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Ionic Zinc May Function as an Endogenous Ligand for the Haloperidol-Sensitive σ_2 Receptor in Rat Brain

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SUMMARY

In the search for an endogenous σ transmitter, whose existence was previously suggested by release studies, we tested the effects of releasable substances known to be present in the hippocampus, and we determined that ionic zinc may function as an endogenous ligand for the haloperidol-sensitive σ_2 site. Zn^{2+} displaced 1,3-di(2-[5-^{3}H]tolyl)guanidine ([^{3}H]DTG) from two binding sites in rat brain membranes, with an IC_{50} for the high affinity site of 110 ± 3 μM and for the low affinity site of 20 ± 4 mM. The σ_1-selective ligand (+)-[^{3}H]pentazocine was only weakly displaced from rat brain membranes by Zn^{2+} (IC_{50} = 1.4 ± 0.05 mM). These results indicate that the Zn^{2+}-sensitive σ binding site corresponds to the σ_2 site. The interaction between Zn^{2+} and the σ_2 site may have physiological significance, because ionic zinc is present in synaptic vesicles in the brain and may function to regulate binding at the σ_2 site. To test this hypothesis, we measured the effects of metallothionein peptide 1, a specific zinc chelator, on the actions of the putative endogenous σ ligand(s) released in the hippocampus by focal electrical stimulation. Release of the endogenous σ ligand(s) was measured by competition with specific radioligand binding in live hippocampal slices. High frequency, focal, electrical stimulation of the zinc-containing mossy fibers in the hilar region of the hippocampus caused a decrease in the specific binding of [^{3}H]DTG, (+)-[^{3}H]3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine, or (+)-[^{3}H]pentazocine to σ sites. The decrease in [^{3}H]DTG binding was largely blocked by metallothionein peptide 1, whereas the decrease in (+)-[^{3}H]pentazocine binding was unaffected. These results suggest that Zn^{2+} may act as an endogenous ligand at σ_2 sites in the rat hippocampus.

Haloperidol-sensitive σ receptors are a class of membrane proteins that recognize a wide variety of psychoactive drugs, including *N*-allylnormetazocine, pentazocine, dextromethorphan, phencyclidine, and haloperidol (see Ref. 1 for review). Evidence that the σ binding site is a functional receptor includes observations that the binding of (+)-3-PPP and (+)-benzomorphans is regulated by guanine nucleotides and pertussis toxin-sensitive GTP-binding proteins (2, 3), that σ drugs inhibit carbachol-stimulated phosphoinositide turnover (4), and that σ drugs can modulate neuronal firing rates with potencies that correlate with their affinities at the σ receptor (5, 6). In addition, we have recently provided evidence that depolarization of brain slices releases an endogenous ligand that competes with the binding of the σ radioligands [^{3}H]DTG and (+)-[^{3}H]3-PPP (7–9). These results support the hypothesis that a form of the σ binding site may act as a receptor for both σ drugs and an endogenous neurotransmitter.

Evidence is also accumulating for σ receptor heterogeneity (1). Two forms of the σ receptor have been distinguished in brain, liver, and cultured neuronal cell lines (10–13). The σ_1 and σ_2 subtypes differ in their affinities for σ drugs, tissue distributions, and GTP sensitivities. For example, DTG binds to σ_1 receptors with moderately higher affinity, compared with σ_2, (+)-3-PPP is somewhat more selective for σ_1, and (+)-pentazocine is a highly selective σ_1 ligand. Brain contains both σ_1 and σ_2 receptors, whereas liver contains a preponderance of σ_2 sites (14). The structural and functional relationships between the σ_1 and σ_2 sites are unclear, as are their relative contributions to the effects outlined above (15). Further characterization of the pharmacological effects of σ drugs requires correlation between binding affinities and functional effects; thus, additional biochemical description of σ receptor subtypes remains important.

In recent experiments, in which stimulation of selected excitatory pathways in the rat hippocampus released a putative endogenous ligand for σ receptors, we found that stimulation in the hilar region was most efficient at releasing the material (8). Fiber tracts that run through the hilar region of the hippocampus contain a number of neurotransmitter substances (including glutamate, norepinephrine, acetylcholine, dynorphin, and serotonin) (16, 17) that are likely to be released by

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ABBREVIATIONS: 3-PPP, 3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine; DTG, 1,3-di(2-tolyl)guanidine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ACSF, artificial cerebrospinal fluid.

electrical stimulation. However, none of the known transmitter candidates have been found to have significant affinity for either σ receptor (1, 18, 19). The mossy fiber tracts of the hilus also contain large amounts of ionic zinc, which has been demonstrated to be released by synaptic stimulation or tissue depolarization (20, 21). In this study, we examined the ability of ionic zinc to displace σ ligands from their binding sites in rat brain and compared its effects with those of other divalent cations.

Materials and Methods

Membrane binding assays. Membranes for equilibrium binding experiments were prepared as previously described (7). Briefly, fresh or frozen whole rat brain was homogenized in assay buffer [either 200 mM Tris, pH 7.8, or ACSF (composition (in mM): NaCl, 124; KCl, 6; $MgCl_2$, 2.4; $CaCl_2$, 2.5; HEPES, 10); pH 7.2] with a Wheaton homogenizer and was centrifuged in a Beckman JA-20 rotor at 18,000 × g for 30 min at 4°. The membrane pellet was resuspended in 15 volumes of incubation buffer and centrifuged as before, and the final pellet was resuspended in assay buffer to make a 5% (w/v) suspension. This corresponded to a protein concentration of approximately 8 mg/ml.

