chronic pain and spread of pain sensation be induced and maintained by glial activation? *Acta Physiol (Oxf)*. 2006; 187:321–7. [PubMed: 16734769]
- He Y, Tian X, Hu X, Porreca F, Wang ZJ. Negative reinforcement reveals non-evoked ongoing pain in mice with tissue or nerve injury. *J Pain*. 2012; 13:598–607. [PubMed: 22609247]
- Hobo S, Eisenach JC, Hayashida K. Up-regulation of spinal glutamate transporters contributes to antihypersensitive effects of valproate in rats after peripheral nerve injury. *Neurosci Lett*. 2011; 502:52–5. [PubMed: 21802494]
- Hoschouer EL, Basso DM, Jakeman LB. Aberrant sensory responses are dependent on lesion severity after spinal cord contusion injury in mice. *Pain*. 2010; 148:328–42. [PubMed: 20022699]
- Hu Y, Li W, Lu L, Cai J, Xian X, Zhang M, Li Q, Li L. An anti-nociceptive role for ceftriaxone in chronic neuropathic pain in rats. *Pain*. 2010; 148:284–301. [PubMed: 20022427]

- Hulsebosch CE. From discovery to clinical trials: treatment strategies for central neuropathic pain after spinal cord injury. *Curr Pharm Des.* 2005; 11:1411–20. [PubMed: 15853671]
- Hulsebosch CE. Gliopathy ensures persistent inflammation and chronic pain after spinal cord injury. *Exp Neurol.* 2008; 214:6–9. [PubMed: 18708053]
- Hulsebosch CE, Hains BC, Crown ED, Carlton SM. Mechanisms of chronic central neuropathic pain after spinal cord injury. *Brain Res Rev.* 2009; 60:202–13. [PubMed: 19154757]
- Inquimbert P, Bartels K, Babaniyi OB, Barrett LB, Tegeder I, Scholz J. Peripheral nerve injury produces a sustained shift in the balance between glutamate release and uptake in the dorsal horn of the spinal cord. *Pain.* 2012; 153:2422–31. [PubMed: 23021150]
- King T, Qu C, Okun A, Melemedjian OK, Mandell EK, Maskaykina IY, Navratilova E, Dussor GO, Ghosh S, Price TJ, Porreca F. Contribution of PKMzeta-dependent and independent amplification to components of experimental neuropathic pain. *Pain.* 2012; 153:1263–73. [PubMed: 22482911]
- Kuner R. Central mechanisms of pathological pain. *Nat Med.* 2010; 16:1258–66. [PubMed: 20948531]
- Lane MA, Fuller DD, White TE, Reier PJ. Respiratory neuroplasticity and cervical spinal cord injury: translational perspectives. *Trends Neurosci.* 2008; 31:538–47. [PubMed: 18775573]
- Latremoliere A, Woolf CJ. Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *J Pain.* 2009; 10:895–926. [PubMed: 19712899]
- Lee JH, Tigchelaar S, Liu J, Stammers AM, Streijger F, Tetzlaff W, Kwon BK. Lack of neuroprotective effects of simvastatin and minocycline in a model of cervical spinal cord injury. *Exp Neurol.* 225:219–30. [PubMed: 20599974]
- Lepore AC, Fischer I. Lineage-restricted neural precursors survive, migrate, and differentiate following transplantation into the injured adult spinal cord. *Exp Neurol.* 2005; 194:230–42. [PubMed: 15899260]
- Lepore AC, Dejea C, Carmen J, Rauck B, Kerr DA, Sofroniew MV, Maragakis NJ. Selective ablation of proliferating astrocytes does not affect disease outcome in either acute or chronic models of motor neuron degeneration. *Exp Neurol.* 2008a; 211:423–32. [PubMed: 18410928]
- Lepore AC, Rauck B, Dejea C, Pardo AC, Rao MS, Rothstein JD, Maragakis NJ. Focal transplantation-based astrocyte replacement is neuroprotective in a model of motor neuron disease. *Nat Neurosci.* 2008b; 11:1294–301. [PubMed: 18931666]
- Lepore AC, O'Donnell J, Bonner JF, Paul C, Miller ME, Rauck B, Kushner RA, Rothstein JD, Fischer I, Maragakis NJ. Spatial and temporal changes in promoter activity of the astrocyte glutamate transporter GLT1 following traumatic spinal cord injury. *J Neurosci Res.* 2011a; 89:1001–17. [PubMed: 21488085]
- Lepore AC, O'Donnell J, Kim AS, Yang EJ, Tuteja A, Haidet-Phillips A, O'Banion CP, Maragakis NJ. Reduction in expression of the astrocyte glutamate transporter, GLT1, worsens functional and histological outcomes following traumatic spinal cord injury. *Glia.* 2011b; 59:1996–2005. [PubMed: 21882244]
- Liaw WJ, Stephens RL Jr, Binns BC, Chu Y, Sepkuty JP, Johns RA, Rothstein JD, Tao YX. Spinal glutamate uptake is critical for maintaining normal sensory transmission in rat spinal cord. *Pain.* 2005; 115:60–70. [PubMed: 15836970]
- Luis-Delgado OE, Barrot M, Rodeau JL, Ulerly PG, Freund-Mercier MJ, Lasbennes F. The transcription factor DeltaFosB is recruited by inflammatory pain. *J Neurochem.* 2006; 98:1423–31. [PubMed: 16787404]
- Maragakis NJ, Rothstein JD. Glutamate transporters: animal models to neurologic disease. *Neurobiol Dis.* 2004; 15:461–73. [PubMed: 15056453]
- Maragakis NJ, Rothstein JD. Mechanisms of Disease: astrocytes in neurodegenerative disease. *Nat Clin Pract Neurol.* 2006; 2:679–89. [PubMed: 17117171]
- McClung CA, Ulerly PG, Perrotti LI, Zachariou V, Berton O, Nestler EJ. DeltaFosB: a molecular switch for long-term adaptation in the brain. *Brain Res Mol Brain Res.* 2004; 132:146–54. [PubMed: 15582154]
- McDonald JW, Becker D. Spinal cord injury: promising interventions and realistic goals. *Am J Phys Med Rehabil.* 2003; 82:S38–49. [PubMed: 14502038]

