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Excitotoxic spinal cord injury induced dysesthesias are associated with enhanced intrinsic growth of sensory neurons

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HIGHLIGHTS

- Excitotoxic spinal cord lesions resulted in enhanced neuronal growth capacity.
- Dysesthesias are associated with neurite growth in small, medium and large neurons.
- Excitotoxic lesions promoted neurite growth several segments caudal to the injury.

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ABSTRACT

Sensory dysesthesias and pain are common sequelae following spinal cord injury (SCI). While efforts to understand the mechanisms involved in SCI pain syndromes have focused on spinal and supraspinal regions, recent evidence suggests that SCI induces pathological responses in primary afferent neurons that may contribute to the development of sensory abnormalities. The purpose of this study was to investigate if excitotoxic spinal lesions lead to abnormal growth responses of cultured dorsal root ganglia (DRG) neurons, and to examine if the degree of neurite growth correlated with the presence of dysesthesias. Long–Evans rats underwent excitotoxic spinal cord lesions by injection of quisqualic acid at spinal level T12. Animals were examined daily for overgrooming behavior. Fourteen days after injury, DRG neurons were removed from at and below the level of injury, cultured and analyzed for soma size and neurite length. Grooming animals showed robust neurite growth in small, medium, and large neurons compared to nongrooming and control animals. Enhanced neuronal growth responses also occurred several segments caudal to the level of injury. This study provides the first evidence that excitotoxic spinal lesions result in DRG neurite outgrowth that correlated with the presence of sensory dysesthesias, providing support for the role of maladaptive peripheral afferent responses contributing to SCI pain syndromes.
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1. Introduction

Spinal cord injury (SCI) results in altered sensation and the development of neuropathic pain in the majority of SCI patients [9,20,24]. At- and below-level pain-associated dysesthesias are categorized as; spontaneous, independent of peripheral stimuli, or evoked, occurring in response to mechanical or thermal stimuli [8,25]. Historically, investigations to understand SCI pain have focused on the injury epicenter and supraspinal (brain) regions [28]. Recent evidence suggests that SCI has an impact on primary afferents that might contribute to the development of sensory dysfunction [2–4,6]. Research on peripheral effects of SCI are limited, but have shown that SCI results in spontaneous activity, chronic hyperexcitability, and structural plasticity in primary afferents that might contribute to pain sensations [2–4,6]. Since neuropathic pain symptoms are refractory to current treatments, understanding the peripheral contributions to these SCI-induced sensory syndromes is needed to identify improved therapeutic strategies [20,26].

It is well-accepted that primary afferent sprouting after peripheral nerve injury contributes to aberrant sensory function and the development of pain [14,19]. Neuropathic SCI pain and associated dysesthesias may also result from abnormal neuroplasticity characterized by enhanced sensory neuronal growth responses that lead to erroneous synaptogenesis and amplification of the pain sensation [5,8,28]. Multiple lines of evidence demonstrate that SCI results in dorsal horn remodeling, where increases in dorsal horn

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nociceptive fiber expression has been interpreted as sprouting of afferent projections [12,13,18]. Further evidence in support of SCI-induced sprouting comes from studies showing that SCI triggers upregulation of growth promoting proteins in central primary afferent terminals [1,18]. Although recent research suggests that dorsal horn injury (central injury) also has peripheral consequences, the direct evidence to support that SCI results in aberrant primary afferent growth responses is limited and the association with the development of dysesthesias is virtually unstudied [2–4].

The purpose of this study was to investigate if enhanced neurite growth of primary afferent (DRG neurons) fibers correlated with at-level dysesthesias (overgrooming) [29]. Using the excitotoxic model of SCI we demonstrated that 14 days after injury, cultured DRG neurons show extensive neurite growth, with the extent of neurite elongation associated with at-level spontaneous dysesthesia. Lesion-induced DRG growth responses also occurred several segments caudal to the level of injury. This study provides further support for the role of maladaptive peripheral neuronal responses that contribute to sensory dysesthesias following CNS injury.

2. Materials and methods

2.1. Animals and surgery

Experiments were approved by the Institutional Animal Care and Use Committee of East Carolina University. Surgery methods were previously described [29]. Briefly, isoflurane was used to anesthetize 16 male Long Evans rats. A posterior midline incision was made along the thoracolumbar junction. One spinous process and vertebral laminae were removed and the dura incised longitudinally and reflected. A 10 μl Hamilton syringe attached to a glass micropipette with 5–10 μm tip diameter was used for injections. The syringe was mounted on a microinjector (Kopf 5000) and micromanipulator. Unilateral intramedullary injections of 125 mM quisqulic acid (QUIS/lesion, $n = 12$) or 1.2 μl of phosphate buffered saline (PBS) (sham, $n = 6$) were made at T12 into the deep dorsal horn of the spinal cord at a depth of 1000 μm. Following surgery, muscles and skin were sutured. Animals were assessed daily for the presence of overgrooming behavior characterized by scratching and biting the dermatomal region associated with the level of injury, defined by removal of hair and damage to the superficial layers of the skin [29]. Fourteen days after surgery animals were euthanized, DRG were removed and prepared for culture.

