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Depletion of vesicular zinc in dorsal horn of spinal cord causes increased neuropathic pain in mice

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Abstract Zinc enriched (ZEN) neurons and terminals are abundant in the rodent spinal cord. Zinc ions have been suggested to modulate the excitability of primary afferent fibers believed to be important in nociceptive transmission. To test the hypothesis that vesicular zinc concentration is related to neuropathic pain we applied Chung's rodent pain model on BALB/c mice, and traced zinc transporter 3 (ZnT3) proteins and zinc ions with immunohistochemistry and autometallography (AMG), respectively. Under anesthesia the left fifth lumbar spinal nerve was ligated in male mice in order to produced neuropathic pain. The animals were then sacrificed 5 days later. The ZnT3 immunoreactivity was found to have decreased significantly in dorsal horn of fourth, fifth, and sixth lumbar segments. In parallel with the depressed ZnT3 immunoreactivity the amount of vesicular zinc decreased perceptibly in superficial gray matters of especially layer I-IV of the same segments. The transection-induced reduction of vesicular zinc in ZEN terminals of the dorsal horn was synchronic to reduced pain threshold, as measured by von Frey method. In a separate study, we observed intensive zinc selenite precipitation in somata of the smaller spinal ganglion cell, but 5 days after spinal nerve transection zinc precipitation was also found in the lager ganglion cells. The present results indicate that zinc may be involved in pain mechanism in the spinal ganglion level. These results support the hypothesis that vesicular zinc might have a modulatory role for neuropathic pain. Thus, increased pain sensitivity might be related to reduce vesicular zinc level in the dorsal spinal gray matter.

Keywords Zinc · Spinal cord · Pain · Zinc transporter · Autometallography

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Introduction

In the mammalian brain, around 10% of the total zinc is localized in synaptic vesicles (Frederickson et al. 2000; Holm et al. 1988). This zinc pool of ionic or loosely bound zinc can be visualized either by autometallography (AMG) (Danscher et al. 2004; Danscher and Stoltenberg 2005) or the toluene sulfonamide quinoline (TSQ) fluorescence method

(Frederickson et al. 1987). In the brain, zinc ions are found ultrastructurally to be located to clear, round vesicles in terminals, making asymmetric synapses and being immunoreactive to glutamate (Martinez-Guijarro et al. 1991; Rubio and Juiz 1998). The AMG detectable zinc ions, also called chelatable zinc, present in a pool of synaptic vesicles, can be released into the synaptic space (Perez-Clausell and Danscher 1985, 1986). In the hippocampus the release has been shown to take place as a result of intense synaptic activity (Assaf and Chung 1984; Howell et al. 1984; Aniksztejn et al. 1987). The Zinc enriched (ZEN) terminals of the spinal cord are primarily GABAergic and only a minor contingent are glutamatergic (Danscher et al. 2001; Wang et al. 2001a, b). Zinc is actively concentrated in the synaptic vesicles. Thus, Palmiter et al. (1996) identified a zinc transporter 3 (ZnT3), which they found to be essential for the transport of zinc into synaptic vesicles in ZEN terminals (Palmiter et al. 1996). It consequently seems reasonable to suggest that zinc is involved in neural transmission.