- Mills CD, Xu GY, Johnson KM, McAdoo DJ, Hulsebosch CE. AIDA reduces glutamate release and attenuates mechanical allodynia after spinal cord injury. *Neuroreport*. 2000; 11:3067–70. [PubMed: 11043525]
- Mills CD, Fullwood SD, Hulsebosch CE. Changes in metabotropic glutamate receptor expression following spinal cord injury. *Exp Neurol*. 2001; 170:244–57. [PubMed: 11476590]
- Mills CD, Hulsebosch CE. Increased expression of metabotropic glutamate receptor subtype 1 on spinothalamic tract neurons following spinal cord injury in the rat. *Neurosci Lett*. 2002; 319:59–62. [PubMed: 11825670]
- Mills CD, Johnson KM, Hulsebosch CE. Role of group II and group III metabotropic glutamate receptors in spinal cord injury. *Exp Neurol*. 2002a; 173:153–67. [PubMed: 11771948]
- Mills CD, Johnson KM, Hulsebosch CE. Group I metabotropic glutamate receptors in spinal cord injury: roles in neuroprotection and the development of chronic central pain. *J Neurotrauma*. 2002b; 19:23–42. [PubMed: 11852976]
- Mogil JS. Animal models of pain: progress and challenges. *Nat Rev Neurosci*. 2009; 10:283–94. [PubMed: 19259101]
- Nestler EJ. Review. Transcriptional mechanisms of addiction: role of DeltaFosB. *Philos. Trans R Soc Lond B Biol Sci*. 2008; 363:3245–55.
- Nicaise C, Hala TJ, Frank DM, Parker JL, Authelet M, Leroy K, Brion JP, Wright MC, Lepore AC. Phrenic motor neuron degeneration compromises phrenic axonal circuitry and diaphragm activity in a unilateral cervical contusion model of spinal cord injury. *Exp Neurol*. 2012a; 235:539–52. [PubMed: 22465264]
- Nicaise C, Putatunda R, Hala TJ, Regan KA, Frank DM, Brion JP, Leroy K, Pochet R, Wright MC, Lepore AC. Degeneration of phrenic motor neurons induces long-term diaphragm deficits following midcervical spinal contusion in mice. *J Neurotrauma*. 2012b; 29:2748–60. [PubMed: 23176637]
- Nicaise C, Frank DM, Hala TJ, Authelet M, Pochet R, Adriaens D, Brion JP, Wright MC, Lepore AC. Early phrenic motor neuron loss and transient respiratory abnormalities following unilateral cervical spinal cord contusion. *J Neurotrauma*. 2013
- Nie H, Weng HR. Impaired glial glutamate uptake induces extrasynaptic glutamate spillover in the spinal sensory synapses of neuropathic rats. *J Neurophysiol*. 2010; 103:2570–80. [PubMed: 20220084]
- Norenberg MD, Smith J, Marcillo A. The pathology of human spinal cord injury: defining the problems. *J Neurotrauma*. 2004; 21:429–40. [PubMed: 15115592]
- Olsen ML, Campbell SC, McFerrin MB, Floyd CL, Sontheimer H. Spinal cord injury causes a widespread, persistent loss of Kir4.1 and glutamate transporter 1: benefit of 17 beta-oestradiol treatment. *Brain*. 2010; 133:1013–25. [PubMed: 20375134]
- Ortinski PI, Dong J, Mungenast A, Yue C, Takano H, Watson DJ, Haydon PG, Coulter DA. Selective induction of astrocytic gliosis generates deficits in neuronal inhibition. *Nat Neurosci*. 2010; 13:584–91. [PubMed: 20418874]
- Pekny M, Nilsson M. Astrocyte activation and reactive gliosis. *Glia*. 2005; 50:427–34. [PubMed: 15846805]
- Regan MR, Huang YH, Kim YS, Dykes-Hoberg MI, Jin L, Watkins AM, Bergles DE, Rothstein JD. Variations in promoter activity reveal a differential expression and physiology of glutamate transporters by glia in the developing and mature CNS. *J Neurosci*. 2007; 27:6607–19. [PubMed: 17581948]
- Ren K. Emerging role of astroglia in pain hypersensitivity. *Jpn Dent Sci Rev*. 2010; 46:86. [PubMed: 20161683]
- Robison AJ, Nestler EJ. Transcriptional and epigenetic mechanisms of addiction. *Nat Rev Neurosci*. 2011; 12:623–37. [PubMed: 21989194]
- Rothstein JD, Patel S, Regan MR, Haenggeli C, Huang YH, Bergles DE, Jin L, Dykes Hoberg M, Vidensky S, Chung DS, Toan SV, Bruijn LI, Su ZZ, Gupta P, Fisher PB. Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. *Nature*. 2005; 433:73–7. [PubMed: 15635412]