The ion competition experiments were performed in 200 mM Tris, pH 7.8, or ACSF, pH 7.2, with a final volume, after all additions, of 500 μl. The assay tubes contained 3 nM [^{3}H]DTG (39–52 Ci/mmol), (+)-[^{3}H]3-PPP (115 Ci/mmol), or 2 nM (+)-[^{3}H]pentazocine (35 Ci/mmol), 2% brain membranes, and the appropriate concentration of the displacing ions. Nonspecific binding was defined by either 10 μM haloperidol or 10 μM (±)-pentazocine (as noted). As also noted, in some experiments with [^{3}H]DTG 100 nM (+)-pentazocine was included to block radioligand binding to σ_1 sites. The assay tubes were incubated at room temperature for 90 min, to allow the components to reach binding equilibrium, and the reactions were terminated by vacuum filtration over GF/C filters that had been presoaked in 0.1% polyethylenimine. The filters were washed with ice-cold H_2O, and the bound radioligand was quantified by liquid scintillation counting (Ecolite, NEN). Counting efficiency was 45%. For studies on the reversibility of cation inhibition of σ radioligand binding, membranes were incubated with the appropriate concentration of ion for 90 min and then washed three times by centrifugation at 18,000 × g for 30 min. Radioligand binding to the treated membranes was then determined as described above.

Protein content of the membrane suspension was determined by the method of Bradford (22), with bovine serum albumin as standard. Binding data were analyzed using the nonlinear curve-fitting program GRAPHpad version 2.0 (Institute for Scientific Information). The equilibrium dissociation constants of the inhibitors (K_i values) were calculated from the Cheng-Prusoff equation, assuming simple competitive interaction between radioligand and displacer, $K_i = IC_{50}/(1 + [L]/K_d)$, where $[L]$ is the concentration of radioligand and K_d is the equilibrium dissociation constant of the radioligand. Confidence intervals were calculated from the standard deviation of the sample means and represent $p = 0.01$.

Slice binding assays. Hippocampal slices (500 μm) were prepared from male Sprague-Dawley rats weighing 100–250 g (Tyler Labs, Bellevue WA). Transverse slices from the hippocampus, excluding the septal and temporal poles, were used for the radioligand displacement assays, which were performed as previously described (7, 8). Each well (including all final additions) contained 500 μl of Krebs bicarbonate buffer [composition (in mM): NaCl, 124; KCl, 4.9; KH_2PO_4, 1.2; $MgCl_2$, 2.4; $CaCl_2$, 2.5; glucose, 10; $NaHCO_3$, 25.6; saturated with 95% O_2/5% CO_2, unless otherwise noted].

Slices were preincubated for 30 min at 34° before the initiation of depolarization by focal electrical stimulation, which was delivered through a narrow, concentric, bipolar electrode (SNE-100; Rhodes Medical Supply) connected to a Grass stimulus isolation unit (PSIU6) controlled by a Grass S11A stimulator. Stimulating electrodes were attached to a micromanipulator (Brinkmann) and positioned manually. Electrical stimulation was initiated 10 min before the addition of [^{3}H]DTG (final concentration, 3 nM) or (+)-[^{3}H]3-PPP (final concentration, 1 nM) and 15 min before the addition of (+)-[^{3}H]pentazocine (final concentration, 2 nM). The slices were stimulated for a total of 30–35 min with trains of 300-μA pulses at 50 Hz, delivered for 1 sec every 10 sec; each pulse lasted 0.3 msec. Mouse metallothionein peptide 1 (100 μM) additions were made 15 min before the depolarization was initiated. Nonspecific binding was determined in parallel wells, in the presence of 10 μM haloperidol. In addition, nonspecific binding of (+)-[^{3}H]pentazocine to opioid receptors was blocked with 1 μM naloxone added to all wells.

After incubation for 20 min in radioligand, each slice was homogenized by removal from the incubation well through a 26-gauge needle, into a 1-ml syringe, and was then ejected onto GF/C filters (Whatman) that had been presoaked in 0.1% polyethylenimine. The total harvest and wash time was <20 sec. Bound radioligand was quantified by liquid scintillation counting; each slice binding assay was performed with one or two slices per condition. Statistical analyses were done using Students t test, with p values of <0.05 being considered significant.

Physiological salts, drugs, and mouse metallothionein peptide 1 (Lys-Cys-Thr-Cys-Cys-Ala) were obtained from Sigma Chemical Co. (St. Louis, MO). Radioligands [^{3}H]DTG, (+)-[^{3}H]3-PPP, and (+)-[^{3}H]pentazocine were obtained from DuPont NEN (Wilmington, DE).

Results

The abilities of different divalent cations to compete with [^{3}H]DTG at its binding sites in rat brain membranes was examined. In 200 mM Tris buffer at pH 7.8, the most potent competitor of [^{3}H]DTG binding was Zn^{2+}, which completely displaced the radioligand with an IC_{50} of 130 ± 3 μM (six experiments) (Fig. 1A). Results were identical for experiments performed with either $ZnSO_4$ or $ZnCl_2$. The slope of the displacement curve was shallower than predicted by simple mass action kinetics. A Hill plot of the competition data for Zn^{2+} versus [^{3}H]DTG had a slope of 0.63 (99% confidence interval, 0.48–0.78; 11 experiments), which indicated that Zn^{2+} was either displacing [^{3}H]DTG from more than one site or displacing [^{3}H]DTG from its binding site(s) in a cooperative manner. The next most potent displacer of [^{3}H]DTG, after Zn^{2+}, was Cd^{2+}, which also displaced [^{3}H]DTG with a shallow slope and which had an IC_{50} of 370 ± 100 μM (three experiments). None of the other cations tested (Pb^{2+}, Co^{2+}, Fe^{3+}, Ca^{2+}, and Mg^{2+}, as chloride or sulfate salts) had a mean IC_{50} of <800 μM, and none appeared to discriminate between σ_1 and σ_2 receptors, although complete inhibition curves were not obtained for all the cations at concentrations below 50 mM.

