Published in final edited form as:

Neurosci Lett. 2008 July 11; 439(2): 212–215. doi:10.1016/j.neulet.2008.05.030.

Prolonged nociceptive responses to hind paw formalin injection in rats with a spinal cord injury

Jeung-Woon Lee, Orion Furmanski, Daniel A. Castellanos, Linda A. Daniels, Aldric T. Hama, and Jacqueline Sagen

Abstract

Unilateral lesioning of the spinal dorsal horn with the excitotoxin quisqualic acid (QUIS) leads to robust degeneration of dorsal horn grey matter, and robust pain-related symptoms, such as cutaneous hypersensitivity, persist long after injury. A possible mechanism that underlies the pain-related symptoms is the disruption of dorsal horn inhibitory neuron function, leading to decreased inhibition of nociceptive neurons. Five percent formalin was injected into the hind paw of rats with either a QUIS lesion or sham lesion. Both QUIS-lesioned and sham-lesioned rats displayed bi-phasic hind paw flinches following formalin injection, but a prolonged response was observed in QUIS-lesioned rats. The expression of the immediate-early gene product Fos in the dorsal horn ipsilateral to formalin injection was similar between QUIS- and sham-lesioned rats. In QUIS-lesioned rats, however, there was a marked absence of dorsal horn neurons, particularly GABAergic neurons, compared to sham-lesioned rats. The prolonged nociceptive response observed with a unilateral QUIS lesion may be due to generalized changes in dorsal horn neuron function including a loss of inhibitory neuron function.

Keywords

Fos; gamma-aminobutyric acid (GABA); quisqualic acid; sensitization

A significant percentage of spinal cord injury (SCI) patients report pain, including pain with neuropathic components [9]. Both spontaneous pain and cutaneous hypersensitivity have been reported above, at and below the level of the SCI and the mechanism of neuropathic SCI pain, particularly below-level, appears to have a few similarities with that of peripheral neuropathic pain. For example, the loss of descending tonic inhibition at the level of the spinal dorsal horn leads to hyper-responsiveness of dorsal horn neurons to peripheral stimulation, increased unevoked, spontaneous firing and elevated transcriptional activity [3,4,10,25]. These neurons may be found several segments distal to the SCI [5,25]. Restoring bulbospinal inhibition is one method of attenuating neuropathic SCI pain [10,11].

A second important source of spinal inhibition is GABAergic neurons found in lamina II. These neurons appear to be particularly sensitive to the excitotoxic events following peripheral nerve injury. The excitotoxic cascade that occurs within the spinal cord following nerve injury includes increased extracellular glutamate, dynorphin and other excitatory neurotransmitters,

*Correspondence to: Aldric T. Hama, Ph.D., The Miami Project to Cure Paralysis, University of Miami, Miller School of Medicine, 1095 NW 14th Terrace (R-48), Miami, FL 33136, USA, Tel: 305-243-5618, Fax: 305-243-4528, Email: E-mail: ahama@med.miami.edu.

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

and a concurrent decrease in postsynaptic inhibition, leading to cutaneous hypersensitivity [7]. Animals with a chronic sciatic nerve injury develop increased hind paw sensitivity to noxious and innocuous stimuli in parallel with a significant reduction in dorsal horn GABAergic immunoreactivity [14].

Since a similar excitatory cascade has been observed following SCI, it is possible that a part of neuropathic SCI pain may be due to a profound dysfunction or loss of inhibitory dorsal horn neurons. To evaluate this possibility, a SCI was induced by unilateral dorsal horn injections of the non-selective glutamate receptor agonist quisqualic acid (QUIS), which leads to persistent neuropathic pain-related behaviors [23]. To induce a prolonged nociceptive stimulus, the hind paw ipsilateral to the lesion was injected with formalin. Formalin injection also evokes the expression of the immediate early gene product Fos, believed to be a marker of neural transcriptional activity [20]. Fos has been implicated in the production of other gene products, such as dynorphin, which maintain the injury cascade and, thus, the pain state [7]. To determine if there is a difference in spinal neural activity between rats that were either QUIS-lesioned or sham-lesioned, spinal Fos immunostaining between these rats was compared. Possible loss of dorsal horn neurons, particularly GABAergic neurons, was also evaluated.

