Original Article
Upregulation of neuregulin-1 reverses signs of neuropathic pain in rats

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Abstract: Background: Peripheral nerve injury can result in neuropathic pain, a chronic condition of unclear cause often poorly responsive to current treatments. One possibility is that nerve injury disrupts large A-fiber-mediated inhibition of C-fiber-evoked responses in spinal dorsal horn neurons, leading to central sensitization. A recent study provided a potential molecular mechanism; large dorsal root ganglion (DRG) neurons secrete neuregulin-1 (NRG1), which binds to erbB4 receptors on interneurons and promotes GABA release to inhibit C-fiber-evoked nociceptive transmission. Thus, reduced NRG1 expression following nerve injury could induce chronic pain by disinhibition. We examined if DRG expression of NRG1 is in fact reduced in a rat model of neuropathic pain and if exogenous NRG1 alleviates behavioral signs of this condition. Methods: Three neuropathic pain models were established in rats: spared nerve injury of the tibial and common peroneal nerves (SNI model), intraplantar injection of complete Freund's adjuvant (CFA model), and subcutaneous formalin injection. NRG1 expression was assessed by immunofluorescent staining, hyperalgesia by paw withdrawal threshold to von Frey filament stimulation, and pain-like behavior by spontaneous flinching. Results: NRG1 protein immunoreactivity was reduced in the rat DRG after SNI. Intrathecal administration of neuregulin-1beta 1 (NRG1-1), a 62 amino acid NRG1 mimetic, transiently increased paw withdrawal threshold in SNI model and reduced flinching in the formalin injection model. Conclusion: Our results are consistent with a model of neuropathic pain whereby peripheral nerve injury reduces NRG1-mediated inhibition of nociceptive signaling. Modulating NRG1 may have therapeutic potential for treating neuropathic pain.

Keywords: Neuregulin-1, GABA, spinal dorsal horn, neuropathic pain, dorsal root ganglion, gate control

Introduction

Neuropathic pain is a clinically heterogeneous condition that may arise following injury to peripheral or central components of the somatosensory system, after surgery, or in association with a variety of systemic diseases causing neuropathy [1-4]. Despite common symptoms like dysesthesia (such as unexplained burning sensations) and allodynia, neuropathic pain is etiologically diverse, which has hampered the development of broadly effective treatments [5]. Prevalence estimates indicate that a substantial minority of adults suffer from chronic pain with neurogenic characteristics, with the prevalences of specific subtypes depending on medical history, age, gender, and a variety of other sociodemographic factors [6-8].

According to gate control theory [4], transmission of nociceptive transmission gated by large A-fiber inputs to spinal interneurons that in turn inhibit spinal projection neurons receiving nociceptive C-fiber inputs. Thus, disruption of this feed-forward inhibitory pathway could lead to central sensitization. However, the exact molecular mechanisms underlying this phenomenon are far from clear. Neuregulin-1 (NRG-1), a multifunction member of the epidermal growth factor family implicated in both cardiac and neural development and plasticity [9, 10], can promote activity-induced GABA release through its cognate receptor erbB4 expressed on spinal interneurons [11].

