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Down-regulation of zinc transporter-1 in astrocytes induces neuropathic pain via the brain-derived neurotrophic factor - K^+-Cl^- co-transporter-2 signaling pathway in the mouse spinal cord

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ABSTRACT

We previously demonstrated, using a DNA microarray analysis, the down-regulated expression of the *slc30a1* gene (zinc transporter 1, ZnT1) in a neuropathic pain model induced by partial sciatic nerve ligation (PSNL). Zinc is an essential trace mineral that plays important roles in physiological functions, and ZnT1 modulates intracellular zinc levels. In the present study, we examined the effects of the down-regulation of the *ZnT1* gene in the spinal cord on tactile allodynia.

The knockdown (KD) of *ZnT1* by the intrathecal administration of siRNA against *ZnT1* to mice induced allodynia, a characteristic syndrome of neuropathic pain, which persisted for at least one month. *ZnT1* KD increased intracellular zinc concentrations in primary astrocyte cultures, and this was followed by enhanced PKCα membrane translocation and NFκB nuclear translocation as well as increases in the levels of IL-6 and BDNF expressed and the phosphorylation of CREB *in vitro*. Neuropathic pain induced by *ZnT1* KD was inhibited by an IL-6, BDNF, and *TrkB* siRNA injection. The down-regulated expression of KCC2 in spinal cord was induced by *ZnT1* KD and prevented by an intrathecal injection of *IL-6*, *BDNF*, and *TrkB* siRNA. These results indicate that PSNL via the down-regulated expression of ZnT1 increases intracellular zinc concentrations, enhances PKCα membrane translocation and NFκB nuclear translocation, up-regulates the expression of IL-6, increases the phosphorylation of CREB, and promotes the BDNF cascade reaction in astrocytes, thereby down-regulating the expression of KCC2 and inducing neuropathic pain *in vivo*. This mechanism is considered to be responsible for the activation of TrkB in neurons through the release of BDNF from astrocytes. The results of the present study also indicate that zinc signaling in astrocytes occurs upstream of the BDNF-TrkB-KCC2 cascade reaction.

Abbreviations: A3, *N*-(2-aminoethyl)-5-chloronaphthalene-1-sulphonamide hydrochloride; ACSF, artificial cerebrospinal fluid; BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester; BDNF, brain-derived neurotrophic factor; CREB, cAMP-response element binding protein; p-CREB, phosphorylated CREB; DMEM, Dulbecco's modified eagle Medium; R-DIOA, R-(+)-[(dihydroindenyl)oxy] alkanoic acid; FCS, fetal calf serum; GABA, γ-amino butyric acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HRP, horseradish peroxidase; HVJ, hemagglutinating virus of the Japan; IL-6, interleukin-6; IκB, inhibitor of κB; KCC2, K^+-Cl^- co-transporter-2; KD, knockdown; MG132, benzyl *N*-[(2*S*)-4-methyl-1-[[(2*S*)- 4-methyl-1-[[(2*S*)-4-methyl-1-oxopentan-2-yl]amino]-1-oxo-pentan-2-yl]amino]-1-oxo-pentan-2-yl]carbamate; MTF1, metal responsive transcription factor 1; NFκB, nuclear factor-κ B; ODN, oligodeoxynucleotide; PKA, protein kinase A; PKC, protein kinase C; PSNL, partial sciatic nerve ligation; siRNA, small interfering RNA; TPEN, tetrakis-(2-pyridylmethyl) ethylenediamine; TrkB, tropomyosin-related kinase B receptor; Zip transporter, zinc (Zn^{2+})-iron (Fe^{2+}) permease zinc transporter; ZnT, zinc transporter.

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1. Introduction

A previous study reported that a reduction in the chloride gradient across the neuronal membrane, which, in turn, decreases the anion reversal potential, may occur in neurons in the superficial dorsal horn following peripheral nerve damage and the change in the chloride driving force contribute to the cellular hyperexcitability producing sensitization to pain (Coull et al., 2003). The mechanisms responsible for the changes in chloride gradient were found to involve the down-regulated expression of K^+-Cl^- co-transporter-2 (KCC2), which is a potassium-chloride exporter, in spinal lamina I (Coull et al., 2003). Brain-derived neurotrophic factor (BDNF) has been shown to change anion gradient and down-regulate the expression of KCC2 by activating the TrkB receptor, and this cascade has been suggested as one of the mechanisms contributing to the production of allodynia and hyperalgesia associated with neuropathic pain (Coull et al., 2005). It is believed that microglia released BDNF. Many reports indicated relationship between microglia and BDNF signaling in neuropathic pain (see review; Trang et al., 2011). It was indicated that microglia played an important role in onset/development of neuropathic pain. However, the upstream signaling of BDNF-TrkB-KCC2 cascade reaction is unclear. Although astrocytes are also considered to contribute to the initiation, rather to the maintenance of neuropathic pain, the role of astrocytes on development of neuropathic pain and underlying signaling cascades are not clear. Considering the long-lasting feature of pathology of neuropathic pain, the present study focused on the upstream of BDNF-TrkB-KCC2 cascade reaction in relation to astroglia.