All procedures were reviewed by the University of Miami Animal Care and Use Committee and followed guidelines outlined in the *Guide for the Care and Use of Laboratory Animals*. Male Sprague-Dawley rats were obtained from Charles River (MA) and were 200–225 g upon arrival. Rats prior to and after surgery were allowed free access to food and water and were housed 2 per cage.

Rats were anesthetized in isoflurane/O₂ and the lumbar area of the back shaved. Using aseptic technique, the dorsal thoraco-lumbar spinal cord was exposed via laminectomy. The dura was cut and 0.4 µl of QUIS (125 mM in phosphate-buffered saline; Sigma-Aldrich Co., MO) was injected three times into the right dorsal horn [24]. The injections were spaced 0.5 mm apart and were made 0.5 mm lateral to the midline at a depth of 1 mm (n = 8 QUIS-lesioned rats). In sham-lesioned rats (n = 8), phosphate-buffered saline was injected into the dorsal horn.

Behavioral testing was performed two weeks after surgery. Rats were briefly restrained in a towel as 50 µl of 5% formalin was injected into the plantar hind paw ipsilateral to the dorsal horn surgery. The number of flinches within one minute were counted at five minute intervals and rats were observed for one hour following formalin injection [4].

Ninety minutes following formalin injection, rats were deeply anesthetized with pentobarbital and perfused with saline followed by phosphate-buffered 4% paraformaldehyde at 4°C [4]. Spinal cords were removed and post-fixed for 24 hours at 4°C. The lumbar segment (L4–L5) was isolated and cut with a cryostat in 10 µm coronal sections. Sections were incubated in rabbit anti-fos antibody (1:5,000; Oncogene Science, MA) overnight at 4°C, rinsed with phosphate-buffered saline and incubated with biotinylated goat anti-rabbit antibody (1:200; Vector Labs, CA). Sections were then incubated in a solution of diaminobenzidine and hydrogen peroxide to visualize the secondary antibody. Profile quantification of 12 randomly selected sections was performed by an observer who was unaware of the source of the sections.

In a subset of randomly selected spinal cord sections from QUIS-lesioned formalin-injected rats, GABA immunocytochemistry was done using a guinea pig polyclonal antibody to anti-GABA (1:100; Protos Immunoresearch, CA) and a monoclonal antibody to neuron-specific nuclear protein (Neu-N; 1:200; Chemicon, CA).

Immunocytochemistry for these antigens were done to qualitatively illustrate the extent of neuronal changes following QUIS injection.

To determine statistical significance, a one-way ANOVA was performed on data from the treatment groups and Dunnett's test was used for *post-hoc* comparisons. Statistical significance was taken at $P < 0.05$.

Following formalin injection, rats with either a QUIS or sham lesion displayed bi-phasic hind paw flinching (Fig. 1). Sham-lesioned rats displayed decreased flinching towards the end of the observation period, whereas QUIS-lesioned rats displayed sustained flinching ($P < 0.05$ vs. sham).

Fos immunoreactivity was visualized as a dark coloration limited to the nucleus of the neuron [4,20]. Ipsilateral to formalin injection, there were numerous Fos immunoreactive profiles in superficial (laminae I–II) and deep (laminae III–V) dorsal horn and around central canal (lamina X) (Fig. 2). In the contralateral spinal cord, there were a few Fos-immunoreactive profiles found in superficial and deep dorsal horn. No significant difference in Fos-immunoreactive profiles was observed between QUIS and sham-lesioned rats (Fig 2; $P > 0.05$).

