Manipulations of Zinc in the Spinal Cord, by Intrathecal Injection of Zinc Chloride, Disodium-Calcium-EDTA, or Dipicolinic Acid, Alter Nociceptive Activity in Mice

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Accepted for publication May 28, 1997

ABSTRACT

Zinc is concentrated in the dorsal horn of the spinal cord and has been proposed to alter excitability of primary afferent C-fibers, structures believed to be important in nociceptive transmission. Based on the inhibitory effect of zinc on the activity of various other neurotransmitters that play a role in nociception, we tested the hypothesis that zinc modulates pain transmission. To test this, we examined the effect of exogenous zinc, administered intrathecally (i.t.), on nociception in the mouse. We also assessed the impact of decreased concentrations of endogenously occurring zinc in the extracellular fluid brought about by an i.t. injection of either ethylenediaminetetraacetic acid disodium-calcium salt (Ca\(^{++}\)EDTA), a calcium-saturated, membrane-impermeable chelator of divalent cations, or of dipicolinic acid, a zinc chelator. Injection of zinc produced a dose-related antinociceptive effect, optimal at 90 min in the writhing assay, but had no effect on tail-flick response latencies. In contrast, injection of either Ca\(^{++}\)EDTA or dipicolinic acid produced a dose-related hyperalgesia in the tail-flick assay at 90 min after injection. Responses induced in the writhing assay were unaffected by Ca\(^{++}\)EDTA. Although zinc had no effect on thermal nociception, the hyperalgesic effect of Ca\(^{++}\)EDTA was antagonized by coadministration of Ca\(^{++}\)EDTA with zinc. Similarly, the antinociceptive effect of zinc on writhing responses was attenuated when coadministered with Ca\(^{++}\)EDTA. Zinc also inhibited primary afferent C-fiber activity because 10 ng of zinc i.t. inhibited the behavioral response induced by injection i.t. of 1 nmol of capsaicin. Neither zinc nor Ca\(^{++}\)EDTA altered writhing or tail-flick latencies, respectively, when injected intracerebroventricularly. These findings support the hypothesis that endogenous zinc, localized in the dorsal horn of the spinal cord, plays a role in the regulation of pain.

Zinc is a molecular building block and structural component of more than 50 enzymes found throughout the body (Vallee and Galdes, 1984). It is also involved in multiple biochemical processes via proteins with which it associates (Frederickson, 1989). Zinc is abundant in the CNS where it exists in three general pools: vesicular, free and protein bound. Vesicular zinc is found in presynaptic vesicles of a special class of zinc-containing neurons (Frederickson and Danscher, 1988), which suggests a specific neurosecretory role in certain CNS neurons. Although vesicular zinc constitutes only a small proportion of the total zinc in the CNS, this pool can be selectively stained histochemically in the neuropil because it appears to be concentrated in synaptic vesicles in axonal terminals.

Zinc-containing neurons are defined as neurons that sequester histochemically reactive zinc in their axonal boutons. The concentration of zinc in these vesicles is estimated to be 200 to 300 \(\mu\)M (Frederickson, 1989). Zinc in boutons can be visualized by various histochemical stains. Silver-amplification methods, for example, Timm-Danscher sulfide-silver and selenium silver, show ultrastructural localization of zinc in boutons (Danscher, 1982, 1984). Although there is a possibility of labeling other metals by this technique, the distribution of stain compares well with the technique that uses more selective, yet less sensitive methods. Dithizone, for example (Frederickson and Howell, 1984), is more specific for zinc but has low sensitivity and resolution, which makes it less useful to detect decreases in zinc content N-(6-methoxy-8-quinolyl)-p-toluene-sulfonamide (TSQ), which is selective for zinc in the presence of physiological concentrations of calcium and magnesium (Frederickson et al., 1987), is also used to visualize zinc (Frederickson et al., 1987; Howell et al., 1991; Mandava et al., 1993).

In addition to active transport, many channels, such as those linked to NMDA and KA receptors, allow influx of zinc.