Zinc is an essential trace mineral that plays important roles in neurodegenerative diseases including spinal cord injuries (Su et al., 2012; Xu et al., 2011). Alterations in extracellular zinc concentrations in the spinal cord modulate pain signaling (Larson and Kitto, 1999; Ma and Zhao, 2001). The chelation of zinc in the extracellular area of the spinal cord was found to suppress the long-term antinociceptive effects of capsaicin and substance P administrated via an intrathecal injection (Larson and Kitto, 1999).

Alterations in zinc transporter (ZnT) proteins for the maintenance of zinc homeostasis induce biochemical and physiological changes. The zinc ion ionophore, zinc-pyrithione inhibits KCC2 activity *in vitro* (Hershfinkel et al., 2009). In other words, an increase in intracellular zinc ion concentrations has a negative impact on KCC2 functions. On the other hand, high synaptic zinc ion concentrations regulated by zinc transporter-3 have been shown to elevate KCC2 activity by activating metabotropic zinc ion sensing receptors (Chorin et al., 2011). These findings suggest that zinc ion concentrations are closely related to KCC2 functions.

Zinc ion levels are tightly regulated by specific ZnT proteins. In mice, 14 *Zip* have been shown to increase, whereas 9 *ZnT* decrease intracellular zinc levels (Fukada and Kambe, 2011). We used a microarray method to show that partial sciatic nerve ligation (PSNL) decreased the mRNA expression of *slc30a1* (zinc transporter 1, ZnT1). ZnT1 is known to play a key role in the modulation of intracellular zinc levels because only ZnT1 effluxes zinc ions from the intracellular to extracellular space (Fukada and Kambe, 2011; Sekler et al., 2002). In the present study, we focused on ZnT1 and investigated whether the down-regulated expression of the *ZnT1* gene affects the development of tactile allodynia and also if a relationship exists between alterations in ZnT1 and the BDNF-TrkB-KCC2 cascade reaction.

2. Materials and methods

2.1. Animals

Male ddY mice (Kyudo, Kumamoto, Japan) weighing 25–30 g were used. All procedures and handling of animals were performed according to the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society as well as the guidelines of Hiroshima University, Hiroshima, Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments of Hiroshima University (Permit Number: A-12-122, A-12-124 and A-12-125). Surgery was performed under isoflurane anesthesia, and all efforts were made to minimize suffering. Animals were used for only one measurement in each experiment.

2.2. Preparation of partial sciatic nerve injury mice

Experiments were conducted using a previously reported method (Morita et al., 2008b). Briefly, mice were anesthetized with isoflurane (1.5% in oxygen). The sciatic nerve was exposed through a small incision in the upper thigh and carefully cleared of surrounding connective tissue distal to the bony prominence of the femur. An 8–0 silk suture was inserted into the nerve using a 3/8 curved, reverse cutting mini-needle and tightly ligated so that the dorsal one-third to one-half of the thickness of the nerve was held within the ligature. The wound was then closed. In sham-operated mice, the nerve was exposed using the same procedure, but without ligation.

2.3. Knockdown (KD) of proteins in the spinal cord

The KD of proteins was performed as described previously (Morita et al., 2008a, b). siRNAs against each gene of *ZnT1*, *IL-6*, *TrkB* and *BDNF* were obtained from Bioneer Corporation (Daejeon, the Republic of Korea). The hemagglutinating virus of the Japan (HVJ) envelope vector system (HVJ Envelope Vector Kit GenomONE; Ishihara Sangyo Kaisha, Ltd., Osaka, Japan) was used for *in vivo* siRNA transfer. This HVJ envelope vector has already been proven to be an effective oligodeoxynucleotide (ODNs) delivery system both *in vitro* and *in vivo* (Kaneda et al., 2002). siRNAs were incorporated into the HVJ envelope vector according to the manufacturer's instructions. Briefly, after mixing 40 μl (1 assay unit, AU) of the HVJ envelope vector with 4 μl of the enclosing factor, the mixture was centrifuged (10,000 × *g*, 4 °C, 10 min) and the pellet was suspended in 10 μl of buffer solution. In this study, a scrambled siRNA was used for generates a negative control for siRNA as a scrambled sequence of the siRNA target sequence. Ten microliters of siRNA solution was added, and the mixture was kept on ice for 5 min. Sterile artificial cerebrospinal fluid (ACSF; composition, in mM: NaCl 142, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, NaH_2PO_4 1.25, glucose 10, HEPES 10) containing synthetic siRNA duplexes (0.45 pmole/5 μl/animal) was injected into the subarachnoid space between the L5 and L6 vertebrae of naive mice under isoflurane anesthesia.

2.4. Pain-related behavioral testing procedures

In order to measure touch-evoked tactile allodynia, each mouse was placed in a separate plastic cage. Tactile allodynia was evaluated by measuring the paw withdrawal threshold in response to probing with a series of calibrated fine filaments (von Frey hairs). After allowing mice to adapt to the environment, numbered von Frey monofilaments were applied perpendicularly to the dorsum