

Sialic acid accelerates the electrophoretic velocity of injured dorsal root ganglion neurons

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Abstract

Peripheral nerve injury has been shown to result in ectopic spontaneous discharges on soma and injured sites of sensory neurons, thereby inducing neuropathic pain. With the increase of membrane proteins on soma and injured site neurons, the negatively charged sialic acids bind to the external domains of membrane proteins, resulting in an increase of this charge. We therefore speculate that the electrophoretic velocity of injured neurons may be faster than non-injured neurons. The present study established rat models of neuropathic pain *via* chronic constriction injury. Results of the cell electrophoresis test revealed that the electrophoretic velocity of injured neuronal cells was faster than that of non-injured (control) cells. We then treated cells with divalent cations of Ca^{2+} and organic compounds with positive charges, polylysine to counteract the negatively charged sialic acids, or neuraminidase to specifically remove sialic acids from the membrane surface of injured neurons. All three treatments significantly reduced the electrophoretic velocity of injured neuronal cells. These findings suggest that enhanced sialic acids on injured neurons may accelerate the electrophoretic velocity of injured neurons.

Key Words: nerve regeneration; peripheral nerve injury; pain sense model; dorsal root ganglion; primary sensory neuron; glycosylated membrane protein; sialic acid; cell electrophoresis; electrophoresis velocity; heat-hyperalgesia behavior; hyperalgesia; neuraminidase; neural regeneration

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Introduction

Pain not only protects the body under normal physiological conditions to avoid injury, but is also a symptom of many diseases (Wall and Devor, 1983; Deng et al., 2014a, b). Chronic constriction injury (CCI) to the sciatic nerve is a model of neuropathic pain and thus has been widely used in pain research. This model demonstrates neuropathic pain such as hyperalgesia, hyperexcitability, paresthesias, and spontaneous pain (Bennett and Xie, 1988). Neuropathic pain has been shown to result from ectopic spontaneous discharge. Ectopic spontaneous discharge originates from the somatic and nerve injury site of sensory neurons in the dorsal root ganglion (DRG) following peripheral nerve injury (Wall and Devor, 1983; Xie and Xiao, 1990; Kajander et al., 1992).

Results from our previous reports combined with those from other studies have concluded that ectopic spontaneous discharge is attributed to excessive expression and abnormal accumulation of Na^+ and Ca^{2+} channel subunits, as well as other functional membrane proteins at the injury site and DRG somata (Luo et al., 2001; Persson et al., 2007). Biochemical studies show that the external domains of these transmembrane proteins are glycosylated, with a large fraction of carbohydrate in the form of negatively charged sialic

acids (McDonaph and Nathan, 1990; Bennett et al., 1997; Bennett, 2002).

We therefore speculate that these membrane proteins may carry more heavily negatively charged sialic acids on the neuronal surface following nerve injury. Sialic acid residues attach to exoplasmic domains of membrane proteins at the terminal ends by N-linked or O-linked bonds (Zhang et al., 2003; Peng et al., 2004; Li et al., 2007). This attachment is likely the reason why injured neurons are more excited than non-injured neurons, hence their hyperexcitability and hyperalgesia (Zhang et al., 2003; Peng et al., 2004; Li et al., 2007).

We also speculate that because more heavily negatively charged sialic acid residues exist on injured neurons, the electrophoretic velocity of injured neurons may be faster than that of non-injured neurons. In the present study, we examined if enhanced sialic acids on injured DRG neurons increase the electrophoretic velocity compared with non-injured DRG neurons.

Materials and Methods

Animals

Sixty healthy, clean, adult male Sprague-Dawley rats, weighing 160–320 g, were purchased from the Institute of Animal

Sciences of Chinese Academy of Medical Sciences in China. All experimental procedures were performed according to the recommendations of the International Association for the Study of Pain. This study was approved by the Animal Ethics Committee of Shanxi Datong University in China.