Neurons that contain zinc ions and process zinc ion transporters (ZnT3) in a population of synaptic vesicles have been termed ZEN neurons (Danscher 1996; Palmiter et al. 1996). The ZEN neurons are also identified by their capacity of retrograde axonal transport of zinc-selenide nanocrystals (quantum dots) created in vivo by local or systemic injection of selenium (Christensen and Frederickson 1998; Danscher et al. 1985; Howell and Frederickson 1990; Sorensen et al. 1995). The distribution of ZEN terminals is well described in mammalian telencephalic structures such as neocortical layers II–III and V, hippocampus (Frederickson and Danscher 1990), amygdala (Perez-Clausell and Danscher 1985), striatum (Mancini et al. 1992), and bulbus olfactorius (Mook Jo et al. 2002). Likewise ZEN terminals are present in cerebellum (Danscher et al. 2001; Mook Jo et al. 2002; Kozma et al. 1981; Wang et al. 2001b, 2005) and brain stem (Smeets et al. 1989; Schroder et al. 2000). The immunohistochemical staining pattern for ZnT3 is identical to that seen with the NeoTimm staining for vesicular zinc in mouse and monkey hippocampus (Cole et al. 1999; Wenzel et al. 1997). The staining patterns show very distinct regional differences indicating different ZEN neuron influence on various neuronal networks. The distribution of ZEN terminals in the spinal cord is described in several papers using AMG techniques and ZnT3 immunohistochemistry ($ZnT3^{IHC}$) (Schroder 1977, 1979; Jo et al. 2000; Schroder et al. 2000). These terminals are found both in the ventral and dorsal horn, but more densely in the dorsal horn layers involved in processing of sensory input. Zinc appears to influence pain perception. Thus, in the rat, zinc ions have been found to alleviate thermal hyperalgesia caused by partial nerve injury (Liu et al. 1999) or peripheral inflammation (Safieh-Garabedian et al. 1996) while chelation with CaEDTA induced peripheral hyperalgesia (Larson and Kitto 1997; Larson et al. 2000).

On this background the present study aimed at investigating possible correlations between pain perception and zinc ions levels in the dorsal horn in the mouse model of neuropathic pain. Our results support the notion that synaptic, vesicular zinc in spinal cord may be involved in dorsal horn processing of pain induced activity.

Materials and methods

Spinal nerve transection

All animal surgeries were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). For this study, we used Chung's neuropathic pain model (Kim and Chung 1992). Twenty-five males BALB/c mice (8–10 weeks old, 30–35 g) were used in this experiment. Mice were anesthetized with 2% isoflurane. A longitudinal incision was made at lower lumbar and sacral levels. Using small scissors, paraspinal muscles were isolated and removed from the L4 spine process to the sacrum. Using a small rounger, the L6 transverse process was removed as completely as possible. After visualization of ventral rami of the L4 and L5 spinal nerves, a piece of 6-0 silk thread was placed around the L5 spinal nerve and tightly ligated and cut immediately distal to the ligation to make sure all fibers were interrupted.

Evaluation of pain threshold

To record the effect of spinal nerve transection on pain threshold, five mice were subjected to the von Frey-test (Chaplan et al. 1994). Pain threshold tests

were performed on the hind paw preoperatively and on the paw on the lesioned side at 1, 3, 7, and 14 days after operation. To compare the pain threshold at the intact and injured side, both sides were tested at 5 days after operation. Unrestrained mice were placed beneath an inverted clear plastic chamber on a meshed metal surface and a series of von Frey filaments (Stoelting, IL, USA) with forces ranging from 0.4 mg to 28.8 g was applied to the plantar surface of the hind paws. Each von Frey filament test was performed five times at an interval of one to a few seconds. If paw-withdrawal due to stimulation was observed, it was registered as a response to a filament. Response threshold was defined as the lowest force of two or more consecutive von Frey filaments that produced a response (Chaplan et al. 1994).

ZnT3-immunohistochemistry ($ZnT3^{IHC}$)

Five control and five spinal nerve transected mice were used for ZnT3 immunostaining. Five days after operation mice were perfused with 4% paraformaldehyde. Then the spinal cord segments L4-6 were selected and postfixed in 30% sucrose and frozen in TissueTek by dry ice. Twenty µm thick cryo sections were cut and incubated for 2 days at 4°C with a ZnT3 antiserum (an affinity-purified polyclonal rabbit antibody specific against ZnT3 provided by R.D. Palmiter) for immunohistochemical localization. An avidin–biotin complex (ABC) method was used as detection system (ABC kit; DAKO, Glostrup, Denmark diluted 1:100 in TBS containing 3% goat serum). Following rinses in TBS containing Triton, the sections were incubated in biotinylated goat anti-rabbit IgG (diluted 1:200) for 1 h at room temperature (22°C). Sections were rinsed in TBS (pH 7.6) and incubated for 15 min in 0.025% 3,3′-diaminobenzidine (DAB) with 0.0033% H_2O_2. Stained sections were rinsed in TBS followed by alcohol dehydration and xylene clearance.