- Sandrow-Feinberg HR, Zhukareva V, Santi L, Miller K, Shumsky JS, Baker DP, Houle JD. PEGylated interferon-beta modulates the acute inflammatory response and recovery when combined with forced exercise following cervical spinal contusion injury. *Exp Neurol*. 223:439–51. [PubMed: 20109445]
- Sandrow-Feinberg HR, Izzi J, Shumsky JS, Zhukareva V, Houle JD. Forced exercise as a rehabilitation strategy after unilateral cervical spinal cord contusion injury. *J Neurotrauma*. 2009; 26:721–31. [PubMed: 19489718]
- Sandrow HR, Shumsky JS, Amin A, Houle JD. Aspiration of a cervical spinal contusion injury in preparation for delayed peripheral nerve grafting does not impair forelimb behavior or axon regeneration. *Exp Neurol*. 2008; 210:489–500. [PubMed: 18295206]
- Scholz J, Woolf CJ. The neuropathic pain triad: neurons, immune cells and glia. *Nat Neurosci*. 2007; 10:1361–8. [PubMed: 17965656]
- Sofroniew MV. Reactive astrocytes in neural repair and protection. *Neuroscientist*. 2005; 11:400–7. [PubMed: 16151042]
- Stamegna JC, Felix MS, Roux-Peyronnet J, Rossi V, Feron F, Gauthier P, Matarazzo V. Nasal OEC transplantation promotes respiratory recovery in a subchronic rat model of cervical spinal cord contusion. *Exp Neurol*.
- Takahashi Y, Nakajima Y. Dermatomes in the rat limbs as determined by antidromic stimulation of sensory C-fibers in spinal nerves. *Pain*. 1996; 67:197–202. [PubMed: 8895248]
- Tao YX, Gu J, Stephens RL Jr. Role of spinal cord glutamate transporter during normal sensory transmission and pathological pain states. *Mol Pain*. 2005; 1:30. [PubMed: 16242033]
- Tawfik VL, Regan MR, Haenggeli C, Lacroix-Fralish ML, Natile-McMenemy N, Perez N, Rothstein JD, DeLeo JA. Propentofylline-induced astrocyte modulation leads to alterations in glial glutamate promoter activation following spinal nerve transection. *Neuroscience*. 2008; 152:1086–92. [PubMed: 18358622]
- Vera-Portocarrero LP, Mills CD, Ye Z, Fullwood SD, McAdoo DJ, Hulsebosch CE, Westlund KN. Rapid changes in expression of glutamate transporters after spinal cord injury. *Brain Res*. 2002; 927:104–10. [PubMed: 11814437]
- Wang MY, O'Shaughnessy B, Haq I, Green BA. Pain following spinal cord injury. *Seminars in Neurosurgery*. 2004; 15:99–105.
- Wang W, Wang Y, Huang J, Wu S, Li YQ. Temporal changes of astrocyte activation and glutamate transporter-1 expression in the spinal cord after spinal nerve ligation-induced neuropathic pain. *Anat Rec (Hoboken)*. 2008; 291:513–8. [PubMed: 18384122]