Injection of 50 μ l of saline into the ipsilateral hind paw in QUIS-lesioned rats did not lead to significant hind paw flinching ($n = 6$; data not shown). Also, hind paw saline injection ipsilateral did not induce Fos immunoreactivity in either QUIS-lesioned or sham-lesioned rats (data not shown). Thus, the induction of Fos was dependent on a robust formalin-mediated nociception [20].

A reduction of Neu-N immunoreactivity confirms previous observations that QUIS injection into the dorsal horn lead to a marked loss of ipsilateral dorsal grey matter, in particular, deep dorsal horn laminae IV–V (Fig. 3; [23,24]). Compared to the contralateral dorsal horn, the reduction of Neu-N immunoreactivity following QUIS injection indicated large-scale destruction of neurons. (Injection of an equal volume of vehicle did not affect either the morphology of deep dorsal horn (data not shown).) With the general loss of neurons, a paucity of GABAergic profiles was also apparent (Fig. 3), compared to the abundance of GABAergic profiles in the contralateral dorsal horn. GABAergic axons were present in both the ipsilateral and contralateral dorsal horn, possibly from bulbospinal neurons, indicating the selectivity of QUIS for soma over axons [18].

Formalin-evoked spinal dorsal horn neuron activation, as indicated by Fos-immunoreactivity, was similar between QUIS- and vehicle-lesioned rats. Thus, despite a lesion that led to a marked decrease in dorsal horn neurons, a considerable number of neurons were still activated by peripheral noxious stimulation. Towards the end of the observation period, significant hind paw flinching was observed in rats with a QUIS lesion, whereas rats with a sham lesion showed decreased flinching. Since the neural activity, suggested by Fos immunoreactivity, was similar between QUIS- and sham-lesioned rats, the sustained duration of flinches could be due to a reduction of spinal inhibition.

The loss of extrinsic and intrinsic inhibition at the level of the dorsal horn following SCI leads to exaggerated responses of dorsal horn neurons and the development of neuropathic SCI pain. A loss of GABAergic tone was clearly evident following a spinal cord contusion injury and a significant decrease in GABAergic neurons is observed after a transient spinal cord ischemia, both of which were accompanied by prominent below-level cutaneous hypersensitivity [6, 26]. In the case of transient ischemia, as the severity of cutaneous hypersensitivity decreases over time, the number of GABAergic neurons in the dorsal horn increased to eventually return to control-uninjured levels. A significant drop in spinal GABA inhibition was observed in other chronic neuropathic pain models, which appears to be reversed with either treatment or over time [14,19].

It is possible that diminished numbers of specific dorsal horn neurons occurred following QUIS lesioning. Since QUIS receptors are found in lamina II and GABAergic neurons are also found in this lamina, QUIS injection may lead to a significant attenuation of GABAergic neurons [15]. In the current study, there was a striking diminution of GABAergic profiles in the ipsilateral dorsal horn. Combined with the decreased Neu-N immunostaining, the data suggest that GABAergic interneurons may be particularly sensitive to QUIS. Alternatively, diminished GABA synthesis, rather than neural death, may have occurred following QUIS injection. Zhang et al. pointed out that following spinal ischemia there was no evidence of “morphological damage” and that the number of GABAergic neurons in their study recovered to pre-injury levels [26]. More extensive morphological and neurochemical studies may be able to determine the extent to which QUIS causes either outright GABAergic cell loss or dysfunction.

In parallel with the loss of inhibition, there is increased activation of dorsal horn neurons in neuropathic SCI pain [23]. Elimination of bulbospinal descending inhibition by a complete thoracic level transection significantly increased formalin-evoked Fos, bilaterally, compared to Fos levels in sham-transected rats [4]. In rats following a severe contusion injury of the thoracic spinal cord, which eliminates most descending tracts, formalin-evoked dorsal horn Fos was greater than in uninjured rats [1,2]. Following either QUIS injection or contusion, abnormal evoked activity and increased unevoked background activity have been noted in dorsal horn neurons, which mediate, in part, below-lesion cutaneous hypersensitivity [6,25]. The Fos-immunoreactive profiles in the current study could be markers of dysfunctional interneurons or supraspinally projecting neurons [22].