ABBREVIATIONS: Ca\(^{++}\)EDTA, ethylenediaminetetraacetic acid disodium-calcium salt; CNS, central nervous system; EAA, excitatory amino acid; ECF, extracellular fluid; KA, kainic acid; i.c.v., intracerebroventricular; i.t., intrathecal; MT, metallothionein; NMDA, N-methyl-D-aspartate; DMSO, dimethyl sulfoxide; TSQ, N-(6-methoxy-8-quinolyl)-p-toluene-sulfonamide.
(Yin and Weiss, 1995). Consistent with this, zinc has been found to be highly colocalized with EAAs in the hippocampus (McGinty et al., 1984). Zinc that is presumed to originate from the vesicular pool has been released from mossy fibers in hippocampal tissue in a calcium-dependent manner in response to either electrical stimulation of granule neurons (Howell et al., 1984) or depolarization with potassium or KA (Assaf and Chung, 1984). Thus, zinc has been shown to be taken up into boutons by high-affinity uptake, sequestered in vesicles and released by exocytosis of the zinc-filled vesicles (Frederickson, 1989).

Although mossy fiber axons of the hippocampus are the most thoroughly studied of zinc-containing fiber systems (Frederickson, 1989), there is histological evidence that zincergic neurons may also be localized in the spinal cord. Injection of sodium selenite is a method of defining zinc-containing systems in the CNS (Frederickson, 1989). Within 2 hr of injection, there is strong selenium stain in the neuropil of the dorsal spinal cord (Danscher, 1982), an area known to be important in sensory processing in general, and pain transmission specifically. This dense selenium stain in the dorsal horn is consistent with similar findings with TSQ and Timm staining and may thus reflect a releasable pool of zinc in the spinal cord.

If zinc is localized along pain-relevant systems, it may contribute to changes in pain transmission in a fashion similar to its proposed modulatory role in the hippocampus. In support of a role for zinc in pain transmission, primary afferent C-fibers have been found to be sensitized in zinc-deficient rats (Izumi et al., 1995). Spontaneous pruritis, a sensation transmitted by primary afferent C-fibers, is common in patients whose plasma zinc level is lowered by repeated hemodialysis (Gilchrest et al., 1982; Stahle-Backdahl, 1989; Stahle-Backdahl et al., 1988). NMDA and KA receptors are both located on primary afferent C-fiber terminals innervating the superficial spinal cord (Liu et al., 1994; Sato et al., 1993), which provides a possible mechanism for accumulation of zinc in small fibers. Like zinc-containing neurons in the hippocampus, primary afferent C-fibers are known to contain EAAs like aspartate and glutamate (DeBiasi and Rustioni, 1988; Tracy et al., 1991; Wanaka et al., 1987) which appear to play a role in the spinal mediation of nociception (King and Lopez-Garcia, 1993; Aanonsen et al., 1990; Raigorodsky and Urca, 1990; Urca and Raigorodsky, 1990; Cotman et al., 1987). Nocuous stimulation induces release of EAAs in vivo (Skilling et al., 1988), whereas antagonists of both NMDA and non-NMDA type EAA receptors inhibit nociception (Näström et al., 1992).

If zinc, presumed to be localized in the spinal cord, plays a role that is redundant with that in the hippocampus, one might speculate that zinc may modulate the transmission of pain when released into the spinal cord area. To test the hypothesis that zinc originating from this area modulates pain transmission, we examined the effect of increased and decreased availability of zinc in the CNS on chemical and thermal nociception in mice. Because the concentration of zinc in the CNS is not readily affected by changes in the availability of zinc in the periphery unless a state of deficiency or excess is produced, the blood-brain barrier was bypassed by injecting zinc chloride i.t. Nociception was tested by use of the writhing assay, which measures the abdominal contractions induced by acetic acid injected intraperitoneally, and the tail-flick assay, which measures the latency of an animal’s response to immersion of the tail in a water bath maintained at 53°C. Decreases in the concentration of zinc in the ECF were similarly produced by an i.t. injection of Ca²⁺-EDTA, a membrane-impermeable chelator of divalent cations, or by injection of dipicolinic acid, a cell-impermeant chelator of zinc.

Materials and Methods

Animals. Male Swiss-Webster mice (20–25 g, Charles River Lab, Portage, MI) were housed 4 per cage and allowed to acclimate for at least 24 hr before use. Mice were allowed free access to food and water. Animals were used strictly in accordance with the Guidelines of the University of Minnesota Animal Care and Use Committee and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [DHEW Publication (NIH) 78–23, revised 1978].