Autometallography (AMG)

Five controls and five spinal nerve transected mice were used for histochemical visualization of zinc. Five days after spinal nerve transection, zinc-selenium AMG staining was performed. Animals were injected sodium selenite (10 mg/kg, ip) under isoflurane anesthesia. One and a half-hours later the animals were sacrificed by transcardial perfusion with 3% glutaraldehyde (GA) solution. The spinal cord segments L4-6 were postfixed, cryoprotected by 30% sucrose immersion, and then 20 µm thick sections were cut which were mounted on slides rinsed with Farmer's solution (Danscher et al. 1985; Danscher and Stoltenberg 2005). The AMG silver enhancement was performed as previously described (Danscher 1982).

Optical density measurements

Five AMG stained sections from each of the lesioned and the control animals were placed in the microscope. Individual images of layers III–IV were captured at a fixed intensity of white light (tungsten) by a CCD camera, digitalized, stored, and used for quantification. The zones in the dorsal horn were digitized as follows: a square (200 × 100 μm^2) of dorsal white matter was used as a reference for the measurements of squares of the same size in gray matter layers III–IV. Optical Density was calculated conventionally (O.D. = ($\log_{10}$ [incident light/transmitted light])), with 'incident light' taken as the intensity of light transmitted through the zinc-free reference zone, and 'transmitted light' taken as the raw intensity reading for individual samples. The OD of sham operated animals was set as 100%. Zinc intensity from the lesioned side of operated animals was compared with non-lesioned site and with the controls.

Statistical analyses

All data are presented as mean ± SEM and data were analyzed by a two-tailed Student's *t*-test. $P < 0.05$ were considered statistically significant.

Results

Pain threshold is lowered after spinal nerve transection

Spinal nerve transection produced a significant reduction of mechanical pain thresholds from about 10 g to a level of about 2 g from 1 day after the surgery. This low level was observed throughout the

observation period, until 14 days after surgery (Fig. 1). Thus, this increased pain sensitivity started immediately after spinal nerve transection and continued for several weeks.

Zinc transporter 3 (ZnT3) immunoreactivity in spinal gray matter is decreased by spinal nerve transection

The $ZnT3^{IHC}$ stained sections revealed a characteristic laminar staining pattern (Fig. 2). In the dorsal horn more labeling was present in the superficial laminae (I, III–IV) than in the deeper laminae (V–VI). Five days after transection of the fifth lumbar spinal nerve the density of ZnT3-immunoreactivity was clearly reduced in the dorsal horn of fourth, fifth, and sixth lumbar segments (Fig. 2).

Vesicular zinc in spinal gray matter is decreased by spinal nerve transection

As we have previously shown (Jo et al. 2000), the AMG patterns were in particular dense in the gray matter of the mouse dorsal horn. More labeling was present in the superficial laminae (I, III–IV) than in the deeper laminae (V–VI). Lamina I contained two or three strands of AMG grains. Lamina II (substantia gelatinosa) appeared as the least stained area of the entire spinal gray. Laminae III and IV were densely stained and the staining were composed primarily of smaller AMG grains. Thus, the borders between laminae III or IV and the neighboring laminae II or V are well distinguishable. The intensity of vesicular zinc in Laminae III and IV 5 days after the spinal nerve transection was significant reduced in the lumbar segments (L4-6) (Fig. 3). The level of stainable zinc on the injured side was reduced 41% compared to the level of the controls. The intensity of zinc from the uninjured side was reduced, too, though only 5% compared to sham operated mice (Fig. 4).

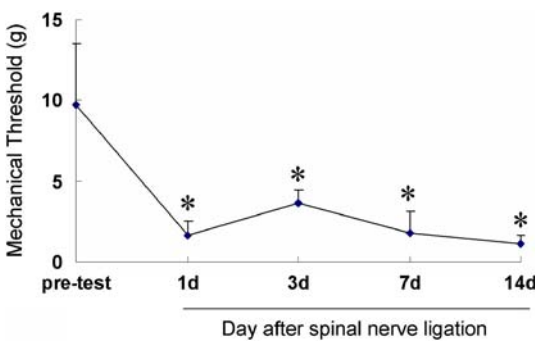

Fig. 1 Changes in pain threshold from the spinal nerve-ligated mice. The neuropathic pain was evaluated from the response to mechanical (von Frey hairs) stimuli at 0, 1, 3, 7, and 14 days after the transection. Total five mice were used in this experiment. Data are expressed as mean + SEM, and *asterisks* indicate values significantly different from corresponding values in the 'pretest' group