[^{3}H]DTG labels both σ_1 and σ_2 sites; thus, we examined the ability of Zn^{2+} to compete with the relatively selective σ_1 receptor radioligand (+)-[^{3}H]3-PPP for binding to rat brain membranes. (+)-[^{3}H]3-PPP has an 85-fold preference for σ_1 sites in guinea pig brain membranes (13). (+)-[^{3}H]3-PPP was displaced by Zn^{2+} from σ binding sites in rat brain with a mean IC_{50} of 890 ± 20 μM (three experiments). The Hill slope for Zn^{2+} displacing (+)-[^{3}H]3-PPP was 0.79 ± 0.075. In addition, we measured the ability of Zn^{2+} to displace the highly σ_1-selective radioligand (+)-[^{3}H]pentazocine from rat brain membranes. Zn^{2+} inhibited the binding of 2 nM (+)-[^{3}H]pentazocine relatively weakly, with a IC_{50} of 2 ± 0.06 mM (five experiments) (Fig. 1C). The Hill slope for Zn^{2+} displacing (+)-[^{3}H]pentazocine was 0.59 ± 0.1, although the binding of (+)-[^{3}H]pentazocine was not completely inhibited by the concentrations of Zn^{2+}

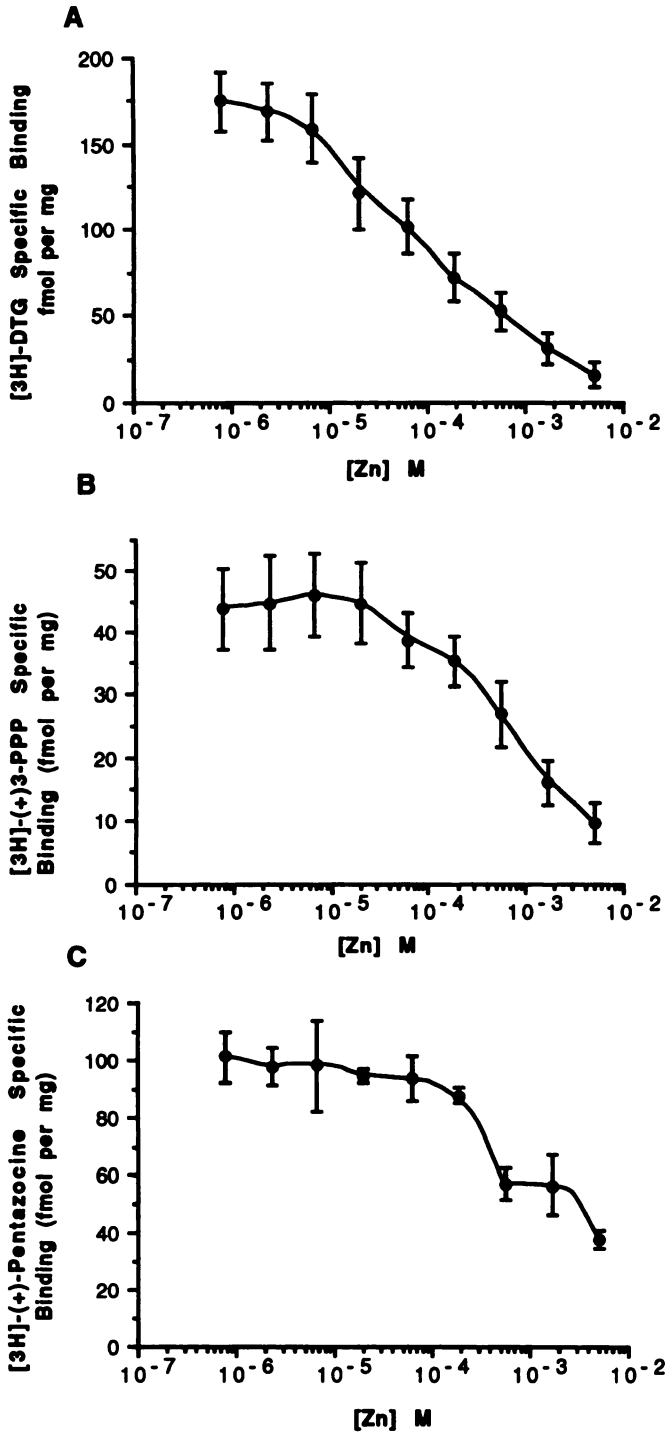


Fig. 1. Zn^{2+} displaces [^{3}H]DTG, (+)-[^{3}H]3-PPP, and (+)-[^{3}H]pentazocine from haloperidol-sensitive σ sites in rat brain membranes. Assays were performed in 200 mM Tris buffer at pH 7.8, as described in Materials and Methods. The concentrations of [^{3}H]DTG and (+)-[^{3}H]3-PPP were 3 nM, and 2 nM (+)-[^{3}H]pentazocine was used. Nonspecific binding was determined in the presence of 10 μM haloperidol. Each *point* represents the mean ± standard error of *n* independent determinations, each performed in duplicate or triplicate. A, $ZnSO_4$ displaces [^{3}H]DTG with an IC_{50} of 130 ± 3 μM (n = 6). Zn^{2+} has no effect on nonspecific binding. B, $ZnSO_4$ displaces (+)-[^{3}H]3-PPP with an IC_{50} of 890 ± 20 μM (n = 3). C, $ZnSO_4$ displaces (+)-[^{3}H]pentazocine from haloperidol-sensitive sites with an IC_{50} of 2 ± 0.06 mM.

used. These results indicate that Zn^{2+} has a lower potency against the σ_1-selective radioligands than against [^{3}H]DTG.

