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Intrathecal Zn^{2+} attenuates morphine antinociception and the development of acute tolerance

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Abstract

Vesicular Zn^{2+}, released in the brain and from small dorsal root ganglion neurons, interacts with opioid as well as *N*-methyl-D-aspartate (NMDA) receptors. We investigated the effect of Zn^{2+} on morphine antinociception in mice (tail flick assay), as well as acute tolerance and dependence, phenomena associated with NMDA activity. Administered intrathecally (i.t.), Zn^{2+} inhibited morphine antinociception in a dose-related fashion. Zn^{2+} also inhibited acute tolerance to morphine antinociception (5 h after 100 mg/kg of morphine). Injection i.t. of di-sodium calcium ethylenediamine tetra acetic acid (Na^+Ca^{2+} EDTA), a chelator of divalent cations, had no effect on analgesia, acute tolerance or acute dependence. However, withdrawal jumps produced by naloxone (1 mg/kg s.c.) in morphine-pellet implanted mice (3 days) were potentiated by injections twice daily of 10 nmol of Na^+Ca^{2+} EDTA, suggesting that endogenous Zn^{2+} tends to inhibit long-term development of withdrawal. These data suggest that the availability of Zn^{2+} is an important factor in opioid activity.

Keywords: Morphine; Zn^{2+}; Intrathecal; Antinociception; Tolerance; Dependence

1. Introduction

Zn^{2+} is an abundant and important divalent cation in the brain, involved in multiple biochemical processes via proteins with which it associates (Frederickson, 1989). Vesicular Zn^{2+}, estimated to be 200–300 μM, can be visualized histologically in many parts of the brain (Frederickson, 1989) and spinal cord (Danscher, 1982; Velázquez et al., 1999). Zn^{2+} is typically co-localized with excitatory amino acids (McGinty et al., 1984) in the mammalian central nervous system (CNS) (Schroder, 1979; Frederickson et al., 1982; Danscher, 1982). When released in response to depolarization (Howell et al., 1984; Assaf and Chung, 1984), Zn^{2+} is believed to modulate the activity of a variety of receptor populations. Physiological concentrations of Zn^{2+} inhibit [^{3}H]naloxone (Hannissian and Tejwani, 1988) and [^{3}H] [D-Ala^2, Met^5] enkephalinamide binding (Stengaard-Pedersen, 1982). Zn^{2+} also acts allosterically as a non-competitive antagonist at NMDA receptors (Westbrook and Mayer, 1987; Forsythe et al., 1988; Peters et al., 1987; Christine and Choi, 1990), an effect that is produced by even lower concentrations of Zn^{2+} in the spinal cord than in the brain (Kovács and Larson, 1997). Based on the importance of opioid receptors in narcotic analgesia and the mediation of tolerance and dependence by NMDA receptors (Trujillo and Akil, 1991; Elliott et al., 1994), we hypothesized that changes in the availability of Zn^{2+} influence opioid activity in vivo. In support of this possibility, dietary Zn^{2+} deficiency in mice decreases the antinociceptive effect of morphine (Dursun et al., 1995). This is clinically important as the concentration of Zn^{2+} in the cerebrospinal fluid (CSF) of ex-heroin addicts is significantly lower than that of controls (Potkin et al., 1982).

Because the blood–brain barrier prevents Zn^{2+} in the periphery from affecting its concentration in the CNS, we manipulated central Zn^{2+} concentrations using intrathecal (i.t.) injections of zinc chloride ($Zn^{2+}Cl_2^-$) or di-sodium calcium ethylenediamine tetra acetic acid (Na^+Ca^{2+}EDTA), a cell-impermeant compound known to chelate non-Ca^{2+}, divalent cations. Because the action of Na^+Ca^{2+} EDTA is restricted to the extracellular fluid

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(ECF) and because Zn^{2+} is the most prevalent divalent cation in this area, $Na^{+}Ca^{2+}$ EDTA decreases the concentration of Zn^{2+} in the extracellular area. This approach has been widely used to study the contribution of Zn^{2+} on receptor activity (Westergaard et al., 1995), transmitter release (Wang and Quastel, 1990), excitotoxicity (Frederickson et al., 1989) and nociceptive processing (Larson and Kitto, 1997). Increases in the latency of the tail flick response of mice were used to monitor opioid antinociceptive activity and acute tolerance to this effect was produced by a single injection of 100 mg/kg of morphine (Yano and Takemori, 1977). This high dose of morphine produces an initial antinociception and behavioral hyperactivity followed by a decreased antinociceptive response to subsequent injections of morphine. Dependence was studied by measuring the number of naloxone-induced withdrawal jumping behaviors produced in mice injected with 100 mg/kg of morphine or implanted s.c. 3 days previously with a morphine pellet.