Abbreviations

SCI	spinal cord injury
CNS	central nervous system
PNS	peripheral nervous system
STT	spinothalamic tract
GLT1	glutamate transporter-1
NMDAR	N-methyl-D-aspartate receptor
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
GABA	gamma-Aminobutyric acid
CGRP	calcitonin gene related peptide
ATP	adenosine triphosphate

NO	nitric oxide
C5, C6, etc.	cervical spinal cord level 5 (6, etc.)
T1	thoracic spinal cord level 1
IHC	immunohistochemistry
GFAP	glial fibrillary acidic protein
Cdk5	cyclin-dependent kinase 5
GluA	glutamate (AMPA) receptor subunit
GluN	glutamate (NMDA) receptor subunit

Highlights

- We characterized two rat models of unilateral cervical contusion spinal cord injury.
- Both models resulted in chronic thermal hyperalgesia in the ipsilateral forepaw.
- Spinothalamic pain transmission neurons were chronically activated in both models.
- Injury resulted in persistent astrocyte activation and reduced GLT1 expression.
- Histological changes were pronounced in superficial laminae of the dorsal horn.

Figure 1. Unilateral cervical contusion SCI resulted in persistent thermal hyperalgesia
 Rats received either a unilateral 200 kilodyne contusion injury at level C5 (A) or C6 (B) using the IH Impactor, while uninjured control animals received laminectomy only. For both contusion paradigms, analysis of immunohistochemistry was conducted on sections from the lesion epicenter and 2.0mm caudal to the epicenter (A-B). Similarly, whole tissue homogenate was sub-dissected from these locations for immunoblotting (from a separate cohort of animals than the rats used for histology). Following both injury paradigms, the right forepaw showed a significant decrease in withdrawal latency as compared to uninjured rats and pre-injury baseline (C); however, there were no significant differences in withdrawal latencies for the left forepaw when compared to uninjured rats and pre-operative baselines (D). WB: Western blotting. n = 9 per group for behavioral testing, ***p < 0.001

Figure 2. Unilateral cervical contusion SCI produced focal cystic cavitation

Histological staining with Eriochrome cyanine and Cresyl violet demonstrated that uninjured rats displayed an intact spinal cord morphology with clearly defined grey and white matter structures (A). However, following either a unilateral C5 (B) or C6 (C) injury, the normal structure of the dorsal horn and lateral white matter tracts was compromised. The gross structure of the spinal cord appeared mostly normal at 2.0mm caudal to the epicenter (D). The lesion extended approximately 2mm in the rostral-caudal axis from the impact epicenter, with no statistical difference in the rostral-caudal spread of the injury between the

two paradigms at both time points (E). $n = 7-8$ per group. For both contusion paradigms, all analyses of immunohistochemistry were conducted in superficial laminae I-II (F-G).