Further investigation of the nature of the interaction between Zn^{2+} and the σ sites in brain membranes revealed that a component of the [^{3}H]DTG binding was irreversibly inhibited by Zn^{2+}. Incubation of the membranes with 200 μM Zn^{2+} for 90 min caused a 55 ± 5% decrease in [^{3}H]DTG binding; after extensive washing to remove the Zn^{2+}, specific [^{3}H]DTG binding to the treated membranes was 49 ± 3% below [^{3}H]DTG binding to control membranes washed in the same manner. The specific binding of (+)-[^{3}H]pentazocine to membranes that had been pretreated with 200 μM Zn^{2+} was unchanged, compared with control. These results demonstrate that Zn^{2+} interacts with the σ_1 receptor only weakly and suggest that the component of [^{3}H]DTG binding that is strongly and irreversibly inhibited by Zn^{2+} in Tris buffer at pH 7.8 is the σ_2 site.

The effects of Cu^{2+} were next compared with those of Zn^{2+}. Initial inspection of the displacement curve for Cu^{2+} against [^{3}H]DTG seemed to show a biphasic displacement of the radioligand from two sites, with widely different sensitivities to the ion (Fig. 2A). However, closer examination revealed that Cu^{2+} was having complex effects on the nonspecific binding of [^{3}H]DTG. At concentrations above 10 μM, Cu^{2+} increased the nonspecific binding of [^{3}H]DTG. The effect of Cu^{2+} on specific binding is shown in Fig. 2B. The maximum increase in nonspecific binding occurred at a concentration of 550 μM Cu^{2+} (175% of control binding in the presence of 10 μM haloperidol). In addition, the effect of Cu^{2+} on [^{3}H]DTG binding was irreversible; membranes treated with 100 μM Cu^{2+} for 90 min and then washed three times by centrifugation showed a decrease in the specific binding of [^{3}H]DTG, from 168 ± 20 fmol/mg to 32 ± 10 fmol/mg of protein (three experiments). In contrast, at concentrations of >10 μM, Cu^{2+} markedly increased both the total and nonspecific (+)-[^{3}H]pentazocine binding to rat brain membranes (Fig. 2C). This effect was apparent when either haloperidol, (±)-pentazocine, or (+)-pentazocine was used to define nonspecific binding (each at a concentration of 10 μM). Despite the increase in overall binding, there was very little change in the specific binding of (+)-[^{3}H]pentazocine at concentrations of Cu^{2+} up to 5 mM (Fig. 2D). Thus, the effects of Cu^{2+} on σ binding were similar to those of Zn^{2+}; both ions had a greater effect on the σ_2 site and that effect was not readily reversible.

In the process of examining the basis for the irreversible effect of Zn^{2+} on σ_2 binding in Tris (pH 7.8) buffer, we found that the inhibition was fully reversible under more physiological buffer conditions. σ binding studies are often performed at pH 7.8–8.0, to increase the specific binding (23). However, in a binding assay using ACSF [composition (in mM): NaCl, 124; KCl, 6; $MgCl_2$, 2.4; $CaCl_2$, 2.5; HEPES, 10] at pH 7.2, Zn^{2+} reversibly inhibited the binding of [^{3}H]DTG to σ sites in rat brain membranes. As shown in Fig. 3, in ACSF, pH 7.2, the IC_{50} was 160 ± 5 μM (eight experiments); the slope of the displacement curve was shallower than that predicted by simple mass action kinetics, and the slope of a Hill plot of the data was 0.60 ± 0.08. The competition data were best fit by a two-site model, where Zn^{2+} inhibited [^{3}H]DTG binding to site 1 with an IC_{50} of 110 ± 3 μM and to site 2 with an IC_{50} of 20 ± 4 mM (eight experiments, $F = 10.73$, $p < 0.01$ versus a one-site fit). Zn^{2+} also displaced (+)-[^{3}H]pentazocine relatively weakly in ACSF, with an IC_{50} of 1.4 ± 0.05 mM and a Hill slope of 0.81