Drug administration. Except where indicated, all injections were made intrathecally in mice at approximately the L5-L6 intervertebral space with use of a 30-gauge, 0.5-inch disposable needle on a 50-μl Luer tip Hamilton syringe. A volume of 5 μl was used for all i.t. injections. Throughout the studies, zinc chloride and Ca²⁺-EDTA were each administered i.t. in saline. Dipicolinic acid was dissolved in 5% DMSO in acidified saline (pH 3.5) because this compound requires an acidified environment to chelate zinc. Control groups were injected with an equivalent volume of each respective vehicle. Capsaicin used for i.t. injection was dissolved (2.6 nmol/5 μl) first in DMSO and diluted with saline to a final concentration of 5% DMSO by volume.

Antinociceptive testing. The abdominal stretch, or writhing assay, was performed by injecting 0.3 ml of 1.0% acetic acid intraperitoneally in manually restrained mice. Immediately after injection, animals were placed in a large glass cylinder containing approximately 2 cm of bedding. The number of abdominal stretches occurring in a 5-min interval was counted beginning 5 min after acetic acid. Values are reported as the mean (± S.E.M.) for each treatment with groups composed of at least eight mice. Treatments that produced a significant decrease in the number of abdominal stretches were considered to be antinociceptive. Mice were sacrificed immediately after testing.

The latency of the tail-flick response to a thermal stimulus was determined by the tail immersion or tail-flick assay. Mice were gently restrained and the tail submerged in the water of a bath maintained at 53°C. A cutoff of 12 sec was used to avoid tissue damage. The mean difference in the latency of response for mice in each group of at least six mice was calculated (postinjection latency of response minus preinjection control latency) and compared with values from saline-injected control mice tested on the same day. Control mice typically responded by the withdrawal of their tail with a latency of response that averaged 4.28 sec over the course of the experiments. Differences in latencies of groups that were significantly less than control values were considered to be hyperalgesic.

Drugs. Dipicolinic acid was purchased from Molecular Probes (Eugene, OR). Ca²⁺-EDTA, zinc chloride and capsaicin were purchased from Sigma Chemical Company (St. Louis, MO). Calcium-EDTA was chosen as an appropriate chelator of zinc for two reasons. First, the membrane-impermeable nature of Ca²⁺-EDTA ensures that it chelates only divalent cations in the extracellular space rather than protein-bound zinc that is necessary for structural purposes or enzymatic activity. Second, EDTA saturated with calcium is used widely as a selective chelator of zinc. Although a variety of trace elements are found in the body, most are associated with protein complexes and located intracellularly. Of all the divalent cations found endogenously, zinc is the only one present in relatively high concentrations both intracellularly as well as in the ECF. Thus, zinc...
is the only divalent cation in the ECF with an affinity that would compete with calcium for the EDTA divalent cation binding site.

Data analysis. Mean differences (± S.E.M.) are presented in the figures. Throughout the experiments, each group represents at least six mice. Statistical analysis of the tail-flick latency results was performed by ANOVA followed by the Scheffé F-test for multiple comparisons. Results from writhing and from capsaicin-induced biting and scratching experiments were analyzed by Kruskal-Wallis nonparametric analysis. In both cases, P values less than .05 were used to indicate a significant difference. Means of the test groups were routinely compared with control values collected the same day.

Results

An i.t. injection of 10 ng of zinc chloride produced an antinociceptive effect in the writhing assay that was optimal at 90 min (fig. 1). The antinociceptive effect of zinc at this time was dose related (fig. 2) such that a dose as small as 0.1 ng of zinc produced a significant inhibition of abdominal stretch behaviors and 30 ng blocked writhing completely. On the other hand, a dose of zinc (10 ng) that inhibited writhing behaviors by about 50% had no effect on tail-flick latencies at 53°C (fig. 3B).

Although increasing the availability of zinc had no effect on tail-flick latencies, injection of Ca<sup>2+</sup>EDTA, which is presumed to chelate endogenous zinc in the ECF making it unavailable for biological activity, produced a dose-related hyperalgesia (fig. 4) that was optimal 90 min after injection of Ca<sup>2+</sup>EDTA (fig. 3A). When tested at times and doses that elicited thermal hyperalgesia, Ca<sup>2+</sup>EDTA had no affect on the number of behaviors induced in the writhing assay (fig. 2).