We speculated that peripheral nerve injury leads to the downregulation of NRG-1 in large neurons of the DRG, resulting in reduced levels of GABA in the spinal cord and disinhibition of nociceptive transmission. To test this hypothesis, we first examined NRG1 expression in the DRG and spinal cord by immunofluorescent staining and then test whether direct intrathe-
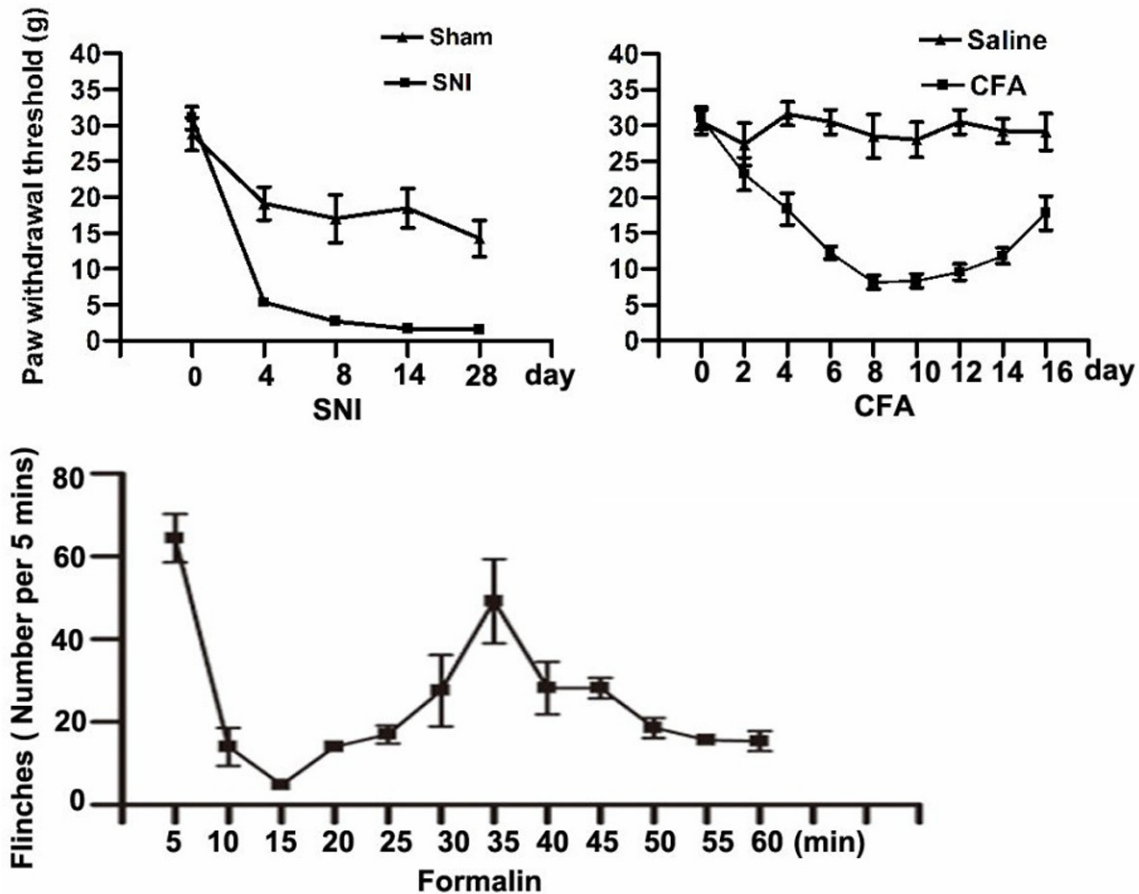
Figure 1. Behavioral changes in the three pain models. A. Spared nerve injury (SNI) pain model. The paw withdrawal threshold in response to calibrated von Frey filaments was measured after injury on the indicated days. B. Complete Freund's adjuvant (CFA) pain model. The paw withdrawal threshold was measured using von Frey filaments for 16 days post-injection. C. Formalin injection model. Following intraplantar injection of 2% formalin, the number of flinches was counted in each successive 5 min period for 1 h. The data are expressed as mean ± standard deviation (SD).

cal injection of a NRG-1 mimetic [12] can suppress behavioral signs of pain in rat neuropathic pain models. Our results strongly suggest that loss of NRG1 release from the DRG into the spinal dorsal horn is indeed suppressed following peripheral nerve injury and so may contribute to this particular form of neuropathic pain.

Materials and methods

Animals

Adult male Sprague-Dawley (SD) rats (body weight: 225-250 g) were obtained from the Laboratory Animal Center of Shanghai Jiaotong University. Rats were housed at 20-25°C and 50 ± 5% humidity with *ad libitum* access to food and water under a 12:12 h light/dark

cycle. All procedures and animal experiments were approved by the Animal Ethical Committee of the Affiliated Sixth People's Hospital, Shanghai Jiaotong University.