2. Materials and methods

2.1. Animals

Male, Swiss Webster mice weighing 20–25 g were purchased from Charles River (Portage, MI) and housed four per cage on 12-h light–dark cycle. Mice were given free access to food and water. Mice were acclimated at least 72 h before experiments commenced. 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 1995].

2.2. Chemicals

Di-sodium calcium ethylenediamine tetra acetic acid ($Na^{+}Ca^{2+}$ EDTA), zinc chloride ($Zn^{2+}Cl_2^{-}$), and naloxone hydrochloride were obtained from Sigma (St. Louis, MO). Morphine sulfate and morphine base pellets were obtained from Mallinckrodt (St. Louis, MO).

2.3. Drug administration

All drugs were diluted to the correct concentration using 0.85% saline solution. A 1-ml disposable syringe with a 1/2-in., 27-gauge needle was used for intraperitoneal (i.p.) and subcutaneous (s.c.) injections. The 100 mg/kg dose of morphine sulfate was injected s.c. in manually restrained mice in a volume of 0.1 ml, as were the 20 and 1 mg/kg doses of naloxone. Morphine sulfate was administered i.p. at a dose of 10 mg/kg in a 0.1 ml volume. Zn^{2+} and $Na^{+}Ca^{2+}$ EDTA were each administered in a 5-μl volume, intrathecally (i.t.), in manually restrained mice using a 30-gauge disposable needle attached to a 50-μl Luer tip Hamilton syringe. This route was used for ease of injection centrally and because of the reported importance of the spinal cord in morphine tolerance development (Gutstein and Trujillo, 1993). Morphine pellets were implanted s.c. in ether-anesthetized mice.

2.4. Protocol for analgesia experiments

The tail flick assay was used to determine antinociceptive activity by manually restraining the mouse while submerging its tail in a waterbath maintained at 53°C. Withdrawal latency was defined as the time (s) for the mouse to withdraw its tail from the water bath. A cut-off time of 12 s was used to prevent tissue damage. In all experiments involving tail flick measurements, baseline latencies were measured at least three times, once per day on 3 consecutive days, before any treatment was started. After treatment, each mouse was tested once, control latencies were pooled and the mean control latency, standard deviation and standard error were calculated.

To measure the effects of Zn^{2+} and of $Na^{+}Ca^{2+}$ EDTA on morphine analgesia, 0.3, 1 and 10 ng Zn^{2+} or 1, 10 and 30 nmol of $Na^{+}Ca^{2+}$ EDTA was administered i.t. 1 h before i.p. injection of 10 mg/kg morphine. Doses of Zn^{2+} and $Na^{+}Ca^{2+}$ EDTA were based on those that have been found to induce antinociceptive effects in the writhing assay (Zn^{2+}) and hyperalgesia in the tail flick assay ($Na^{+}Ca^{2+}$ EDTA) when administered by this route (Larson and Kitto, 1997). One-half hour later, mice were tested for their tail flick latency. This pretreatment interval was used as Zn^{2+}, injected i.t., has been found to produce a maximal antinociceptive effect at 90 min when tested in the writhing assay, suggesting optimal availability along nociceptive pathways at this time (Larson and Kitto, 1997). Injection of Zn^{2+} alone, 90 min prior to testing, was found to have no effect on tail flick latencies while $Na^{+}Ca^{2+}$ EDTA injected i.t. is hyperalgesic at this time (Larson and Kitto, 1997).

2.5. Protocol for tolerance experiments

Zn^{2+}, in doses of 1, 3 and 10 ng, or $Na^{+}Ca^{2+}$ EDTA in doses of 1, 10 and 30 nmol, were administered i.t. 30 min before injection of 100 mg/kg of morphine, which was administered s.c. 4 h before tail flick measurements. One-half hour after the tail flick test, a challenge dose of 10 mg/kg of morphine was administered i.p. and followed 30 min later by a second tail flick test. Decreases in the antinociceptive effect of morphine, indicative of the development of acute tolerance, were determined by comparing the mean tail flick latency before the 10 mg/kg morphine challenge to that determined 30 min after the morphine challenge.