2.2. DRG cultures

Neuronal cultures were performed as previously described [22]. Briefly, 2 DRG were extracted from QUIS-injected and sham-injected animals. Because the excitotoxic injury model employs unilateral injections, DRG ipsilateral to injection and overgrooming behavior were collected from both at (T11–12) and below (L4–L5) the level of the lesion. DRG were washed in media (DMEM/F12, N2, glutamine, horse serum, penicillin, streptomycin), disassociated via microsnipping and incubated with collagenase and trypsin. Cells were plated at low density onto 12 mm poly-L-lysine/laminin coated coverslips and incubated at 37.0 °C. Cell density 1 day after dissociation was 52 ± 15 cells/coverslip.

2.3. Morphological analysis

Twenty hours after plating, cultured DRG neurons were fixed with 4% paraformeldehyde. Cells were permeabalized with 0.2% Triton, blocked with 10% bovine serum albumin and stained with a neuronal growth marker; rabbit anti-tubulin III (1:75 dilution, Sigma) antibody conjugated to secondary Cy3 (1:300 dilution, Jackson ImmunoResearch). Coverslips were mounted onto slides using ProLong Gold anti-fade (Invitrogen). Images were acquired using the Leica DM4000 microscope and Q-imaging Retiga 2000R camera. Morphological analysis was performed with Image Pro Express software using the tracing feature to determine the maximum length of the longest neurite. Cells with neurites >10 μm were measured for neurite length. DRG from each of the experimental groups (groomers, nongroomers, sham) were pooled at and below the level of the lesion. A minimum of 5 coverslips and more than 50 neurons were counted in each condition. The average number of neurons with neurites and average length of the longest neurite was reported. Soma diameter measurements were classified as follows: small (<30.4 μm), medium (30.5–45.4 μm), and large (>45.5 μm) [10]. All measurements were performed by an investigator that was blinded to the conditions.

2.4. Statistical analysis

Data were expressed as mean ± SEM and analyzed using GraphPad Prism version 5.0. One-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test for between group comparisons or paired t-tests were used to identify differences between the means. For categorical data (% neurons with neurites), Pearson Chi-Square analysis method was used. Significance was set at $p \leq 0.05$.

3. Results

3.1. Excitotoxic spinal lesions result in enhanced neurite growth that is correlated with at-level dysesthesias

The growth response of cultured DRG neurons from QUIS-injected animals 14 days after injury was assessed and correlated with the presence of overgrooming behavior. Five of the lesioned animals (42%) developed overgrooming behavior graded as class II: extensive hair removal with signs of damage to the superficial skin [29]. Morphological analysis of DRG neurons cultured from the level of injury showed that lesioned animals had a greater number of neurons with neurites vs. sham animals (Fig. 1). The presence of the overgrooming behavior was associated with enhanced neurite length. Quantitative analysis revealed that groomers had a significant increase ($p < 0.0001$) in the number of neurons with neurites (40.0%, n = number of neurons/cells, 212) compared to nongroomers (31.0%, $n = 296$) and sham animals (20.6%, $n = 247$) (Fig. 2A). The average maximum length of neurites from groomers (138.8 μm ± 23.8 μm, $n = 92$) was significantly greater ($p < 0.0001$) compared to sham (17.2 μm ± 1.9 μm, $n = 61$) and nongrooming (75.5 μm ± 11.8 μm, $n = 122$) animals, while there was no significant difference between sham and nongroomers (Fig. 2C). These results indicate that injury to the dorsal horn resulted in enhanced neuronal growth, with the extent of neurite elongation correlating with the occurrence of the at-level dysesthesia, overgrooming.

3.2. Excitotoxic spinal lesions promote enhanced neurite growth in small, medium and large diameter neurons

Aberrant sprouting of both nociceptive (small) and mechanoreceptive (large) fibers has been implicated in the development of SCI pain [8,18]. Therefore, we sought to determine the type of DRG neurons that exhibit growth responses associated with sensory disturbances following excitotoxic lesion. Soma size analysis from cultured DRG 14 days after injury showed that overgrooming animals had a significant increase ($p < 0.05$) in the percent of neurons with neurites in small (31.3%) and large diameter neurons (48.1%) compared to sham (small 13.3%; large 23.2%) (Fig. 3A). Comparing