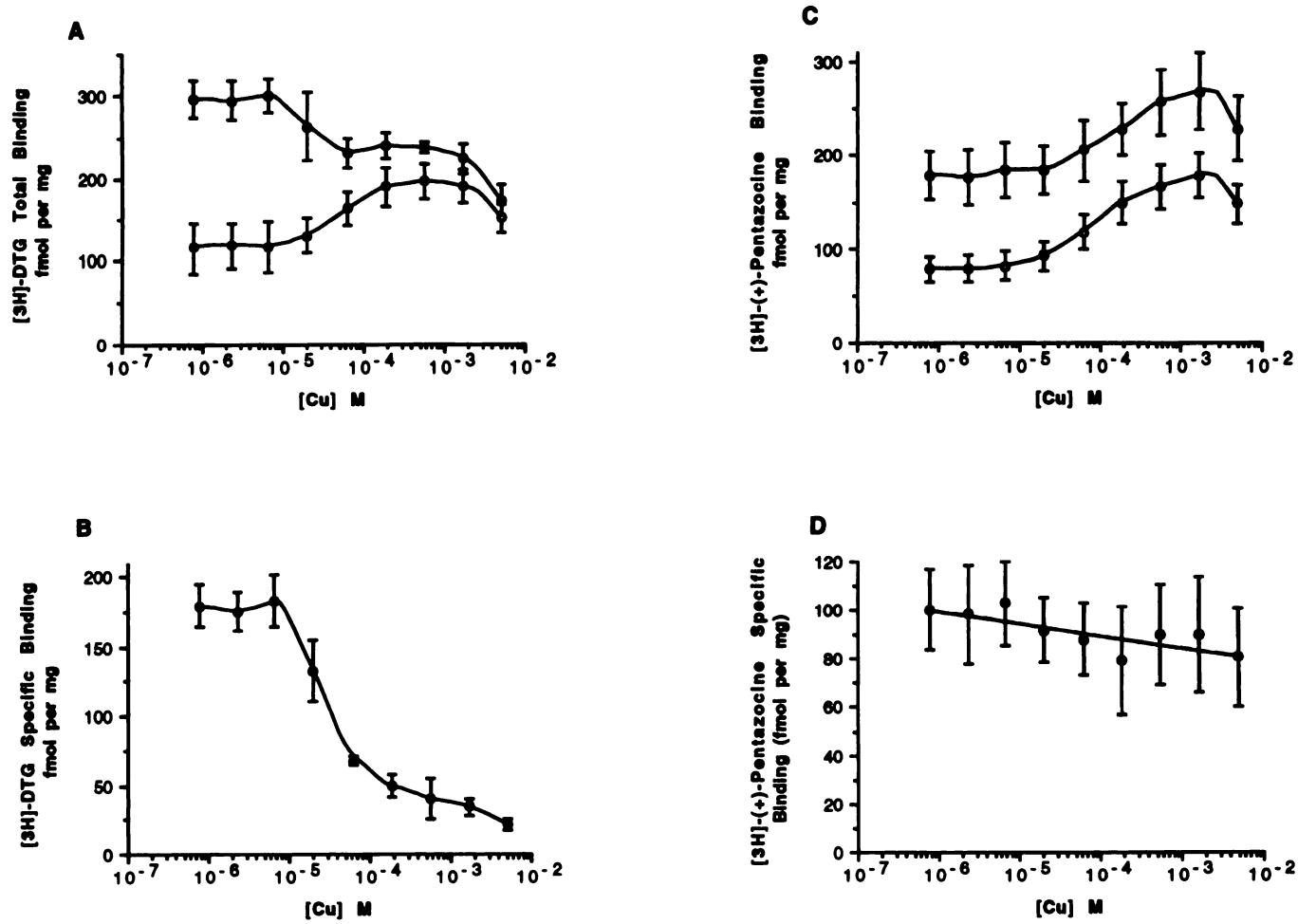


Fig. 2. Cu^{2+} has complex effects on the binding of [^{3}H]DTG and (+)-[^{3}H]pentazocine to haloperidol-sensitive σ sites in rat brain membranes. Assays were performed in 200 mM Tris, as described in Materials and Methods. Radioligand concentration was 3 nM [^{3}H]DTG or 2 nM (+)-[^{3}H]pentazocine. Nonspecific binding was determined using either 10 μM haloperidol or, for some of the experiments with (+)-[^{3}H]pentazocine, 10 μM (±)-pentazocine. Results were the same with either unlabeled ligand. Each *point* represents the mean ± standard error of *n* experiments, each performed in duplicate. A, $CuSO_4$ inhibits the binding of [^{3}H]DTG to rat brain membranes; however, at concentrations of Cu^{2+} above 10 μM there is both a decrease in total binding (●) and an increase in nonspecific binding (○) of the radioligand (n = 3). B, The effect of Cu^{2+} on the specific binding of [^{3}H]DTG, the observed decrease in [^{3}H]DTG binding is irreversible (see text) (n = 3). C, $CuSO_4$ potentiates the binding of (+)-[^{3}H]pentazocine to rat brain membranes, although both the total (●) and nonspecific (○) binding appear to increase in parallel (n = 5). D, $CuSO_4$ affects the haloperidol-sensitive binding of (+)-[^{3}H]pentazocine very weakly, despite increasing the total binding of the radioligand to rat brain membranes (n = 5).

± 0.05 (Fig. 3). The low potency of Zn^{2+} against (+)-[^{3}H] pentazocine binding supports the conclusion that the σ binding site is more sensitive to Zn^{2+} is the σ_2 site. Although the values obtained in ACSF, pH 7.2, were similar to those obtained in Tris, pH 7.8, the interpretation of the former is more straightforward, because of the reversible nature of the interaction.