Dorsal horn Fos levels were similar between QUIS- and sham-lesioned rats following formalin injection which contrasts to observations from other types of SCI. There are at least two possibilities that could explain the lack of difference in the current study. First, although the number of dorsal horn inhibitory neurons may have diminished following QUIS lesioning, descending tracts were sufficiently intact to allow for some inhibition at the level of the dorsal horn, thus suppressing a potential additional increase in Fos expression [24]. However, QUIS-lesioned rats displayed a prolonged duration of hind paw flinching, which suggests that descending inhibition may have not been entirely effective in suppressing sustained neural activation due to injury-induced plasticity of the (e.g.) serotonergic system [10].

The second possibility is that Fos does not directly reflect the actual nociceptive state and that other molecular markers may more direct indicators. For example, in spinally transected rats, formalin-evoked Fos was greatly increased but flinching was significantly less compared to control rats [4]. In contusion-injured rats, although Fos was increased following formalin injection, it is not known if behavioral response were greater or less than that of uninjured rats (rats were anesthetized) [2]. The lack of Fos expression in QUIS-lesioned rats injected in the hind paw with saline suggests a lack of basal activity of dorsal horn neurons, yet robust spontaneous activity and prolonged after-discharges to innocuous cutaneous stimulation have been reported in these rats [25]. Indeed, vigorous stimulation, such as visceral distention and electrical stimulation, in addition to formalin injection, is needed to induce dorsal horn Fos expression [2,4,16]. By contrast, extracellular recordings demonstrated that hind paw brushing evoked significant exaggerated neural responses, but the same stimulus did not evoke Fos expression [2,6,25]. Similar levels of Fos in spinal dorsal horn have been reported following innocuous mechanical stimulation and no stimulation above the contusion injury [21]. Also, studies in non-SCI rats which directly suppressed spinal Fos expression demonstrated conflicting behavioral responses to formalin [12,13]. Other molecular markers, such as extracellular signal-regulated kinase (ERK), may display greater distinction between the injured and uninjured state to a given stimulus and also reveal the involvement of non-neural cells and the associated injury cascade in neuropathic SCI pain [5,27].

The current data demonstrated a prolonged hind paw response to noxious stimulation in rats with a dorsal horn QUIS lesion. Based on findings in other SCI models, the exaggerated responses to noxious stimuli in the current study and in clinical SCI suggest a significant loss of segmental inhibition in conjunction with increased excitation [17]. Thus, to restore inhibitory tone as a long-term method of reducing neuropathic SCI pain, one could consider either enhancing recovery of intrinsic inhibitory neurons or transplanting GABA secreting cells [8, 14].

Acknowledgements

We thank Ms. C.D. Carrasco and Dr. S. Chen for expert technical assistance. Sponsored by The Miami Project to Cure Paralysis and by NIH grant NS51667.