Experiments were also designed to confirm the ability of Ca<sup>2+</sup>EDTA to chelate zinc and negate its biological effect, as well as the ability of zinc to overcome the effects of Ca<sup>2+</sup>EDTA. We tested the ability of zinc, which is inactive in the tail-flick assay, to reverse the hyperalgesic effect of Ca<sup>2+</sup>EDTA and the ability of Ca<sup>2+</sup>EDTA, which is inactive in the writhing assay, to reverse the antinociceptive effect of zinc. As indicated in figures 5 and 6, the effect of each compound was inhibited by coadministration with the other when compared with the effects of zinc only (fig. 5) or to the effect of Ca<sup>2+</sup>EDTA only (fig. 6) at the same dose and on the same day.

The slow onset of action (90 min) of zinc, Ca<sup>2+</sup>EDTA and dipicolinic acid suggested that the site of action of these compounds might be in the brain rather than the spinal cord. To examine this possibility we injected mice i.c.v. with a dose of 10 ng of zinc that is antinociceptive in the writhing assay when injected i.t., and with a dose of 10 nmol of Ca<sup>2+</sup>EDTA, which is hyperalgesic in the tail-flick assay when injected i.t. Ten nanograms of zinc injected i.c.v. failed to inhibit the number of writhing responses in mice when examined at 30, 90 or 180 min after injection compared with their respective saline-injected control groups. Compared with saline-injected control groups, injection of 10 nmol of Ca<sup>2+</sup>EDTA also failed to alter the tail-flick latency when tested 30, 60 and 180 min after injection, times that would encompass its hyperalgesic effect when injected i.t.

We also tested dipicolinic acid, a zinc chelator that is structurally distinct from Ca<sup>2+</sup>EDTA and has a greater selectivity for zinc than Ca<sup>2+</sup>EDTA. Injection of 0.01 to 3 nmol of dipicolinic acid i.t. in mice 90 min before testing in the tail-flick assay resulted in a dose-related hyperalgesia (fig. 7). Dipicolinic acid must be dissolved in an acidified solution to activate its zinc-chelating activity. Consistent with this, dissolution of 1 nmol of dipicolinic acid in normal saline (pH 7) rather than acidified saline failed to induce hyperalgesia, which indicates that zinc chelation, rather than other pharmacological actions induced by the structure of dipicolinic acid, is responsible for hyperalgesia.

To determine the influence of zinc on primary afferent C-fiber activity, structures believed to play an important role in nociceptive transmission, we examined the ability of zinc and of Ca<sup>2+</sup>EDTA to affect the behavioral biting and scratching response to an i.t. injection of 1 nmol of capsaicin, a compound that depolarizes primary afferent C-fibers. Consistent with its antinociceptive effect, pretreatment with 10
ng of zinc inhibited the mean number of biting and scratching behavioral responses during a 2-min interval after injection of 1 nmol of capsaicin (25.3 ± 3.3 S.E.M.) when compared with the number of capsaicin-induced behaviors in vehicle-pretreated control mice (42.8 ± 4.2 S.E.M.). When tested in an identical fashion, 10 nmol of Ca\(^{11}\)EDTA had no affect on the mean number of capsaicin-induced biting and scratching behaviors (40.3 ± 3.4).

To determine whether zinc merely duplicates the effect of another commonly occurring divalent cation, we tested the effect of 10 ng of calcium chloride injected i.t. 90 min before testing. Treatment with calcium failed to alter the tail-flick latency and the number of writhing behaviors. This dose of calcium also failed to mimic the ability of zinc to reverse the hyperalgesic effect of Ca\(^{11}\)EDTA.

**Discussion**

To test the hypothesis that zinc, originating from the dorsal spinal cord, plays a role in the modulation of pain trans-
mission, we examined the effect of increased and decreased availability of zinc in the spinal cord on chemical and thermal nociception in mice. Intrathecal injections of zinc appeared to inhibit chemical nociception, as measured by the writhing assay. However, when tested at times and doses that were found to attenuate acetic acid-induced writhing, zinc had no effect on thermal nociception measured by the tail-flick assay. Thus, if zinc localized in the neuropil of the dorsal spinal cord is released in response to depolarization, as previously observed in the hippocampus (Assaf and Chung, 1984; Frederickson, 1989; Howell et al., 1984), one would expect that one physiological action of this pool of zinc might be to inhibit chemical nociceptive transmission.