2.6. Protocol for dependence experiments

To determine the effect of Zn^{2+} on the development of acute dependence, doses of 1, 3 and 10 ng of Zn^{2+}, or doses of 1, 10 and 30 nmol of $Na^{+}Ca^{2+}$ EDTA was administered i.t. followed 30 min later by a s.c. injection of 100 mg/kg of morphine. Four hours later, the number of withdrawal jumps in response to injection (s.c.) of 20 mg/kg naloxone was monitored. To measure the effect of Zn^{2+} or $Na^{+}Ca^{2+}$ EDTA on chronic dependence, doses of 10 and 30 ng of Zn^{2+} or doses of 10 and 30 nmol of $Na^{+}Ca^{2+}$ EDTA were injected i.t. twice daily over a three-day period in animals implanted with a 75 mg morphine base pellet. On day 4, the number of withdrawal jumps in response to a 1 mg/kg dose of naloxone (s.c.) was measured. Doses of naloxone in each model were chosen to ensure an adequate number of behavioral responses in morphine pretreated control mice to monitor decreases or increases in these behaviors in test groups. Mice were observed in an 8-l clear plastic container over a 15-min interval beginning immediately after injection of naloxone. A jump was counted only when all four feet left the bedding.

2.7. Data analysis

In tail flick experiments, the mean latency of response (±S.E.M.) was calculated for each group before morphine challenge and then compared to the mean latency measured 30 min after morphine. In withdrawal experiments, the mean number of jumps (±S.E.M.) in response to a given dose of naloxone in groups pretreated with morphine only were compared to that in groups receiving no morphine and to groups receiving morphine plus either Zn^{2+} or $Na^{+}Ca^{2+}$ EDTA. Statistics were calculated using Macintosh StatView II with analysis of variance (ANOVA) followed by the Fischer test for statistical significance. *P* values for significance were set at ≤ 0.05.

3. Results

3.1. Effect of Zn^{2+} and $Na^{+}Ca^{2+}$ EDTA on morphine antinociception

Injection of 10 mg/kg of morphine in mice pretreated i.t. with saline 90 min prior to testing, produced an increase in tail flick latency, indicative of antinociception. Pretreatment with 0.3, 1 and 10 ng of Zn^{2+} 60 min prior to morphine (90 min prior to tail flick) resulted in a dose-related inhibition of the antinociceptive effect (Fig. 1). Injection of Zn^{2+} alone 90 min prior to testing was found to have no effect on tail flick latencies (data not shown), consistent with previous data from our laboratory (Larson and Kitto, 1997).

In contrast to the inhibitory effect of Zn^{2+} on morphine antinociception, removal of Zn^{2+} in the ECF by injection of 1, 10 and 30 nmol of the cell-impermeant chelator of divalent cations, $Na^{+}Ca^{2+}$ EDTA, had no effect on morphine analgesia (data not shown).

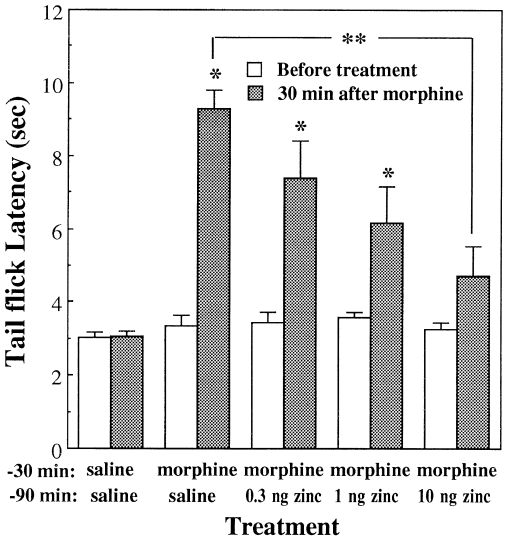

Fig. 1. Effect of Zn^{2+} on morphine analgesia in mice. Zn^{2+} was injected i.t. 60 min before an i.p. injection of 10 mg/kg of morphine sulfate. Thirty minutes after morphine, tail flick latencies were determined using a bath maintained at 53°C. Treatment of each group is indicated above relative to the time of the tail flick assay. Throughout the figures, values are expressed as the mean (±S.E.M.) derived from groups of a minimum of five mice per group. Statistical analysis was performed as described in Section 2. In all figures, a single asterisk represents values that are significantly different ($P < 0.05$) from saline-injected control values, whereas double asterisks indicate a significant difference ($P < 0.05$) between the groups indicated.