CCI of the sciatic nerve and experimental groups

The rats were equally and randomly divided into the model and sham-control groups. According to Bennett and Xie's study (1988), the site of ligation on the sciatic nerve was moved up to the location where the left sciatic nerve is not divided into sural and tibial nerve. Normal control rats underwent a sham surgery, which involved an identical surgical procedure except for the ligation of the sciatic nerve.

Confirmation of CCI using the pain-induced paw withdrawal reflex test

To confirm CCI, the latency of the pain-induced paw withdrawal reflex was measured after a noxious radiant heat source was focused onto both heels of hindpaws of all rats 3–17 days post-surgery. Based on the paw withdrawal latency, rats that exhibited heat-hyperalgesia were identified as having CCI. In these rats, the paw withdrawal latency was significantly shorter than that in control rats. Rats that were identified with hyperalgesia were then given the cell electrophoresis test.

Treatment of DRG neurons with Ca^{2+} , polylysine, or neuraminidase

To expose the L_4 or L_5 ganglia, a partial laminectomy on anesthetized rats from both groups was performed, according to previous studies (Mironov and Dolgaya, 1985; Li and Liu, 2009). Small parts of L_4 or L_5 DRG neurons were carefully removed under microscopy by fine forceps, and then placed in standard electrophoresis solution. These neurons were then immediately dispersed into single cells using the blunt mechanical isolation method.

Divalent cations of Ca^{2+} and organic compounds with positive charges, polylysine (Type VII-B), were dissolved in electrophoresis solution at 10 mM and 5 mM, respectively, for 3–5 minutes. Neuraminidase (2 U/mL, type V; Sigma, St. Louis, MO, USA) was then added to the surface of exposed DRG neuronal cells for 2 hours. Electrophoresis was then applied (100 V at 2 mA) (Apparatus from Beijing Ruihua Apparatus Making Factory, Beijing, China). During this procedure, the cells in the quadrate capillary moved to the anode under electrostatic field for a specific distance (d ; μm). The time (t , seconds) it took to cover this distance was then recorded, and the conductance (g) of the electrophoresis solution was then measured. The mean electrophoretic velocity (W) was calculated according the following formula: W ($\mu\text{m}\cdot\text{cm}/\text{V}\cdot\text{second}$) = $d/(t \times g \times s)/I$ (where s is the area (cm^2) of cross section of the quadrate capillary). 30–40 neurons from each DRG were observed.

Statistical analysis

All data are expressed as the mean \pm SD and analyzed by

Student's t -test using SPSS 13.0 software (SPSS, Chicago, IL, USA). Significance was reached at $P < 0.01$.

Results

Electrophoretic velocity was increased in CCI rats

On postoperative days 7–14, eight CCI rats showed typical hyperalgesia and hyperexcitability. Cell electrophoresis showed that on the injured side (left) of these rats, the mean electrophoretic velocity of neurons (number of neurons = 240) was $0.90 \pm 0.11 \mu\text{m}\cdot\text{cm}/\text{V}\cdot\text{second}$, which was significantly ($P < 0.01$) higher than in control rats with a mean electrophoretic velocity of neurons (number of neurons = 150) of $0.62 \pm 0.06 \mu\text{m}\cdot\text{cm}/\text{V}\cdot\text{second}$. These results suggested that neuronal hypersensitivity carried more heavily negative charges following peripheral nerve injury.

Electrophoretic velocity of injured DRG neurons was reduced with Ca^{2+} or polylysine

Injured neurons of 12 CCI rats that showed hyperalgesia and hyperexcitability had a significantly ($P < 0.01$) lower electrophoretic velocity after they were treated with divalent cations of Ca^{2+} (10 mM) or polylysine (5 mM). However, electrophoretic velocity did not change in control rats when exposed to these treatments (Figure 1). Electrophysiological recordings have shown that after the addition of Ca^{2+} or polylysine to the surface of exposed DRG neurons, ectopic discharges disappeared. After 20 minutes of rinsing Ca^{2+} and polylysine by physiological saline, the ectopic discharges reappeared (Zhang et al., 2003; Li et al., 2007). Our results indicate that Ca^{2+} or polylysine connected to the surface of injured neuronal cells counteracts negative charges carried by sialic acids. The reduction of electrophoretic velocity in injured neurons by Ca^{2+} or polylysine was consistent with ectopic spontaneous discharges.