Although increasing the availability of zinc in the spinal cord area had no effect on thermal nociception, decreases in the concentration of zinc in the ECF, that are presumed to occur after an i.t. injection of Ca"EDTA or dipicolinic acid, produced hyperalgesia in the tail-flick assay, as indicated by decreased latencies of response to noxious thermal stimulation at 53°C. When tested at times and doses that elicited thermal hyperalgesia, Ca"EDTA had no effect on writhing behaviors. These data suggest that an endogenously occurring divalent cation, such as zinc, localized in the ECF, is important in normal thermal nociceptive responses.

Although sodium EDTA has a high affinity for calcium and magnesium, once saturated with calcium, the only cations with which it would be predicted to bind would be cobalt, cesium, copper, nickel, lead and zinc. Of these, only zinc is found in abundance in the normal CNS. Most zinc and various divalent cations present in the CNS are localized intracellularly where they are protein bound and serve biochemical and structural functions, unavailable for chelation with Ca"EDTA administered i.t. Zinc released from a histochemically reactive pool is the only divalent cation that would be predicted to be found in the ECF, based on the proposed role of zinc as a neuromodulator that is released from zinc-containing neurons. Based on the selectivity of Ca"EDTA for a limited group of divalent cations and its impermeability across cell membranes, it is widely used as a selective chelator of zinc in the ECF to study the contribution of zinc on receptor activity (Westergaard et al., 1995), on transmitter release (Wang and Quastel, 1990) and during excitotoxicity induced by EAAs (Frederickson et al., 1989). The ability of Ca"EDTA to chelate zinc when injected in vivo has been previously demonstrated by the ability of 500 nmol of Ca"EDTA, injected i.c.v. in rats, to protect against zinc translocation and neuronal death associated with transient global ischemia (Gwag et al., 1995). Based on this evidence, we presume that the action of i.t. administered Ca"EDTA in the present study is to chelate zinc making it unavailable for neuromodulation. Consistent with this, injection of dipicolinic acid, a cell-impermeant zinc chelator, also induced hyperalgesia 90 min after its injection i.t., which supports the action of endogenous zinc in thermal nociceptive transmission.

The effect produced by changing the concentration of zinc appears to depend on the nociceptive pathway tested. Because thermal hyperalgesia is produced by decreasing the concentration of zinc in the ECF whereas chemical antinociception results from increasing the availability of zinc, the resting concentration of endogenous zinc in the ECF appears to be necessary for normal thermal nociceptive response latencies, but insufficient to affect chemical nociceptive responses. The difference in the sensitivity of these two models of nociception to the effect of zinc suggests that there are two distinct mechanisms responsible for the chemical antinociceptive and thermal antihyperalgesic effects of zinc.

Although these data are consistent with the hypothesis that zinc plays a role in the modulation of nociceptive transmission, the mechanisms of the antinociceptive and antihyperalgesic effects of zinc are not known. The ability of zinc, injected i.t., to inhibit capsaicin-induced biting and scratching behaviors is consistent with a general stabilization of primary afferent C-fibers previously proposed by Izumi et al. (1995). This conclusion was reached by Izumi’s group based on the tendency for primary afferent C-fibers in zinc-deficient rats to be hypersensitive to stimulation. However, the concentration of zinc in the periphery does not usually reflect that in the brain, and vice versa, until relatively extreme states of zinc deficiency or excess. Thus the increased sensitivity of primary afferent C-fibers observed in their studies may reflect changes in the concentration of zinc along peripheral afferent processes, whereas our manipulations would be expected to affect a central pool. This difference in experimental design may explain the failure of Ca"EDTA, injected i.t., to alter the depolarizing effect of capsaicin. Based on the work by Izumi et al. (1995), one would expect Ca"EDTA to produce a local zinc deficiency and increase C-fiber responsivity. Although Ca"EDTA did cause thermal hyperalgesia, the inability of Ca"EDTA to affect capsaicin-induced responses indicates that the concentration of endogenously occurring zinc in the ECF is not sufficient to tonically inhibit primary afferent C-fibers. However, based on the sensitivity to zinc of both acetic acid- and capsaicin-induced behaviors, higher concentrations of zinc may stabilize primary afferent C-fibers.

