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Rac1-regulated dendritic spine remodeling contributes to neuropathic pain after peripheral nerve injury

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ABSTRACT

Although prior studies have implicated maladaptive remodeling of dendritic spines on wide-dynamic range dorsal horn neurons as a contributor to pain after spinal cord injury, there have been no studies on dendritic spines after peripheral nerve injury. To determine whether dendritic spine remodeling contributes to neuronal hyperexcitability and neuropathic pain after peripheral nerve injury, we analyzed dendritic spine morphology and functional influence in lamina IV–V dorsal horn neurons after sham, chronic constriction injury (CCI) of the sciatic nerve, and CCI treatment with NSC23766, a selective inhibitor of Rac1, which has been implicated in dendritic spine development. 10 days after CCI, spine density increased with mature, mushroom-shaped spines preferentially distributed along dendritic branch regions closer to the cell body. Because spine morphology is strongly correlated with synaptic function and transmission, we recorded the response of single units to innocuous and noxious peripheral stimuli and performed behavioral assays for tactile allodynia and thermal hyperalgesia. Wide dynamic range dorsal horn neurons of CCI animals exhibited hyperexcitable responses to a range of stimuli. They also showed reduced nociceptive thresholds in the ipsilateral hind paw. 3-day treatment with NSC23766 significantly reduced post-CCI spine dimensions and densities, and attenuated injury-induced hyperexcitability. Drug treatment reduced behavioral measures of tactile allodynia, but not for thermal hyperalgesia. Together, our results demonstrate that peripheral nerve injury induces Rac1-regulated remodeling of dendritic spines on dorsal horn neurons, and suggest that this spine remodeling contributes to neuropathic pain.

Introduction

Neuropathic pain is broadly defined as chronic pain arising as a consequence of disease or dysfunction of the somatosensory system, and may arise from a spectrum of traumatic insults to the nervous system, including peripheral nerve injury (Treede et al., 2008). Neuropathic pain is often refractory to clinical therapies, and a major challenge of pain research today is to identify the underlying pathological mechanisms of neuropathic pain. A wide body of evidence has shown that many factors can contribute to the development of neuropathic pain, including the loss of inhibitory neurotransmission (Cordero-Erausquin et al., 2005; Coull et al., 2003), inflammation (Hains and Waxman, 2006), and aberrant sodium ion channel expression (Hains et al., 2004; Waxman et al., 1999). Although primary afferent plasticity has been implicated in pain after peripheral nerve injury (Woolf and Salter, 2000; Woolf et al., 1992), it is unknown whether second-order dorsal horn nociceptive neurons also exhibit maladaptive structural plasticity.

Dendritic spines are micron-sized protrusions on dendritic branches, which represent modifiable sites of synaptic contact. In the normal nervous system, dendritic spines subserve several adaptive functions, including experience-dependent circuit reorganization and memory formation (Calabrese et al., 2006). In pathology, emerging evidence has suggested that dendritic spines have an important role, and change shape, size, and number in response to various injury and disease insults (Halpain et al., 2005). In a model for Fragile X mental retardation, mice with Fmr1 protein knockout show impaired long-term potentiation (Wilson and Cox, 2007), decreased neuropathic allodynia (Price et al., 2007), and malformed dendritic spines due to dysfunctional Rac1 signaling (Chen et al., 2010). Similarly, spinal cord injury (SCI) results in dendritic spine alterations on motor cortex neurons (Kim et al., 2006), and induces Rac1-regulated spine remodeling on nociceptive dorsal horn neurons that contributes to neuropathic pain (Tan et al., 2008, 2009a).

In the present study we show that dendritic spine remodeling occurs within dorsal horn neurons after chronic constriction injury (CCI), a well-established peripheral nerve injury model of neuropathic pain. We reasoned that if dendritic spine remodeling has a role in

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neuropathic pain states, then disrupting spine structure should attenuate injury-induced pain. Because *in vitro* and *in vivo* studies have shown that Rac1, a small kinase, can modulate dendritic spine structure and function (Tashiro and Yuste, 2004, 2008), we treated peripheral nerve injured animals with the Rac1-specific inhibitor, NSC23766 (Gao et al., 2004). Targeted inhibition of Rac1 signaling reduced injury-induced spine malformation, decreased neuronal excitability, and improved nociceptive behavioral thresholds. These findings demonstrate that peripheral nerve injury can promote dendritic spine remodeling on nociceptive dorsal horn neurons, and suggest a role for Rac1-dendritic spine remodeling in chronic neuropathic pain after nerve injury.

Materials and methods

Animals

Experiments were performed in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*. All animal protocols were approved by the Yale University Institutional Animal Use Committee. Adult male Sprague–Dawley rats (200–225 g) were used for this study. Animals were housed under a 12 h light/dark cycle in a pathogen-free area with water and food provided *ad libitum*.

Peripheral nerve injury

Chronic constriction injury (CCI) was performed as described previously (Zhao et al., 2006; Hains et al., 2004). Rats ($n=35$) were deeply ANESTHETIZED with ketamine/xylazine (80/5 mg/kg i.p.) and the left sciatic nerve exposed at the mid-thigh level by blunt dissection of the biceps femoris. For chronic constriction injury (CCI), the nerve was ligated with four chromic gut (size 4-0) sutures about 1 mm apart, proximal to its trifurcation. Sham animals ($n=16$) underwent the same procedure, but the sciatic nerve was left intact. After surgery, the overlying muscles and skin were closed with 4-0 prolene sutures, respectively. Animals were allowed to recover post-operatively on an electric heating blanket and received saline for rehydration as necessary (5.0 cc s.c.).