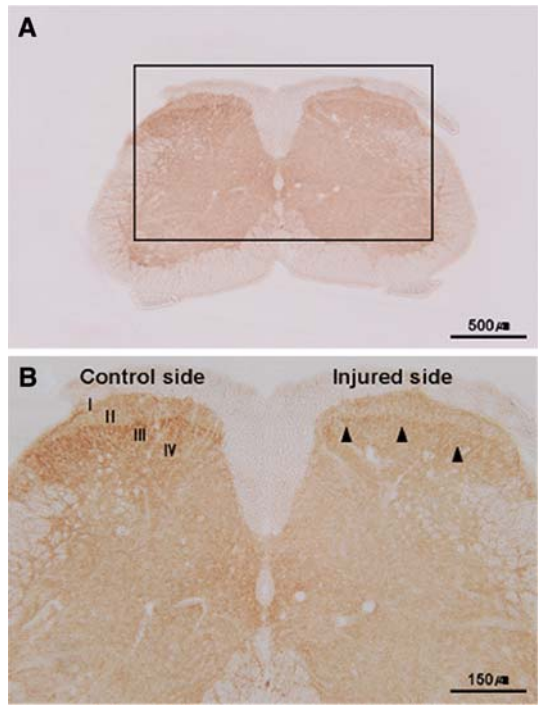

Fig. 2 Light micrograph image taken from $ZnT3^{IHC}$ stained-30 μm-thickness section of mouse spinal cord 5 days after unilateral spinal nerve transection. A decrease in $ZnT3^{IHC}$ stain density in the superficial dorsal horn is seen on the injured side (arrow heads) when compared to the contralateral side (Intact)

Zinc selenite precipitate in spinal ganglion increased by spinal nerve transection

Spinal ganglion cells vary in size from 15 to 100 μm. In general, these cells fall into two size groups. The smaller neurons are strongly stained with AMG, whereas the larger cells are almost unstained. All ganglion cells are surrounded by Schwann cells showing apparent AMG stainability. When studied 5 days after the spinal nerve transection (Fig. 5) AMG

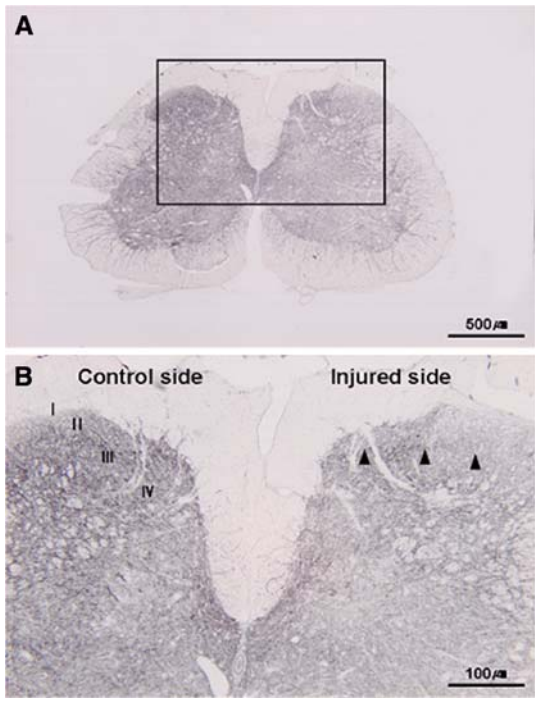

Fig. 3 Light micrograph image taken from $ZnSe^{AMG}$ stained L5 lumbar spinal segment at 5 days after unilateral spinal nerve transection. Apparent decrease in the staining density is seen on the injured side (arrow heads) compared to the intact side