Spared nerve injury (SNI) pain model

To establish the spared nerve injury (SNI) rat model [13], the tibial and common peroneal nerves were tightly ligated with a silk suture and transected distally. For sham controls, the sciatic nerve and branches were exposed but not ligated or transected. Rats were allowed to recover in their normal environment for 4 days, and the paw withdrawal threshold in response to calibrated von Frey filaments, a measure of allodynia, was measured 0, 4, 8, 14, and 28 days after SNI or sham treatment.
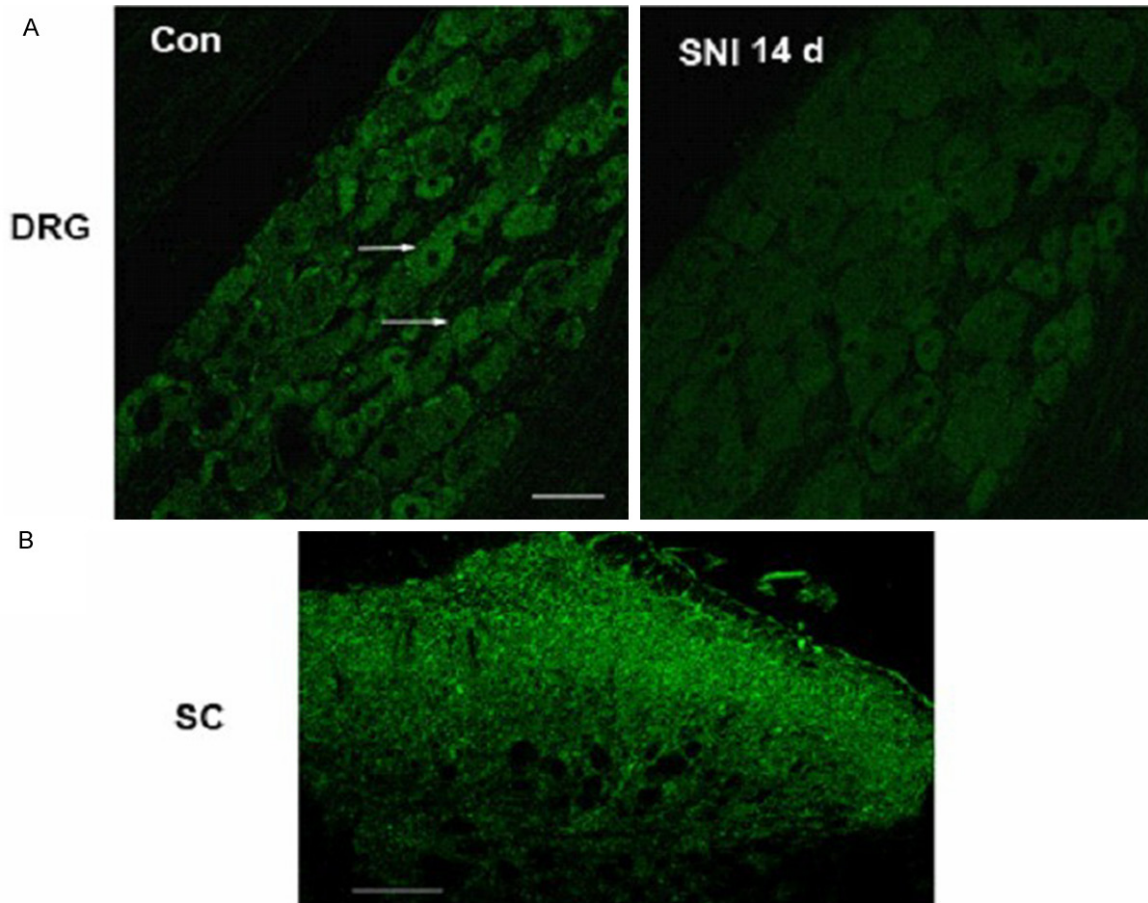
Figure 2. Immunofluorescence staining for (A) NRG1 in dorsal root ganglion (DRG) and (B) erbB5 in spinal cord (SC). (A) Left Panel: Robust NRG1 expression in the DRG of a sham control (Con) group rat (left panel). Arrows: dorsal root neuronal cell bodies. Right Panel: Lower NRG1 expression in DRG 14 days post SNI (SNI 14 d). Bar: 50 µm. (C) Expression of the NRG1 receptor erbB4 was localized to lamina II and III of the spinal dorsal horn. Bar: 100 µm.

Complete Freund's adjuvant (CFA) pain model

To establish the complete Freund's adjuvant (CFA) rat model, the intraplantar right paw was injected with 50-100 µl CFA or equal volume PBS (control). Paw withdrawal thresholds were measured 0, 2, 4, 6, 8, 10, 12, 14, and 16 days after CFA or PBS injection.

