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Tumor necrosis factor-mediated downregulation of spinal astrocytic connexin43 leads to increased glutamatergic neurotransmission and neuropathic pain in mice

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ABSTRACT

Spinal cord astrocytes are critical in the maintenance of neuropathic pain. Connexin 43 (Cx43) expressed on spinal dorsal horn astrocytes modulates synaptic neurotransmission, but its role in nociceptive transduction has yet to be fully elaborated. In mice, Cx43 is mainly expressed in astrocytes, not neurons or microglia, in the spinal dorsal horn. Hind paw mechanical hypersensitivity was observed beginning 3 days after partial sciatic nerve ligation (PSNL), but a persistent downregulation of astrocytic Cx43 in ipsilateral lumbar spinal dorsal horn was not observed until 7 days post-PSNL, suggesting that Cx43 downregulation mediates the maintenance and not the initiation of nerve injury-induced hypersensitivity. Downregulation of Cx43 expression by intrathecal treatment with Cx43 siRNA also induced mechanical hypersensitivity. Conversely, restoring Cx43 by an adenovirus vector expressing Cx43 (Ad-Cx43) ameliorated PSNL-induced mechanical hypersensitivity. The sensitized state following PSNL is likely maintained by dysfunctional glutamatergic neurotransmission, as Cx43 siRNA-induced mechanical hypersensitivity was attenuated with intrathecal treatment of glutamate receptor antagonists MK801 and CNQX, but not neurokinin-1 receptor antagonist CP96345 or the Ca^{2+} channel subunit $\alpha_2\delta_1$ blocker gabapentin. The source of this dysfunctional glutamatergic neurotransmission is likely decreased clearance of glutamate from the synapse rather than increased glutamate release into the synapse. Astrocytic expression of glutamate transporter GLT-1, but not GLAST, and activity of glutamate transport were markedly decreased in mice intrathecally injected with Cx43-targeting siRNA but not non-targeting siRNA. Glutamate release from spinal synaptosomes prepared from mice treated with either Cx43-targeting siRNA or non-targeting siRNA was unchanged. Intrathecal injection of Ad-Cx43 in PSNL mice restored astrocytic GLT-1 expression. The cytokine tumor necrosis factor (TNF) has been implicated in the induction of central sensitization, particularly through its actions on astrocytes, in the spinal cord following peripheral injury. Intrathecal injection of TNF in naïve mice induced the downregulation of both Cx43 and GLT-1 in spinal dorsal horn, as well as hind paw mechanical hypersensitivity, as observed in PSNL mice. Conversely, intrathecal treatment of PSNL mice with the TNF inhibitor etanercept prevented not only mechanical hypersensitivity but also the downregulation of Cx43 and GLT-1 expression in astrocytes. The current findings indicate that spinal astrocytic Cx43 are essential for the maintenance of neuropathic pain following peripheral nerve injury and suggest modulation of Cx43 as a novel target for developing analgesics for neuropathic pain.

1. Introduction

Activated spinal astrocytes contribute to long-lasting nociceptive hypersensitivity. Dysfunctional, hyperactive astrocytes are observed in the spinal cord dorsal horn following induction of a painful peripheral neuropathy in rodents (Gao et al., 2009; Maeda et al., 2008a; Shibata et al., 2011; Zhuang et al., 2005). Activated astrocytes contribute to the maintenance of the

Abbreviations: Ad-Cx43, adenovirus vector expressing Cx43; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Cx43, connexin 43; DHK, dihydrokainic acid; GFAP, glial fibrillary acidic protein; HVJ, hemagglutinating virus of the Japan; NMDA, N-methyl-D-aspartate; PSNL, partial sciatic nerve ligation; siRNA, small interference RNA; TNF, tumor necrosis factor.

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neuropathic state by synthesis and release of pro-inflammatory cytokines such as tumor necrosis factor (TNF) (Ohtori et al., 2004). Intrathecal treatment with fluorocitrate, an inhibitor of astrocytic metabolism, significantly improves abnormal pain-related behavior in peripheral neuropathy models (Shibata et al., 2011; Zhang et al., 2012). Thus, reducing or eliminating abnormal astrocytic activity greatly attenuates the expression of proinflammatory substances that maintain the neuropathic state. However, there are other significantly altered astrocytic functions that could be important in the maintenance of neuropathic pain.

Astrocytic intercellular communication through gap junctions is crucial not only in regulating astroglial function but also in maintaining homeostasis of the neuronal network (Giaume et al., 2010; Pannasch and Rouach, 2013). Gap junctions are formed by two connexins expressed in neighboring cells, which consist of a hexamer of connexin (Cx) proteins. Gap junctions have important functions including buffering extracellular Na^+ and K^+, supplying sources of energy between neighboring cells, exchanging molecular substances and intercellular communication, passing signaling molecules such as glutamate, ATP and second messengers (Herrero-González et al., 2009; Langer et al., 2012; Saez et al., 2003; Steinhäuser et al., 2012). Several types of Cx, including Cx30, Cx36 and Cx43, have been identified in the spinal cord (Bautista et al., 2012; Nagy et al., 1999; Rash et al., 2001). Cx43 is preferentially and mainly expressed in astrocytes (Giaume et al., 1991) and altered astrocytic Cx43 expression is associated with various neurological disorders (Chew et al., 2010; Karpuk et al., 2011). For example, decreased astrocytic expression of Cx43 protein enhances neuronal excitability, which contributes to the initiation of the neuroinflammation observed in multiple sclerosis (Brand-Schieber et al., 2005). Small interference RNA (siRNA)-induced knockdown of Cx43 in the trigeminal ganglion of naïve rats evokes facial mechanical hypersensitivity (Ohara et al., 2008). By contrast, spinal Cx43 is upregulated in a number of animal chronic pain models and acute reduction of Cx43 activity by intrathecal injection of Cx43-specific siRNA or gap junction inhibitor carbenoxolone ameliorates pain-related behavior (Spataro et al., 2004; Xu et al., 2014a; Yoon et al., 2013). The relationship between astrocytic Cx43 expression levels and changes in pain perception, particularly between Cx43 expression and neuronal excitability, is still controversial. In addition, the endogenous molecule that initiates the change in astrocytic Cx43 expression and the mechanism related to the induction of neuropathic pain after alteration of Cx43 expression have yet to be identified.