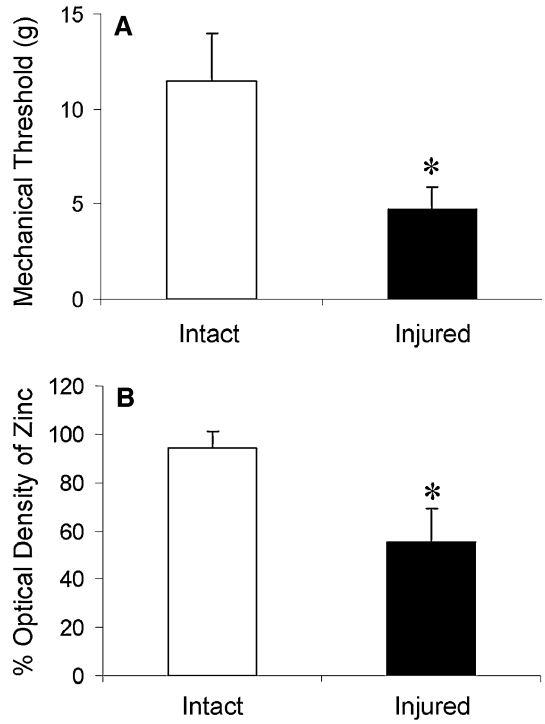

Fig. 4 *Graphs* represent reduced pain threshold and reduced zinc density after fifth lumbar spinal never transection at 5 days after spinal never transection. **A** The neuropathic pain was evaluated from the response to mechanical (von Frey hairs) stimuli. The mechanical threshold of the injured paw was significantly lower than intact side. **B** Vesicular zinc intensity was significantly decreased in injured side of superficial dorsal horn (laminae III–IV) at 5 days after spinal nerve transection. *Graph* represents mean + SEM. *Asterisk* denotes difference from intact side of dorsal horn at $P < 0.05$

granules were increased in the smaller ganglion cells and granules became visible in large ganglion cells.

Discussion

Zinc-enriched terminals form a well-organized pattern in the gray matter of the mouse spinal cord. The superficial dorsal horn (laminae I, III, IV) and lamina X, involved in sensory transmission, contain relatively high concentrations of ZEN terminals (Jo et al. 2000). A high percentage of the ZEN terminals in the spinal cord of rodents are GABAergic while only a small fraction being glutamatergic (Schroder et al. 2000; Danscher et al. 2001; Wang et al. 2001a). This is different from the neocortex and retina, where glutamatergic ZEN terminals are dominant (Beaulieu et al. 1992). This histological distribution of ZEN terminals identified by ZnT3 and AMG detectable zinc suggests that zinc ions may be involved in neurotransmission or long-term potentiation in the spinal cord (Ma and Zhao 2001). Biochemical and physiological studies suggest that zinc ions might be involved in sensory transmission at the spinal cord level (Larson and Kitto 1997, 1999), and demonstration of the presynaptic localization of ZEN terminals on dendrites and somata in the motor nuclei of the ventral horn indicate that ZEN terminals are also involved in motor control (Schroder 1979). Several electrophysiological studies have shown that Zn^{2+} acts on some ligand- and voltage-gated ion channels to modulate excitability of peripheral and central neurons (Busselberg et al. 1992; Li et al. 1997; Gingrich and Burkat 1998; Choi and Lipton 1999). Zn^{2+} released from hippocampal mossy fiber terminals inhibits activation of NMDA receptors in a high-affinity voltage-independent and a low-affinity voltage-dependent manner (Paoletti et al. 1997; Choi and Lipton 1999). Exogenous and endogenous Zn^{2+}