Formalin pain model

For the formalin test, 2% formalin or saline was injected subcutaneously into the center of the right hind paw. The number of flinches was determined within each 5 min epoch following injection for a total of 60 min.

Intrathecal injection

Neuregulin-1beta1 (NRG1-1) or PBS vehicle was administered by intrathecal injection at 10 mg/kg to both SNI and formalin model rats. For SNI rats and matched controls, paw withdrawal threshold tests were conducted 0.5, 1, 2, 3, 4, and 5 h after NRG1-1 or PBS injection. Alternatively, 2% formalin was injected into the right paw of naïve rats 30 min after intrathecal NRG1-1 or PBS injection, and the number of flinches counted as described.

Immunofluorescence staining

Rats were anesthetized with sodium pentobarbital (200 mg/kg, intraperitoneal injection). Cardiac regions were exposed via thoracotomy, and left ventricular cannulation extending to the ascending aorta was performed. Rats were perfused with 200 ml PBS (100 mM, pH 7.4) to flush the blood, followed by 700 ml of 4% paraformaldehyde in PBS for perfusion fixation. Following complete perfusion, all rats were decapitated and L4-L5 DRG and spinal cord
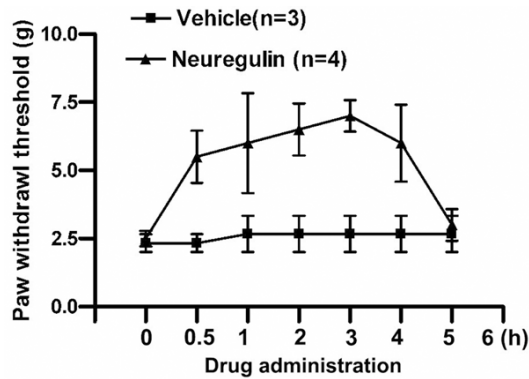
Figure 3. Intrathecal administration of NRG1-1 peptide transiently suppressed neuropathic pain induced by SNI as evidenced by the increase in paw withdrawal threshold to von Frey filaments. In contrast, intrathecal injection of vehicle induced no change in withdrawal threshold. NRG1-1 group (n = 4), PBS group (n = 3). Data are expressed as mean ± SD.

Figure 4. Intrathecal administration of NRG1-1 peptide inhibited formalin-induced flinching. NRG1-1 was administrated at a dose of 10 mg/kg. Thirty minutes later, 2% formalin was injected into the right paw. The number of flinches, an index of pain, was significantly reduced by intrathecal administration of NRG1-1. Data are expressed as mean ± SD. *$P <$ 0.05 *vs.* PBS-treated controls.

removed, fixed in fresh 4% paraformaldehyde overnight at 4°C, placed in 25% sucrose in PBS for 24 h at 4°C, embedded in OCT compound (Miles, Elkhart, IN), and sectioned at 25 μm. Sections were then blocked in normal serum and incubated with NRG1 and erbB4 primary antibody overnight at 4°C. Sections of DRG were washed three times with PBS (5 min/ wash) and stained with FITC-labeled secondary antibody in the dark for 20 min at 37°C. To localize NRG1 protein in DRG and erbB4 in spinal cord, sections were observed under a fluorescence microscope (Olympus, Japan).

Statistical analysis

All data are expressed as mean ± standard deviation (SD). Behavioral data were analyzed by repeated measures two-way ANOVA using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). A *P*-value less than 0.05 were considered statistically significant ($P < 0.05$).