To examine the selectivities of the radioligands under the more physiological conditions, additional competition studies were performed in ACSF, pH 7.2. σ_2 binding of [^{3}H]DTG [measured in the presence of 100 nM (+)-pentazocine, to block σ_1 binding] showed that unlabeled DTG displaced 3 nM [^{3}H] DTG with a K_i of 114 ± 14 nM (Fig. 4A). (+)-Pentazocine displaced [^{3}H]DTG from rat brain membranes with an IC_{50} of 1.7 ± 0.03 μM and a Hill slope of 0.58 ± 0.06. The competition data were best fit by a two-site model, with (+)-pentazocine having a K_i for site 1 of 2.7 ± 0.03 μM and a K_i for site 2 of 6.5 ± 0.3 nM (four experiments, $F = 8.1$, $p < 0.01$ versus a one-site fit). Site 1 comprised 80 ± 2% of total [^{3}H]DTG specific binding, whereas site 2 comprised 20 ± 2%. These data suggest that site 2 corresponds to the σ_1 receptor and that the majority of sites labeled by [^{3}H]DTG in ACSF, pH 7.2, were σ_2 binding sites.

(+)-[^{3}H]Pentazocine was displaced by unlabeled (+)-pentazocine with an IC_{50} of 14.8 ± 0.2 nM and a Hill slope of 0.74 ± 0.7 (Fig. 4B). The competition curve was best fit by a two-site model, with (+)-pentazocine having a K_i for the high affinity site of 6.6 ± 0.07 nM and a K_i for the second site of 440 ± 30 nM (three experiments, $F = 4.38$, $p < 0.05$). The high affinity site comprised 85% of the specific binding and presumably corresponds to the σ_1 binding site; the low affinity site comprised 15% of specific binding. DTG displaced (+)-[^{3}H]pentazocine weakly (Fig. 4B) in ACSF, with a K_i of 286 ± 5 nM and a Hill slope of 1.00 ± 0.1. These results indicate that the affinity of DTG for σ_1 sites was also greatly reduced in ACSF. The principal effect of changing from Tris, pH 7.8, to ACSF, pH 7.2, was a 3-fold reduction in potency for DTG at σ_2 sites and a 10-fold reduction in potency for DTG at σ_1 sites. Thus, under physiological conditions, [^{3}H]DTG mainly labels σ_2 sites.

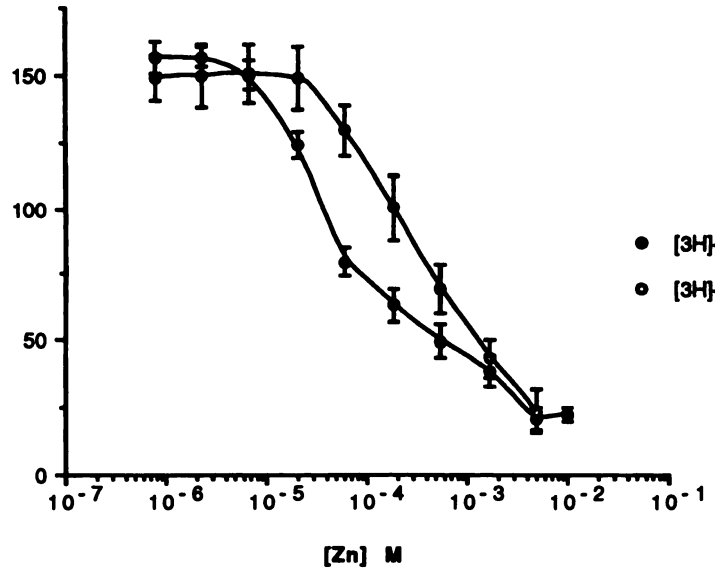
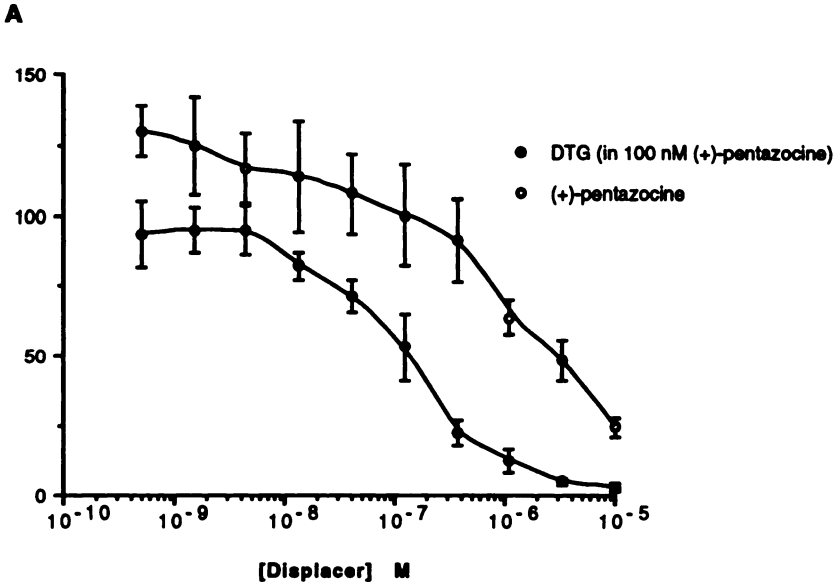


Fig. 3. Zn^{2+} displaces [^{3}H]DTG and (+)-[^{3}H]pentazocine from haloperidol-sensitive σ sites in rat brain membranes. Assays were performed in ACSF at pH 7.2, as described in Materials and Methods. The concentration of [^{3}H]DTG and was 3 nM, and 2 nM (+)-[^{3}H]pentazocine was used. Nonspecific binding was determined in the presence of 10 μM haloperidol. Each *point* represents the mean ± standard error of *n* independent determinations, each performed in triplicate. $ZnSO_4$ displaces [^{3}H]DTG from two sites, with an IC_{50} for site 1 of 110 ± 3 μM and for site 2 of 20 ± 4 mM ($n = 8$, $p < 0.01$ versus a one-site fit). $ZnSO_4$ weakly displaces (+)-[^{3}H]pentazocine from haloperidol-sensitive sites, with a IC_{50} of 1.4 ± 0.05 mM.