Neuraminidase reduced the electrophoretic velocity of hypersensitive neurons

In six CCI rats with typical hyperalgesia, the L_4 and L_5 DRG neurons were exposed and 2 U/mL neuraminidase applied to remove sialic acids from the membrane surface of the neurons. The electrophoretic velocity of injured neurons is shown in Figure 2. Compared with injured neuronal cells before treatment, neuraminidase significantly ($P < 0.01$) decreased the electrophoretic velocity of these cells. However, in the sham-control group, the electrophoretic velocity remained unchanged after neuraminidase treatment, suggesting that the sialylation level in non-injured neurons was lower. We also observed that Ca^{2+} did not further decrease the electrophoretic velocity of injured neurons if the neurons were digested by neuraminidase beforehand.

Discussion

Previous studies have shown that after nerve injury, the negative charge on injured neurons increases, and this rise leads to a faster electrophoretic velocity than that in non-injured neurons (Li and Liu, 2009). We therefore speculated that the mass of sialic acids, which carry negative charges, is located

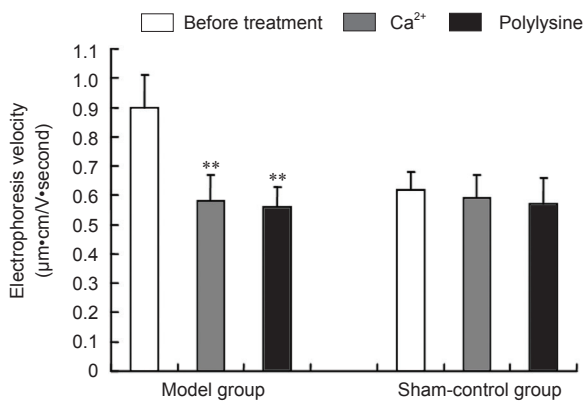


Figure 1 Ca²⁺ and polylysine reduced the electrophoretic velocity of injured neurons in chronic constriction injury rats, but did not affect normal rats.

Compared with before treatment, Ca²⁺ (10 mM, $n = 120$) or polylysine (5 mM, $n = 120$) significantly (** $P < 0.01$) reduced the electrophoretic velocity in injured dorsal root ganglion neurons. However, electrophoretic velocity in the sham-control group remained unchanged with either Ca²⁺ ($n = 100$) or polylysine ($n = 100$) treatment.

on the exoplasmic side of injured DRG neurons, and is thus the reason why the negative charges increased. In the present study, we treated cells with Ca²⁺ or polylysine to counteract the negatively charged sialic acids. Results showed that the electrophoretic velocity of injured neurons was significantly reduced with either treatment. We also used neuraminidase to remove sialic acids from the membrane surface of the injured neurons, and showed that the electrophoretic velocity was reduced.

Overall, these results suggest that sialic acid enhances the negative charges on the membrane surface of injured neurons. Furthermore, the electrophoretic velocity of injured neurons is decreased with Ca²⁺, polylysine, or neuraminidase, because these compounds counteract the negative charges carried by sialic acids or remove sialic acids from the membrane surface of injured neurons.