3.2. Effect of Zn^{2+} and $Na^{+}Ca^{2+}$ EDTA on morphine tolerance

The antinociceptive effect produced by 100 mg/kg of morphine was greatly attenuated 4 h after injection (Fig. 2). In addition, the antinociceptive effect produced 30 min after a challenge dose of 10 mg/kg of morphine was not observed in mice pretreated (4.5 h) with 100 mg/kg of morphine, consistent with acute tolerance originally described by Yano and Takemori (1977). These data document the acute development of tolerance to the antinociceptive effect of morphine. Using this model, we tested the influence of Zn^{2+} on the development of acute tolerance. When 1 or 3 ng of Zn^{2+} was administered 30 min before the 100 mg/kg dose of morphine, tail flick latencies 30 min after morphine (10 mg/kg) challenge (5 h after injection of Zn^{2+}) were increased compared to their pre-challenge control values. This indicates the preservation of morphine's antinociceptive effect and, thus, attenuation of morphine tolerance compared to mice pretreated with saline. In contrast, tail flick responses 4.5 h after a higher dose of Zn^{2+} (10 ng) were no different than those 4 h later, resulting in a U-shaped dose–response curve for the effect of Zn^{2+} on tolerance (Fig. 2).

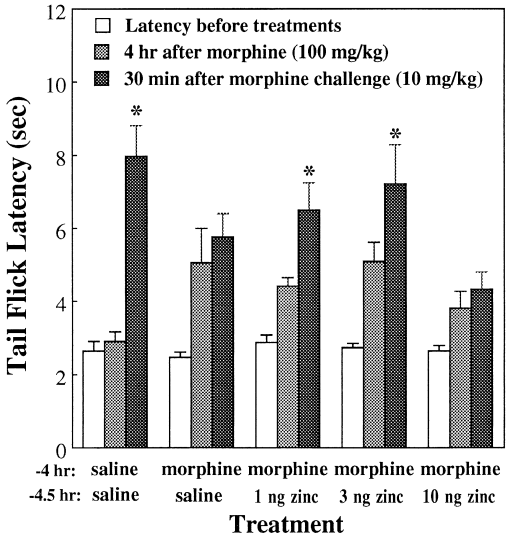

Fig. 2. Effect of Zn^{2+} on acute morphine tolerance. Zn^{2+} was injected i.t. 30 min before pretreatment s.c. with 100 mg/kg of morphine sulfate. Four hours later, tail flick latencies were determined and mice were immediately injected i.p. with a challenge dose of 10 mg/kg of morphine. Tail flick latencies were again determined 30 min after morphine challenge and compared to those values obtained immediately prior to morphine challenge. Treatments are indicated relative to the time of the final tail flick test. Data are expressed as indicated in Fig. 1. Asterisks indicate a significant difference between that latency and the one immediately preceding it within the same treatment group.

To determine whether the 10 ng dose of Zn^{2+} was sufficiently high to inhibit the antinociceptive effect of the morphine challenge, we measured antinociception produced 30 min after injection of morphine and 5.5 h after administration of 1, 3 and 10 ng of Zn^{2+} i.t. (Fig. 3).

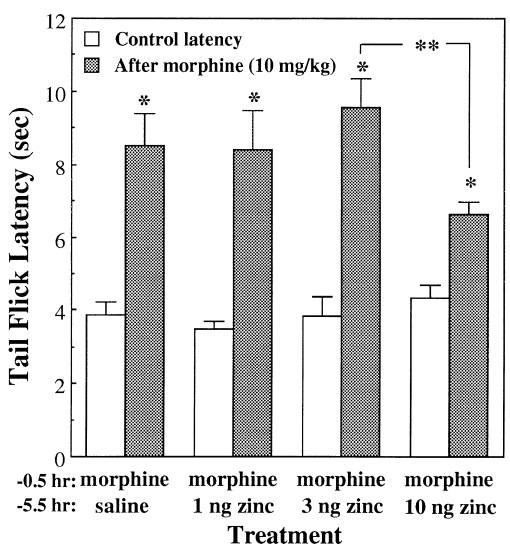

Fig. 3. Persistence of the inhibitory effect of Zn^{2+} on morphine analgesia. Zn^{2+} was administered i.t. 5 h before injection of 10 mg/kg of morphine s.c. The tail flick latency was determined 30 min later and compared to control values determine immediately prior to treatment. Groups were injected as indicated relative in time to the final tail flick assay. Data are expressed as indicated in Fig. 1.