References

1. Basso DM, Beattie MS, Bresnahan JC. Graded histological and locomotor outcomes after spinal cord contusion using the NYU weight-drop device versus transection. *Exp Neurol* 1996;139:244–256. [PubMed: 8654527]
2. Berrocal YA, Pearse DD, Andrade CM, Hechtman JF, Puentes R, Eaton MJ. Increased spinal c-Fos expression with noxious and non-noxious peripheral stimulation after severe spinal contusion. *Neurosci Lett* 2007;413:58–62. [PubMed: 17161529]
3. Bruce JC, Oatway MA, Weaver LC. Chronic pain after clip-compression injury of the rat spinal cord. *Exp Neurol* 2002;178:33–48. [PubMed: 12460606]
4. Castellanos DA, Daniels LA, Morales MP, Hama AT, Sagen J. Expansion of formalin-evoked Fos-immunoreactivity in rats with a spinal cord injury. *Neurosci Res* 2007;58:386–393. [PubMed: 17531342]
5. Crown ED, Ye Z, Johnson KM, Xu GY, McAdoo DJ, Hulsebosch CE. Increases in the activated forms of ERK 1/2, p38 MAPK, and CREB are correlated with the expression of at-level mechanical allodynia following spinal cord injury. *Exp Neurol* 2006;199:397–407. [PubMed: 16478624]
6. Drew GM, Siddall PJ, Duggan AW. Mechanical allodynia following contusion injury of the rat spinal cord is associated with loss of GABAergic inhibition in the dorsal horn. *Pain* 2004;109:379–388. [PubMed: 15157699]
7. Dubner R, Ruda MA. Activity-dependent neuronal plasticity following tissue injury and inflammation. *Trends Neurosci* 1992;15:96–103. [PubMed: 1373925]
8. Eaton MJ, Wolfe SQ, Martinez M, Hernandez M, Furst C, Huang J, Frydel BR, Gomez-Marin O. Subarachnoid transplant of a human neuronal cell line attenuates chronic allodynia and hyperalgesia after excitotoxic spinal cord injury in the rat. *J Pain* 2007;8:33–50. [PubMed: 17207742]
9. Eide PK. Pathophysiological mechanisms of central neuropathic pain after spinal cord injury. *Spinal Cord* 1998;36:601–612. [PubMed: 9773443]
10. Hains BC, Everhart AW, Fullwood SD, Hulsebosch CE. Changes in serotonin, serotonin transporter expression and serotonin denervation supersensitivity: involvement in chronic central pain after spinal hemisection in the rat. *Exp Neurol* 2002;175:347–362. [PubMed: 12061865]
11. Hains BC, Johnson KM, Eaton MJ, Willis WD, Hulsebosch CE. Serotonergic neural precursor cell grafts attenuate bilateral hyperexcitability of dorsal horn neurons after spinal hemisection in rat. *Neuroscience* 2003;116:1097–1110. [PubMed: 12617951]
12. Hou WY, Shyu BC, Chen TM, Lee JW, Shieh JY, Sun WZ. Intrathecally administered c-fos antisense oligodeoxynucleotide decreases formalin-induced nociceptive behavior in adult rats. *Eur J Pharmacol* 1997;329:17–26. [PubMed: 9218679]
13. Hunter JC, Woodburn VL, Durieux C, Pettersson EK, Poat JA, Hughes J. c-fos antisense oligodeoxynucleotide increases formalin-induced nociception and regulates preprodynorphin expression. *Neuroscience* 1995;65:485–492. [PubMed: 7777163]
14. Ibuki T, Hama AT, Wang XT, Pappas GD, Sagen J. Loss of GABA-immunoreactivity in the spinal dorsal horn of rats with peripheral nerve injury and promotion of recovery by adrenal medullary grafts. *Neuroscience* 1997;76:845–858. [PubMed: 9135056]