The selective localization of zinc in the dorsal spinal cord suggests a physiological role at the spinal cord level. Our studies establish that changes in the availability of zinc that originate at the spinal cord level can impact on nociception. Although 90 min is a sufficient interval for compounds to diffuse from an i.t. injection to the brain, effects produced by
i.t. injections of zinc or Ca\(^{2+}\)EDTA are not likely brought about by actions in the brain, because i.c.v. injections of these compounds failed to alter nociception. There are several mechanisms by which zinc may modulate pain transmission within the spinal cord itself. In addition to stabilization of primary afferent C-fibers, as discussed above, antinociception may result from zinc's ability to modulate transmitter activity at receptors believed to be important in nociception. EAs, like glutamate and aspartate, are released during nociception (Skilling \et al., 1988; Sorkin \et al., 1992). Zinc has been postulated to be co-released with glutamate at central synapses in response to potassium, electrical stimulation or seizure activity (Assaf and Chung, 1984; Howell \et al., 1984; Aniksztejn \et al., 1987; Frederickson \et al., 1988; Sloviter, 1985; Frederickson, 1989). Whether a similar co-release occurs in the spinal cord is not known. EAs are presumed to activate NMDA and non-NMDA type EAA receptors involved in nociception (Cordero and Melzack, 1992; Haley \et al., 1990). Zinc is able to decrease NMDA activity (reviewed by Smart \et al., 1994) believed to play a role in hyperalgesia. Ion channels gated by NMDA receptors are antagonized by zinc (Peters \et al., 1987) at a site distinct from that of magnesium (Westbrook and Mayer, 1987). Zinc noncompetitively prevents glycine binding on the NMDA receptor complex, thus attenuating glycine-dependent activation of the NMDA receptor-ion channel complex (Yeh \et al., 1990). Thus, one mechanism by which zinc in the ECF may induce antinociception is by inhibiting NMDA receptors. On the other hand, decreases in zinc in the ECF, such as after injection of Ca\(^{2+}\)EDTA, may attenuate the inhibitory influence of zinc on NMDA receptor activity and thereby lead to hyperalgesia. One might speculate that NMDA receptors located on primary afferent C-fiber terminals (Liu \et al., 1994) may underlie the ability of zinc to alter primary afferent C-fiber excitability.

Regulation of zinc involves MT, a protein that is postulated to play a physiological role in the transport and storage of zinc (Brenner, 1987), as reviewed by Ebadi \et al., (1995). MT has a high affinity for zinc and is typically co-localized with zinc. MT-I and MT-II have been localized immunohistochemically in glial cells, ependyma and pia mater in the brains of rats and mice (Nishimura \et al., 1992; Nakajima and Suzuki, 1995; Hao \et al., 1994). The concentration of MT-I mRNA is higher in the spinal cord than in any other area of the mouse brain (Kinningham \et al., 1995). MT-III, originally referred to as growth inhibitory factor (Kobayashi \et al., 1993), is found only in the CNS where it is highly concentrated in the cell bodies of neurons whose processes sequester zinc in synaptic vesicles (Palmiter \et al., 1992; Masters \et al., 1994). Transfection experiments suggest that MT-III participates in the utilization of zinc as a neuromodulator by enhancing the sequestration of zinc into histologically reactive pools (Erickson \et al., 1995). The concentration of MT-III mRNA in the spinal cord is second only to the hippocampus (Masters \et al., 1994), which supports the localization of neurons in the spinal cord that contain a releasable pool of zinc. Zinc binding to MT depends on the oxidative state of the protein (Maret, 1994). Thus compounds like nitric oxide, found along nociceptive pathways and thought to play a role in the development of hyperalgesia (Meller \et al., 1992a,b), including hyperalgesia after an i.t. injection of NMDA (Kitto \et al., 1992), might do so by influencing zinc binding to MT.

Our data, together with the literature, support the existence of a releasable pool of zinc in the spinal cord. The inhibitory effect of zinc on chemical nociception and the production of thermal hyperalgesia after sequestration of zinc by use of Ca\(^{2+}\)EDTA are consistent with modulation of nociception by endogenously occurring zinc, perhaps by stabilization of primary afferent C-fibers or an inhibitory effect on NMDA receptors involved in nociceptive transmission.

**References**


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