Morphine-induced antinociception was not affected by doses of 1 or 3 ng of Zn^{2+} administered 5.5 h prior to testing. However, the antinociceptive effect of morphine was significantly less after injection of 10 ng of Zn^{2+} than after 3 ng, confirming that an inhibitory effect of Zn^{2+} on morphine-induced antinociception remains at this dose and time.

In contrast to the ability of Zn^{2+} to protect against the development of acute morphine tolerance, decreasing the availability of Zn^{2+} in the ECF by injection of 1, 10 and 30 nmol of $Na^{+}Ca^{2+}$ EDTA i.t. 30 min prior to 100 mg/kg of morphine had no effect on tolerance compared to groups pretreated with saline and morphine (data not shown).

3.3. Effect of Zn^{2+} and $Na^{+}Ca^{2+}$ EDTA on acute and chronic morphine dependence

In studies of acute dependence, injection of 20 mg/kg naloxone 4 h after 100 mg/kg morphine, produced withdrawal responses characteristic of acute dependence in mice (Trujillo and Akil, 1991). Zn^{2+} (1, 3 and 10 ng) and $Na^{+}Ca^{2+}$ EDTA (1 and 10 ng) failed to elicit any change in the number of withdrawal jumps when these compounds were administered i.t. 30 min prior to the 100 mg/kg dose of morphine (data not shown).

Control mice injected with 1 mg/kg of naloxone on the 4th day after implantation of a 75-mg morphine base pellet exhibited withdrawal jumping behavior characteristic of chronic morphine dependence in mice (Fig. 4). When animals were pretreated with 10 nmol of $Na^{+}Ca^{2+}$ EDTA

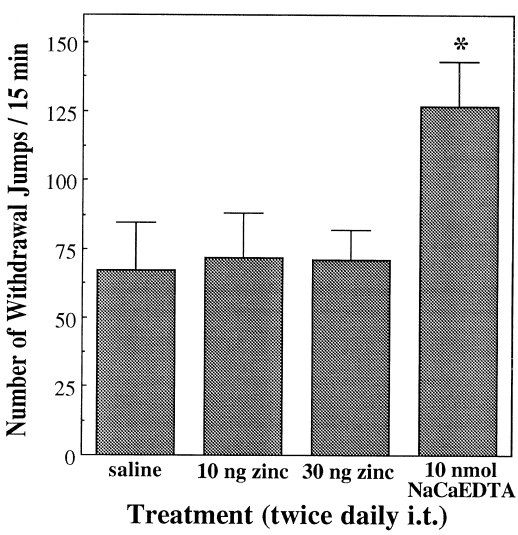

Fig. 4. Effect of Zn^{2+} on chronic morphine dependence. Zn^{2+} was injected i.t. twice daily during days 1–3 in animals implanted with a 75 mg morphine base pellet on day 1. On day 4, mice were injected s.c. with 1 mg/kg of naloxone and immediately thereafter the number of withdrawal jumps counted over a 15-min interval. Data are expressed as indicated in Fig. 1. Asterisks indicate a significant difference between the group indicated and the group injected with saline prior to morphine pellet implantation and naloxone challenge.

twice daily during days 1–3, the number of withdrawal jumps in response to naloxone was potentiated. Groups of mice injected i.t. twice daily with 10 or 30 ng of Zn^{2+} did not show any change in the number of withdrawal jumping behaviors compared to saline-injected control mice (Fig. 4).

4. Discussion

We hypothesized that Zn^{2+} modulates opioid analgesia as well as the development of tolerance and dependence. Our data indicate that increasing the availability of Zn^{2+} inhibits morphine-induced antinociception. Dietary Zn^{2+} deficiency in mice has also been shown to decrease the antinociceptive effect of morphine (Dursun et al., 1995). However, in spite of its tendency to produced hyperalgesia (Larson and Kitto, 1997), decreasing Zn^{2+} in the ECF by injection of $Na^{+}Ca^{2+}$ EDTA had no effect on morphine-induced antinociception. It is unclear whether diet and chelation affect different pools of Zn^{2+} or whether dietary manipulations result in additional extenuating circumstances.