Previous studies have confirmed that ectopic afferent discharges originate at the nerve injury site (Wall and Devor, 1983; Xie and Xiao, 1990; Kajander et al., 1992). Furthermore, the primary sensory neurons have been suggested to play a pivotal role in the generation and maintenance of neuropathic pain such as hyperalgesia, paresthesias, and spontaneous pain (Zhang et al. 2003; Peng et al. 2004; Li et al. 2007). The ectopic spontaneous discharges in injured primary sensory neurons have been shown to be attributed to the change of expression and heavy accumulation of various functional membrane proteins such as voltage-gated channels. This has been observed for the genes *Nav1.2*, *Nav1.3*, and *Nav1.7*, which encode for the Na⁺ channel subunits (Bennett, 2002; Huang et al., 2014; Laedermann et al., 2014). Changes in the expression for the Ca²⁺ channel subunits have also been reported (Luo et al., 2001; Pan et al., 2014), as well as various other receptors and carrier proteins (Watanabe et al., 2003; Kiguchi et al., 2013; Drummond et al., 2014), particularly the Na⁺ channels. These proteins are heavily glycosylated, with a large fraction of carbohydrate in the form

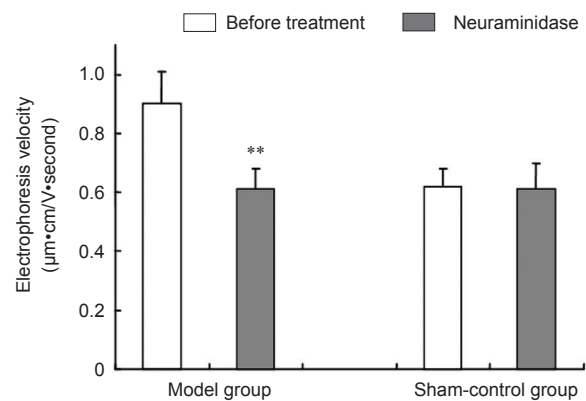


Figure 2 Neuraminidase reduced the electrophoretic velocity of injured neurons in chronic constriction injury rats, but did not affect non-injured neurons.

Compared with before treatment ($n = 240$), the electrophoretic velocity of injured neurons was significantly (** $P < 0.01$) reduced in injured dorsal root ganglion neurons ($n = 100$) with 2 U/mL neuraminidase. These results suggest that neuraminidase reduces sialic acids, thereby decreasing the electrophoretic velocity of injured neurons. In sham-control rats, the electrophoretic velocity remained unchanged before and after neuraminidase treatment, respectively ($n = 100$ or 150).

of negatively charged sialic acid residues (Prince and Welsh, 1998; Shrimal et al., 2013). Under physiological conditions, the density and distribution of these membrane proteins are regulated *via* metabolism (Mironov and Dolgaya, 1985). However, nerve injury interferes with the normal metabolism and distribution of proteins. Therefore, these proteins are upregulated and ectopically accumulated at the site of nerve injury or in the somata, thereby enhancing the conductance of the membrane and initiating the formation of ectopic pacemakers (Prince LS and Welsh, 1998; Shrimal et al., 2013). Sialic acid residues carry an abundance of negative charges (Recio-Pinto, et al., 1990; Zhang, et al., 2003; Peng et al., 2004; Li et al., 2007). In the present study, the increased negative charges carried by sialic acid residues on injured DRG neurons not only resulted in hyperalgesia in CCI rats, but also accelerated the electrophoretic velocity. Neuraminidase was used to remove sialic acids from injured neurons, which resulted in the reduction of the electrophoretic velocity compared with the neuronal cells before treatment. Previous studies have shown that ectopic spontaneous discharges are silenced simultaneously (Zhang, et al., 2003; Peng et al., 2004; Li et al., 2007).

Injured hyperexcitable neurons in an electrostatic field have been shown to rapidly move to the anode compared with control neurons. Furthermore, preventing neuropathic pain in rats results in a significant reduction in the electrophoretic velocity of injured neurons (Li and Liu, 2009). We conclude that in CCI rats, the acceleration of electrophoretic velocity in injured neurons is consistent with its supersensitive state as well as hyperalgesia. Therefore, hypersensitive injured neurons in rats with hyperalgesia may be used as a cell model in which analgesia can be monitored by observing the change in electrophoretic velocity.

Author contributions: CXL was in charge of the funding and

designed the study, analyzed the data as well as wrote the paper. GYM, MFG and YL participated in the study and provided technical support. All authors approved the final version of the paper.

Conflicts of interest: None declared.

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