Intrathecal catheterization and drug delivery

7 days post-CCI, under ketamine/xylazine anesthesia, a sterile 32 gauge intrathecal catheter (ReCathCo, Allison Park, PA) was inserted through a slit in the atlanto-occipital membrane, between the base of the skull and spinal vertebra C1, threaded caudally to the lumbar enlargement and secured by suturing. The rostral opening of the exposed end of the catheter was heat sealed by pinching the tip with a sufficiently heated forcep to prevent CSF leakage and infection. The catheter tip location was confirmed within the intrathecal space after the animals were sacrificed. Either control vehicle (Veh) (0.9% saline; $n=20$) or NSC23766 (1 mg/ml; $n=15$), a Rac1 GTPase-specific inhibitor (EMD Chemicals, Darmstadt, Germany) (Gao et al., 2004) were infused (5 μl volume; twice daily) through the catheter by injection with a Hamilton syringe using a 32 gauge needle (Hamilton Co., Reno, NV), followed by sterile saline flush (10 μl) for 3 days (on post-CCI days 8, 9, and 10).

Dendritic spine/image analysis

Dorsal horn neuron culture images were taken with a Zeiss LSM 510 Meta confocal microscope. Cells were sampled only if the nucleus was visible within the plane of section and if cell profiles exhibited distinctly delineated borders. Spinal cord tissue section images were captured with a Nikon Eclipse E800 light microscope equipped with epifluorescence and Nomarski optics, using a Photometrics CoolSnap HQ camera (Roper Scientific, Tucson, AZ) and MetaVue v6.2r6 software (Universal Imaging Corporation, Downingtown, PA). Control and experimental conditions were evaluated in identical manners. As validation for our microscopy approach for analyzing dendritic spines, a literature search revealed that the spine measurement values we report here for sham animals fall within the published range of spine dimensions (Bonhoeffer and Yuste, 2002; Bourne and Harris, 2007; Harris and Kater, 1994; Kim et al., 2006; Tan et al., 2008).

Because we were interested in the potential role glia may have in altering dorsal horn neuron morphology and function, we analyzed microglial profiles within an ipsilateral (to peripheral injury) region of interest in the spinal cord dorsal horn. A square bin (500×500 μm) was aligned with a virtual horizontal (medio-lateral) and vertical (dorso-lateral) line passing through the central canal. This region closely coincides with lamina IV–V, the general location of wide dynamic range (WDR) neurons (Woolf and King, 1987). Expression of glial staining was quantified using equivalently thresholded images converted into black and white using NIH ImageJ software (http://rsb.info.nih.gov/nih-image/). The pixel density within each region was measured using the same software. For comparison, we averaged the pixel density within each ipsilateral region-of-interest for each treatment group, and then compared mean densities across treatment groups.

Golgi-stained coronal spinal cord tissue sections and dorsal horn neurons stained with MAP-2 (1:1000, EnCor, Gainesville, Fl) were examined by transmitted light or immunofluorescence microscopy, respectively. To sample and analyze whole cells in spinal cord tissue sections, we used five criteria outlined previously (Tan et al., 2008), which allowed us to identify cells with morphology similar to those observed for WDR neurons identified by Woolf (Woolf and King, 1987): (1) neurons were located within Rexed lamina 4 and 5; (2) Golgi-stained neurons must have had dendrites and spines that were completely impregnated, appearing as a continuous length; (3) at least one dendrite extended into an adjacent lamina relative to the origin of the cell body; (4) at least one-half of the primary dendritic branches remained within the thickness of the tissue section, such that their endings were not cut and instead appeared to taper into an ending; and (5) the cell body diameter fell between 20 and 50 μm. As a note, these criteria do not imply the physiological characterization of the neurons we analyzed, and instead control for the morphological diversity of spinal cord dorsal horn neurons. We identified a total of 55 neurons (Sham = 20; Vehicle + CCI = 16; NSC23766 + CCI = 19) in this manner for inclusion in our analysis.

We used specific morphological characteristics to identify dendritic spines (Kim et al., 2006). We considered a protrusion without a visible neck structure—from the main dendritic shaft—a spine only if there was a visible indentation on either side of protrusion's junction from the dendrite branch. A spine neck was defined as the structure between the base of the spine, the interface between the parent dendritic branch, and the base of the spine head where the appearance of the spine began to swell distally. Spine head structure varied greatly from spine to spine, but we defined these generally as the visible bulb-like structures located at the ends of protrusions as in our previous study (Tan et al., 2008). Thin- and mushroom-shaped spines were classified as follows: thin spines have head-like enlargements with diameters less than the length of the neck. Mushroom spines have head diameters greater than the length of the neck. Three supporting rationales support the use of these geometric classifications: (1) two spine shapes allowed us to use simple but strict rules in classifying spine morphology. (2) The number of spines we included in our study (total spines counted = 21,986) precluded discrimination of the subtle variations in spine shape. (3) There is a large body of literature describing the different physiological characteristics associated with the two morphologies of thin- and mushroom-shaped spines (Calabrese et al., 2006).

To reconstruct and analyze the complete appearance of dorsal horn neurons, we used Neurolucida software (version 9.0) (MicroBrightField, Williston, VT). We analyzed complete three-dimensional reconstructions