Intrathecal injection of Zn^{2+} also inhibited the development of acute morphine tolerance, perhaps by decreasing the efficacy of the morphine pretreatment. Decreasing Zn^{2+} in the ECF, by injection of $Na^{+}Ca^{2+}$ EDTA, had no effect on acute morphine tolerance. The effect of these manipulations on dependence varied depending on the pretreatment schedule. Neither increasing nor decreasing Zn^{2+} altered acute dependence. Yet twice daily injections of 10 ng of EDTA, a dose that is maximally hyperalgesic when administered alone in the tail flick assay (Larson and Kitto, 1997), increased withdrawal jumping in response to naloxone in morphine pellet-implanted mice. This inhibition of chronic but not acute morphine dependence suggests a generally negative modulatory role for Zn^{2+} in the production of both opioid tolerance and dependence. These data raise the possibility that the reduced concentration of Zn^{2+} in the CSF of ex-heroin addicts (Potkin et al., 1982) contributes to a long-term state of dependence in these individuals.

Inhibition of morphine-induced antinociception and attenuation of both acute tolerance and dependence may all be due to the inhibitory effect of Zn^{2+} on opioid receptor binding (Hannissian and Tejwani, 1988; Stengaard-Pedersen, 1982). Zn^{2+} oxidizes thiol groups on opiate receptors, decreasing the receptor's affinity for its associated ligands. Binding of agonists at μ opioid receptors is inhibited by Zn^{2+} more than that at δ or κ opioid sites in the rat brain (Tejwani and Hanissian, 1990). Alternatively, Zn^{2+} has been proposed as an endogenous ligand for the σ_2 binding sites as Zn^{2+} displaces 1,3-di(2-[5-^{3}H]tolyl)guanidine, ([^{3}H]DTG) binding from σ_2 receptors while only weakly interacting with σ_1 sites in the rat brain (Connor and Chavkin, 1992). These binding sites are antagonized by haloperidol, well known clinically to potentiate morphine-induced antinociception. Thus, σ_2 receptor activity in vivo may be responsible for the inhibition of morphine antinociception by Zn^{2+}.

Zn^{2+} is also a well-known non-competitive antagonist of NMDA receptor activity (Peters et al., 1987; Westbrook and Mayer, 1987; Forsythe et al.,1988; Christine and Choi, 1990). Zn^{2+} allosterically inhibits [^{3}H]glycine binding to a positive modulatory site on the NMDA receptor complex (Yeh et al., 1990), and in doing so, rapidly and reversibly blocks membrane depolarization produced by NMDA (Peters et al., 1987) and binding of [^{3}H]MK-801 to the NMDA receptor complex (Enomoto et al., 1992). Compounds that block NMDA activity, such as 5-methyl-10,11,-dihydro-5*H*-ebenzo(a,d) cyclohepten-5,10-imine maleate (MK-801) and LY274614, attenuate the development of tolerance and dependence (Trujillo and Akil, 1991; Elliott et al., 1994). ACEA-1328, a glycine antagonist selective for the glycine binding site on the NMDA receptor complex, inhibits the development of morphine tolerance in mice (Lutfy et al., 1995). As Zn^{2+} also inhibits glycine activity at this site, Zn^{2+} may attenuate acute tolerance and $Na^{+}Ca^{2+}$ EDTA enhance chronic dependence by its action on NMDA receptor activity in vivo.

One might expect Zn^{2+} and $Na^{+}Ca^{2+}$ EDTA to produce consistently opposite effects. However, minimal or maximal concentrations of Zn^{2+} may already be present in the CNS. In addition, Zn^{2+} and $Na^{+}Ca^{2+}$ EDTA likely distribute into different intracellular and extracellular compartments. A final but important caveat is that $Na^{+}Ca^{2+}$ EDTA may influence the concentration of a divalent cation other than Zn^{2+}.

In summary, exogenous Zn^{2+} inhibited morphine-induced analgesia and acute tolerance while $Na^{+}Ca^{2+}$ EDTA potentiated withdrawal behaviors in a model of chronic dependence. These data reveal an inhibitory influence of Zn^{2+} on opioid activity in vivo and suggest that the CNS Zn^{2+} deficiency in opiate addicts likely contributes to their state of dependence. While further studies are required, Zn^{2+} may produce these effects by its interaction with μ opioid, σ, and/or NMDA receptors.

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