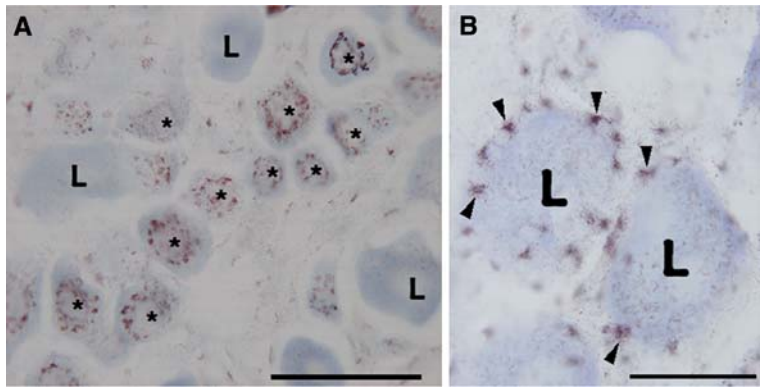

Fig. 5 Light micrographic images taken from rat spinal ganglion after AMG staining. **A** Represents an image from uninjured spinal ganglion and **B** represents an image from a spinal ganglion 5 days after the spinal nerve transection. **A** Shows that the smaller neurons (*asterisks*) are strongly stained but the larger cells (*L*) is not stained with AMG granules. **B** Shows that two large ganglion cells (*L*) have apparently AMG granules in the cytoplasm (*arrowheads*) after spinal cord transection. *Bars* in **B** and **B** indicate 100 and 50 μm, respectively

prevented LTP in either CA1 or CA3 regions of the hippocampus (Quinta-Ferreira and Matias 2005; Xie and Smart 1994; Vogt et al. 2000). Therefore, Zn^{2+} has been considered an important modulator of NMDA receptor function (Peters et al. 1987; Westbrook and Mayer 1987). A recent study on the spinal cord showed that Zn^{2+} might contribute to the modulation of the NMDA receptor-mediated spinal LTP, too (Ma and Zhao 2001). Furthermore, Zn^{2+} reduced inflammatory or neuropathic hyperalgesia, a function which was possibly mediated by inhibiting NMDA receptors or by altering the conformation of nerve growth factor (NGF) in the spinal dorsal horn (Safieh-Garabedian et al. 1996; Larson and Kitto 1997, 1999; Liu et al. 1999; Larson et al. 2000). Evidence thus exists that the inhibitory effect of Zn^{2+} on NMDA receptors may occur also in the spinal cord. Recently, Takeda et al. published interesting results using in vivo microdialysis. Glutamate concentration in the perfusate was significantly decreased by perfusion with $ZnCl_2$, suggesting that presynaptic release of glutamate is inhibited by zinc in the hippocampus. In contrast GABA concentrations in the perfusate were increased by the perfusion with zinc (Takeda et al. 2004). Dietary deficiency of zinc appeared to have the opposite effect (Takeda et al. 2003). Taken together, these studies suggest that Zn^{2+} may be a modulator of NMDA receptors and alter glutamate and GABA release in the central nervous system. This suggests that reduced concentration of zinc ion in the spinal cord, e.g., due to a decrease in vesicular zinc available for release in the synaptic clefts, could increase NMDA receptor sensitivity, increase glutamate excitation, and reduce inhibition from GABA. In the dorsal horn the result expected would be enhancement of sensory input. The present finding of development of neuropathic pain parallel to a depletion of vesicular zinc in ZEN terminals in the dorsal horn fits with this.

Concerning the origin of the dorsal horn ZEN terminals some is known. In the present study, it is shown that lesion of the peripheral branch of the spinal ganglion axons leads to accumulation of zinc in the somata. This accumulation might result from a generally compromised transport of zinc in the ganglion cells and thus affect the central branch in the spinal cord, too. Previously we have shown that a dorsal horn ZEN interneuron system exits (Wang et al. 2001b). These neurons are due to their localization supposed to be involved in sensory transmission. A change in sensory input could therefore influence the zinc levels in the terminals of these interneurons, a reduced sensory input due to spinal nerve lesion could result in a compensatory increased spinal activity provided by a trans-neuronal effect on the ZEN neurons leading to a decreased zinc level. The spinal ZEN neurons project both ipsi- and contra-laterally which could explain the bilateral effect of the spinal nerve lesions.

The present study, investigated alterations in vesicular zinc concentration after spinal nerve

transection. Spinal nerve transection induced significant loss of ZnT3 immunoreactivity and zinc as demonstrated by the AMG method in nerve terminals. Parallel to the reduction in zinc the spinal nerve transected mice developed a lower pain threshold. Therefore, the present results suggest that zinc ions might be involved in modulation of nociception in the spinal cord, and that chronic depletion of zinc due to spinal injury may contribute to pathological hyperalgesia.

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