Figure 3. Dorsal horn neuron activation was chronically increased following unilateral cervical contusion SCI

FosB-expressing neurons exhibit a defined pattern of nuclear staining (A). As compared to laminectomy-only controls (B), there was an increase in the numbers of FosB-expressing neurons in the superficial dorsal horn at both 2 (C) and 6 weeks (D) post-injury in both C5 (not shown) and C6 (C-D) contusion paradigms. Both injury paradigms showed statistically significant increases in the dorsal horn at the injury epicenter and at areas caudal to the injury (F). Arrowheads denote FosB-expressing nuclei. $n = 7-8$ per group, $*p < 0.05$, $\# p < 0.01$, $\% p < 0.001$

Figure 4. Astrocytes were chronically activated following unilateral cervical contusion SCI
Following cervical contusion SCI, there was a pronounced activation of astrocytes in laminae I-II of the superficial dorsal horn (both at the lesion epicenter and in intact caudal spinal cord) at 2 and 6 weeks in both injury paradigms, which was characterized by significantly increased expression of GFAP in individual astrocytes and an increase in the total numbers of astrocytes (A-B). Compared to laminectomy-only controls (C), there was an increase in GFAP expression at 2 weeks and 6 weeks post-injury in both C5 and C6 contusion paradigms. These statistically significant changes in GFAP expression for both the C5 and C6 injuries were observed in the superficial dorsal horn at the level of the

epicenter (D) and at areas caudal to the injury (E) (quantification of all analyses shown in F).
n = 7-8 per group, *p < 0.05, # p < 0.01, % p < 0.001

Figure 5. Astrocyte proliferation following cervical contusion SCI

Following both injury paradigms, large numbers of proliferating astrocytes were observed in the superficial dorsal horn (A). At both 2 and 6 weeks post-injury in both the C5 and C6 contusion paradigms, there was a significant increase in the number of total proliferating cells in the superficial dorsal horn compared to uninjured animals, as assessed with Ki67 (E). This was observed both at the ipsilateral lesion epicenter (C) and in ipsilateral caudal spinal cord (D). In the dorsal horn of the intact spinal cord of laminectomy-only control rats, almost no proliferation was observed (B). A large percentage of these Ki67⁺ cells were

GFAP⁺ astrocytes at both 2 and 6 weeks post-injury (A, F). n = 7-8 per group, *p < 0.05, # p < 0.01, % p < 0.001

Figure 6. GLT1 expression was reduced in the superficial dorsal horn following cervical contusion SCI

Confocal analysis shows that in superficial dorsal horn laminae of uninjured rats virtually all GFAP⁺ astrocytes expressed GLT1 (A). Following cervical contusion SCI, there was a pronounced activation of astrocytes in the superficial dorsal horn (both at the lesion epicenter and in intact caudal spinal cord) at all time points characterized by significantly increased expression of GFAP in individual astrocytes, as well as an increase in the total numbers of astrocytes (B). However, the majority of activated astrocytes following injury did not express GLT1 (B). GLT1 protein expression was spatiotemporally quantified following injury using immunostaining. Laminectomy-only uninjured rats showed greater expression levels of GLT1 in the superficial dorsal horn compared to white matter and more

ventral gray matter locations (C). However, following C6 injury, GLT1 expression decreased in superficial dorsal horn, both at the lesion epicenter (D) and at caudal locations (E). This decrease in total GLT1 protein expression in laminae I-II was statistically significant in the dorsal horn at the injury epicenter and caudal to the injury (F). $n = 3$ per group, $*p < 0.05$, $\# p < 0.01$, $\% p < 0.001$

Immunoblotting analysis was conducted on whole-tissue spinal cord homogenate that was sub-dissected from ipsilateral spinal cord segments at the level of the lesion epicenter and one segment caudal to the epicenter. Western blotting results illustrate that total spinal cord GLT1 protein levels were reduced at both 2 and 6 weeks post-C6 injury, compared to uninjured laminectomy-only control (G). Specifically, decreases were significant at both 2 and 6 weeks at the lesion epicenter, but were only significant at 6 weeks at the caudal location (H). $n = 5-6$ per group, $*p < 0.05$