Results

Behavioral changes in the three pain models

We first established three rat pain models: SNI, intraplantar injection of CFA, and subcutaneous 2% formalin. Allodynia, a common symptom of neuropathic pain, was assessed by measuring changes in the paw withdrawal threshold pressure (in grams) in response to calibrate von Frey filament stimulation. As expected, paw
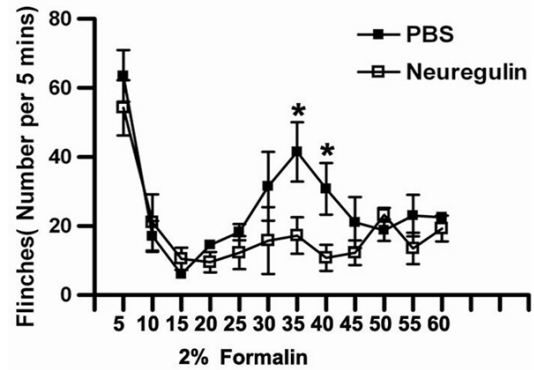
withdrawal thresholds were significantly reduced in SNI (**Figure 1A**) and CFA model rats (**Figure 1B**) compared to sham or vehicle-treated controls. Formalin injection into the paw produced a reliable pain-like response which we quantified by measuring the number of spontaneous flinches within each successive 5 min bin over one hour post-injection (**Figure 1C**). After the initial peak in flinches (60) at 5 minutes post-injection, the number of flinches peaked again (at 50) 35 min post-injection (**Figure 1C**).

Expression of NRG1 protein in DRG and erbB4 protein in spinal cord tissues

We next examined the expression of NRG1 protein in the DRG and erbB4 protein in spinal cord by immunofluorescence labeling. NRG1 immunofluorescence was broadly distributed in large DRG neurons from sham-treated rats, but barely detectable in DGR neurons 14 days after SNI (**Figure 2A**). Expression of the NRG1 receptor erbB4 was localized to lamina II and III of the spinal dorsal horn (**Figure 2B**).

Effects of intrathecal NRG1-1 on pain-like behavior in the two animal models

If reduced NRG1 expression is involved in neuropathic pain, exogenous administration would be expected to reduce behavioral signs of allodynia and spontaneous pain-like behaviors in model rats. In SNI animals, intrathecal adminis-

tration of the mimetic NRG1-1 significantly increased the paw withdrawal threshold for several hours compared to vehicle injection (**Figure 3**), indicating reduced allodynia. The average threshold in PBS-treated SNI model rats remained at 2.5 g, while the threshold in NRG1-1-treated rats reached a maximum of 7 g at 3 h after intrathecal injection. Furthermore, intrathecal injection of NRG1-1 to naïve rats significantly reduced the number of flinches recorded at 35 and 40 min after formalin injection compared to PBS-treated controls ($P < 0.05$) (**Figure 4**).

Discussion

Neurogenic pain following peripheral nerve injury may result from disinhibition of nociceptive transmission. Neuregulin 1 was expressed mainly in large neurons of the DRG, while the NRG1 receptor erbB4 was mainly expressed in Lamina II and III of spinal cord. Thus, NTG1 release from large A-fiber DRG neurons may release NRG1 into the spinal cord. In the dorsal horn, erbB4 stimulation enhances the release of GABA from interneurons, which may suppress nociceptive transmission [11]. In the spared nerve injury (SNI) model [1], NRG1 expression levels were dramatically reduced after injury, suggesting possible disinhibition of nociceptive transmission. Indeed, both spontaneous pain-like behaviors and mechanical hypersensitivity as measured by von Frey filaments were transiently reversed by intrathecal administration of NRG1-1.

This antinociceptive response to NRG1 receptor stimulation is at odds with previous reports showing that NRG1 promotes hypersensitivity and exacerbates pain responses [14-16]. It is clear, however, that SNI induces complex neuroplastic changes in A-fiber transmission [17], so these effects may be time dependent. Moreover, multiple pathogenic processes may progress in parallel following peripheral nerve injury. Nonetheless, these results suggest that NRG1 can suppress neuropathic pain in several rat models, possibly by restoring A-fiber-mediated inhibition of C-fiber-evoked responses in dorsal horn neurons. Further electrophysiological recordings from dorsal horn neurons in response to A- and C-fiber stimulation are necessary to test this hypothesis. It is possible that SNI will decrease and NRG1 (or recombinant NRG1 mimetics) restore A-fiber-mediated inhibition of C-fiber evoked EPSCs.

Regardless of the mechanism, intrathecal administration of NRG1 suppressed spontaneous pain-like behaviors and mechanical hypersensitivity in rat neuropathic pain models. In fact, NRG1 analogues are currently being tested in clinical studies for efficacy in chronic heart failure [12]. Our results suggest that the modulation of NRG1 signaling also has therapeutic value for the treatment of neuropathic pain.

Disclosure of conflict of interest

None.

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