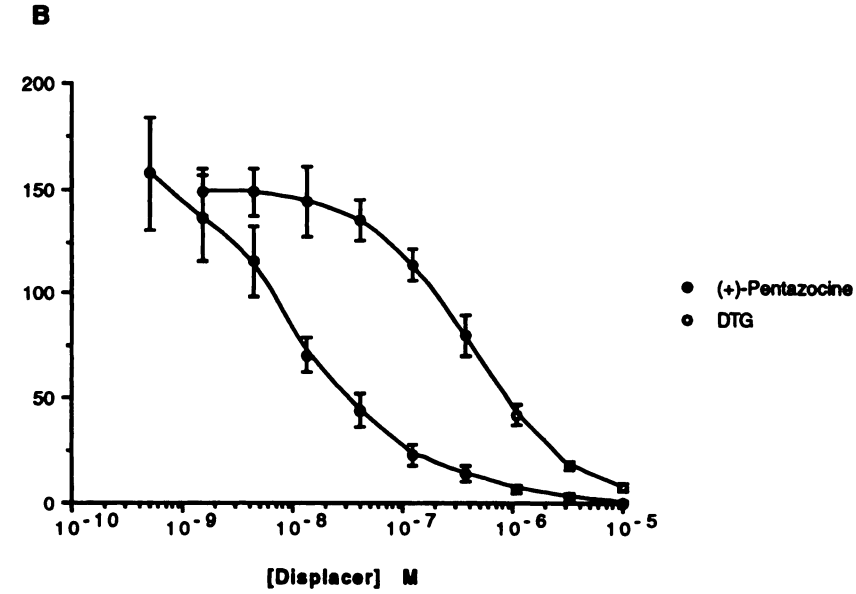


Fig. 4. Affinity of [^{3}H]DTG for σ binding sites in rat brain membranes is reduced in ACSF. Experiments were performed in ACSF, pH 7.2, as described in Materials and Methods. σ sites were labeled with 2 nM (+)-[^{3}H]pentazocine or 3 nM [^{3}H]DTG; nonspecific binding was determined in the presence of 10 μM haloperidol. (+)-Pentazocine was included at 100 nM in the DTG versus [^{3}H]DTG competition curves, to block [^{3}H]DTG binding to σ_1 receptors. Each *point* represents the mean ± standard error of three to five independent experiments, with each point determined in duplicate or triplicate. A, DTG (●) displaces [^{3}H]DTG from σ_2 receptors with a K_i of 114 ± 14 μM; (+)-pentazocine (○) inhibits [^{3}H]DTG binding in manner best fit by a two-site competition model, where [^{3}H]DTG labeling of site 1 is inhibited by (+)-pentazocine with a K_i of 2.7 ± 0.03 μM and [^{3}H]DTG labeling of site 2 is inhibited with a K_i of 6.5 ± 0.3 nM. Site 1 comprises 80% of the total [^{3}H]DTG binding and site 2 accounts for 20%. B, (+)-Pentazocine displaces (+)-[^{3}H]pentazocine (●) in manner best fit by a two-site model of competition, where (+)-[^{3}H]pentazocine has an affinity of 6.6 ± 0.07 nM for site 1 and an affinity of 440 ± 30 nM for site 2. Site 1 comprises 85% of (+)-[^{3}H]pentazocine specific binding and site 2 accounts (for 15%. DTG displaces (+)-[^{3}H]pentazocine (○) with a K_i of 286 ± 5 nM and a Hill slope of 1.00 ± 0.1.

The low affinity of [^{3}H]DTG for σ receptors in ACSF, pH 7.2, makes accurate quantitation of binding association and dissociation rates at these sites difficult. Significant errors are introduced by the dissociation of radioligand during membrane filtration and wash, and the allowable separation time for a ligand of this potency is on the order of 0.1 sec (24). The low affinity of [^{3}H]DTG in ACSF, pH 7.2, made it impractical to determine whether the interaction between Zn^{2+} and [^{3}H]DTG was competitive or noncompetitive.

Previously, we demonstrated a decrease in the binding of [^{3}H]DTG in the hippocampal slice after stimulation in the hilar region of the hippocampus (8). Synaptic stimulation in this area of the hippocampus also releases Zn^{2+} (21). Accordingly, we repeated the focal electrical stimulation experiments in the presence of a fragment of a mouse metallothionein peptide that binds Zn^{2+} (25). As seen previously, high frequency stimulation in the hilus (300 μA pulses lasting 0.3 msec, in 50 Hz trains delivered for 1 sec every 10 sec) resulted in a significant decrease in the specific binding of 3 nM [^{3}H]DTG to the slice. Specific binding decreased from 76 ± 10 fmol/slice in unstimulated tissue to 33 ± 5 fmol/slice in stimulated slices (11 experiments, $p < 0.05$). Preincubation with 100 μM metallothionein peptide had no effect on the specific binding of [^{3}H]DTG to unstimulated slices. However, the metallothionein peptide blocked the reduction in specific binding caused by focal stimulation of the hippocampal slice (Fig. 5A). In the presence of the metallothionein peptide, specific binding of [^{3}H]DTG in slices stimulated as described above was 65 ± 9 fmol/slice (11 experiments), which is not significantly different from the unstimulated control slices. These results suggest that Zn^{2+} released from the mossy fibers may be responsible for most of the decrease in [^{3}H]DTG binding seen after high frequency stimulation of the hilar region of the hippocampus.