Tumor necrosis factor induces abnormal nociception directly by altering astrocytic function and evoking the expression of inflammatory substances which in turn affect neural as well as astrocytic functioning (Gao et al., 2009). Several intracellular mechanisms have been proposed that relate TNF with facilitating nociceptive neurotransmission, however, none have considered an in vivo role between TNF and astrocytic Cx43 function—specifically, the effect of modulating Cx43 function on synaptic neurotransmission in neuropathic pain. Tumor necrosis factor reduces the expression and functioning of Cx43 in cultured brain and spinal astrocytes (Même et al., 2006; Zhang et al., 2013). Thus, TNF could be a key molecule in the downregulation of astrocytic Cx43 expression after PSNL. It is possible that decreased astrocytic Cx43 expression influences synaptic neurotransmission, thereby leading to neuropathic pain.

In the current study, the effects of altered spinal astrocytic Cx43 expression on glutamatergic neurotransmission and pain-related behavior following a peripheral nerve injury were explored. Additionally, a possible role of TNF in regulating astrocytic Cx43 expression was defined. The current results suggest that a complex interaction between signaling molecules such as TNF, astrocytic Cx43 and synaptic neurotransmission maintains the neuropathic pain state.

2. Materials and methods

2.1. Animals

Male ddy mice, 5 weeks of age, were used. The fewest number of mice possible were used in each experiment. Mice were maintained in a vivarium, with the room temperature set at 22 ± 2 °C and 12 h light/dark cycle (lights on/off at 8:00 AM/8:00 PM), and given access to food and water available *ad libitum* during the experimental period. All experiments utilizing animals were conducted in accordance with the "Guidelines for the Care and Use of Laboratory Animals" established by Japanese Pharmacological Society and Hiroshima University, and procedures were reviewed and approved by the Committee of Research Facilities for Laboratory Animal Science of Hiroshima University.

2.2. Partial sciatic nerve ligation (PSNL) in mice

Under sodium pentobarbital (50 mg/kg, i.p.) anesthesia, a tight ligation of approximately one-third to one-half of the diameter of the left sciatic nerve (ipsilateral) was performed with 8–0 silk suture as described previously (Nakamura et al., 2013). A control "sham" group, wherein the sciatic nerve was identified, but no ligation was performed, was generated to determine if the surgery significantly altered the outcome measures of the current study.

2.3. Mouse intrathecal injection

Intrathecal injections were performed on unanesthetized mice (Hylden and Wilcox, 1980; Nakamura et al., 2014). In brief, mice were restrained the left hand and the injection was performed with the right hand. The vertebral landmarks for L5 and L6 vertebrate were identified by palpation. An injection into the subarachnoid space between the L5 and the L6 vertebrae was done via a 27-gage needle. Entry of the needle was confirmed with the presence of a tail flick. Etanercept (TNF blocker, Takeda Pharmaceutical Co. Ltd., Osaka, Japan) was intrathecally injected a total of four times: immediately after sciatic nerve injury, and 2, 4, and 6 days following PSNL. All other drugs were injected only once.

2.4. Hind paw sensitivity to mechanical stimulation

The withdrawal threshold (in grams) of the hind paw to mechanical stimulation was determined using von Frey filaments (Nakamura et al., 2013). In brief, the von Frey filament was pressed against mid-planter surface of the hind paw such that the filament bent slightly. The lowest force that caused responses such as lifting and licking of the hind paw was assigned as the withdrawal threshold. Each hind paw was tested three times. All behavioral tests were performed blinded. Withdrawal thresholds were measured prior to and 3, 7, 14, and 21 days after either PSNL or sham surgery (Day 3, $n = 7$/group; Day 7, $n = 10$/group; Day 14, $n = 10$/group; Day 21, $n = 7$/group; total number of mice = 68).

2.5. Knockdown of lumbar spinal cord Cx43

To evaluate whether specifically down-regulating Cx43 in spinal lumbar dorsal horn leads to mechanical hypersensitivity, the effect of Cx43 knockdown by siRNA transfer on hind paw withdrawal threshold was investigated. Knockdown of Cx43 in naïve mice was performed by using the hemagglutinating virus of the Japan (HVJ) envelop vector system (HVJ Envelop Vector kit GenomONE-Si, Ishihara Sangyo Kaisya, Ltd., Osaka, Japan). This vector is widely used for in vivo siRNA transfer (Kaneda et al., 2002; Morita et al., 2008). Either siRNA targeting mouse Cx43

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