15. Jansen KL, Faull RL, Dragunow M, Waldvogel H. Autoradiographic localisation of NMDA, quisqualate and kainic acid receptors in human spinal cord. *Neurosci Lett* 1990;108:53–57. [PubMed: 1689476]
16. Landrum LM, Jones SL, Blair RW. The expression of Fos-labeled spinal neurons in response to colorectal distension is enhanced after chronic spinal cord transection in the rat. *Neuroscience* 2002;110:569–578. [PubMed: 11906794]
17. Loeser JD, Ward AA Jr, White LE Jr. Chronic deafferentation of human spinal cord neurons. *J Neurosurg* 1968;29:48–50. [PubMed: 5244625]
18. Millan MJ. Descending control of pain. *Prog Neurobiol* 2002;66:355–474. [PubMed: 12034378]
19. Moore KA, Kohno T, Karchewski LA, Scholz J, Baba H, Woolf CJ. Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord. *J Neurosci* 2002;22:6724–6731. [PubMed: 12151551]
20. Presley RW, Menetrey D, Levine JD, Basbaum AI. Systemic morphine suppresses noxious stimulus-evoked Fos protein-like immunoreactivity in the rat spinal cord. *J Neurosci* 1990;10:323–335. [PubMed: 1688935]
21. Siddall PJ, Xu CL, Floyd N, Keay KA. C-fos expression in the spinal cord of rats exhibiting allodynia following contusive spinal cord injury. *Brain Res* 1999;851:281–286. [PubMed: 10642858]
22. Todd AJ, Spike RC, Brodbelt AR, Price RF, Shehab SA. Some inhibitory neurons in the spinal cord develop c-fos-immunoreactivity after noxious stimulation. *Neuroscience* 1994;63:805–816. [PubMed: 7898680]
23. Yezierski, RP. Pain following spinal cord injury: pathophysiology and central mechanisms. In: Sandkühler, J.; Bromm, B.; Gebhart, GF., editors. *Prog Brain Res*. Vol. 129. Elsevier; New York: 2000. p. 429-449.
24. Yezierski RP, Liu S, Ruenes GL, Kajander KJ, Brewer KL. Excitotoxic spinal cord injury: behavioral and morphological characteristics of a central pain model. *Pain* 1998;75:141–155. [PubMed: 9539683]
25. Yezierski RP, Park SH. The mechanosensitivity of spinal sensory neurons following intraspinal injections of quisqualic acid in the rat. *Neurosci Lett* 1993;157:115–119. [PubMed: 8233021]
26. Zhang AL, Hao JX, Seiger A, Xu XJ, Wiesenfeld-Hallin Z, Grant G, Aldskogius H. Decreased GABA immunoreactivity in spinal cord dorsal horn neurons after transient spinal cord ischemia in the rat. *Brain Res* 1994;656:187–190. [PubMed: 7804836]
27. Zhuang ZY, Gerner P, Woolf CJ, Ji RR. ERK is sequentially activated in neurons, microglia, and astrocytes by spinal nerve ligation and contributes to mechanical allodynia in this neuropathic pain model. *Pain* 2005;114:149–159. [PubMed: 15733640]

Fig. 1. Formalin-evoked hind paw flinches in rats with either a QUIIS or sham spinal dorsal horn lesion. Two weeks after either a QUIIS or sham (phosphate-buffered saline)-dorsal horn lesion, rats were injected with 5% formalin into the ipsilateral hind paw. The number of flinches was counted for one min at five min intervals. Data are expressed as mean $\pm$ S.E.M. $n = 8$ /group. * $P < 0.05$ vs. sham.

Fig. 2.

Total number of Fos-immunoreactive (IR) profiles in rats with either a QUIS or sham spinal dorsal horn lesion. Ninety min following hind paw formalin injection, rats were perfused and the lumbar spinal cords processed for Fos immunocytochemistry. Fos-IR profiles were counted within defined dorsal horn laminae (I-II, III-V and X) contralateral and ipsilateral to either QUIS or sham lesion. Data are presented as total (mean $\pm$ S.E.M) of 12 sections. $n = 3/\text{group}$.

Fig. 3.

Representative fluorescence photomicrographs of spinal dorsal horn cord cross-sections from a rat previously injected with QUIS. Spinal dorsal horn sections were immunostained for GABA (top row) and for neuronal-specific nuclear protein (Neu-N) (bottom row). In the unlesioned contralateral dorsal horn, there are numerous GABA-ergic profiles (arrowheads) and axons. By contrast, in the QUIS-lesioned dorsal horn, there are few, if any, visible GABA-ergic profiles. GABA-ergic axons remain in the QUIS-lesioned dorsal horn. Note the decrease in grey matter in the QUIS-lesioned deep dorsal horn (between arrows) compared to the contralateral dorsal horn. In the contralateral dorsal horn, there are numerous neurons immunoreactive (IR) for Neu-N. On the ipsilateral dorsal horn, however, there is a general decrease in Neu-N IR, particularly in the deep dorsal horn (between arrows). Scale bars = 100 μ m.