We next extended the stimulation experiments to include two other ligands for haloperidol-sensitive σ receptors, (+)-[^{3}H]3-PPP and (+)-[^{3}H]pentazocine. At low concentrations (+)-[^{3}H]3-PPP labels mainly σ_1 receptors, although it is not selective (13). The specific binding of 1 nM (+)-[^{3}H]3-PPP in unstimulated hippocampal slices was 31 ± 3 fmol/slice, and specific binding after high frequency stimulation of the hilar region was 6 ± 2 fmol/slice, a significant decrease from control (17 experiments, $p < 0.001$) (Fig. 5B). After preincubation of the slices with 100 μM metallothionein peptide, the specific binding of (+)-[^{3}H]3-PPP in stimulated slices was 20 ± 2 fmol/slice (17 experiments); the peptide partially inhibited the stimulated displacement of (+)-[^{3}H]3-PPP. This value is significantly different ($p < 0.05$) from both the specific binding in unstimulated slices and that in slices stimulated in the absence of the peptide. In the absence of stimulation, the metallothionein peptide had no effect on specific or nonspecific binding.

Focal stimulation of the hippocampus, as described above, also caused a reduction in the binding of (+)-[^{3}H]pentazocine, a highly selective σ_1 receptor ligand (26). Specific binding of 2 nM (+)-[^{3}H]pentazocine to the hippocampal slices was 17 ± 2 fmol/slice in the presence of 1 μM naloxone, and binding was reduced to 11 ± 1 fmol/slice after focal electrical stimulation of the hilus (14 experiments, $p < 0.025$). Specific binding of (+)-[^{3}H]pentazocine to slices stimulated after preincubation with 100 μM metallothionein peptide was 10 ± 2 fmol/slice (11 experiments). The specific binding was significantly different from control ($p < 0.02$) but did not differ from specific binding to slices stimulated in the absence of the peptide. Metallothionein peptide (100 μM) had no effect on the specific binding of (+)-[^{3}H]pentazocine in unstimulated tissue.

Discussion

There are two principal conclusions from the data presented in this study. Firstly, our results suggest that the cation Zn^{2+} has a 50-fold greater affinity for σ_2 binding sites than σ_1 sites and, secondly, they imply that ionic zinc may act as an endogenous ligand at the σ_2 sites. This is the first demonstration of selective effects of a divalent cation on radioligand binding to the σ receptor subtypes and provides evidence that endogenous Zn^{2+} may be responsible for previously observed decreases in the binding of [^{3}H]DTG to hippocampal slices after focal electrical stimulation of the hilar region of the slice *in vitro*.

It has been reported previously that some cations affect the binding of radioligands to the σ receptor. Monovalent ions, such as Li^+, K^+, Rb^+, and Na^+ moderately inhibit the binding of σ radioligands at concentrations of 100 mM (27, 28). Some divalent cations have been shown to be somewhat more potent. In one study (13), Zn^{2+}, Cd^{2+}, and Ni^{2+} all had IC_{50} values of <1 mM against [^{3}H]DTG in guinea pig brain membranes; although detailed examination of their competition with [^{3}H]DTG was not presented, the ions were held not to discriminate between the σ_1 and σ_2 sites. Consistent with previous reports, Mg^{2+}, Ca^{2+}, Ba^{2+}, and Co^{2+} were found in this study to be relatively impotent at displacing a variety of σ receptor ligands, with IC_{50} values in excess of 1 mM (13, 27–29). However, closer examination of the displacement of selected σ radioligands by Zn^{2+} and Cu^{2+} revealed potentially significant interactions between these ions and σ_2 receptors.

The mossy fibers of the hilar region of the hippocampus contain relatively high concentrations of Zn^{2+}, localized to synaptic vesicles (30). The synaptic Zn^{2+} has been shown to be released, in a Ca^{2+}-dependent manner, by both pharmacological and electrical stimulation (20, 21). Extracellular concentrations of free Zn^{2+} after tissue depolarization have been reported to range from 30 μM (21) to 300 μM (20); the K_i of 114 μM for Zn^{2+} at the σ_2 site in rat brain membranes is well within this range. The physiological role of the released Zn^{2+} is not yet known, but Zn^{2+} has been shown to affect transmitter binding (31) and to modulate *N*-methyl-D-aspartate receptor function (32). The results from this study suggest that it may also modulate σ_2 receptor function.

The existence of an endogenous ligand for the σ binding sites has been suggested by the presence of components in brain extracts that can compete with specific radioligands for σ receptor binding (9, 33–35) and by the demonstration that depolarization of live brain slices could release a putative σ receptor transmitter (7–9). In the release studies, we reported that either pharmacological depolarization or focal electrical stimulation caused the Ca^{2+}-dependent decrease in the binding of the σ radioligand [^{3}H]DTG to the hippocampal slice *in vitro*. Tissue stimulation was also able to release a factor from brain slices, into the incubation medium, that could inhibit σ radioligand binding to rat brain membranes (9). The decrease in [^{3}H]DTG binding was thought to reflect the competition between [^{3}H]DTG and endogenous ligands for the σ receptor released by tissue stimulation.

An interesting feature of the release studies was that, with focal stimulation, the hilar region of the hippocampus was the

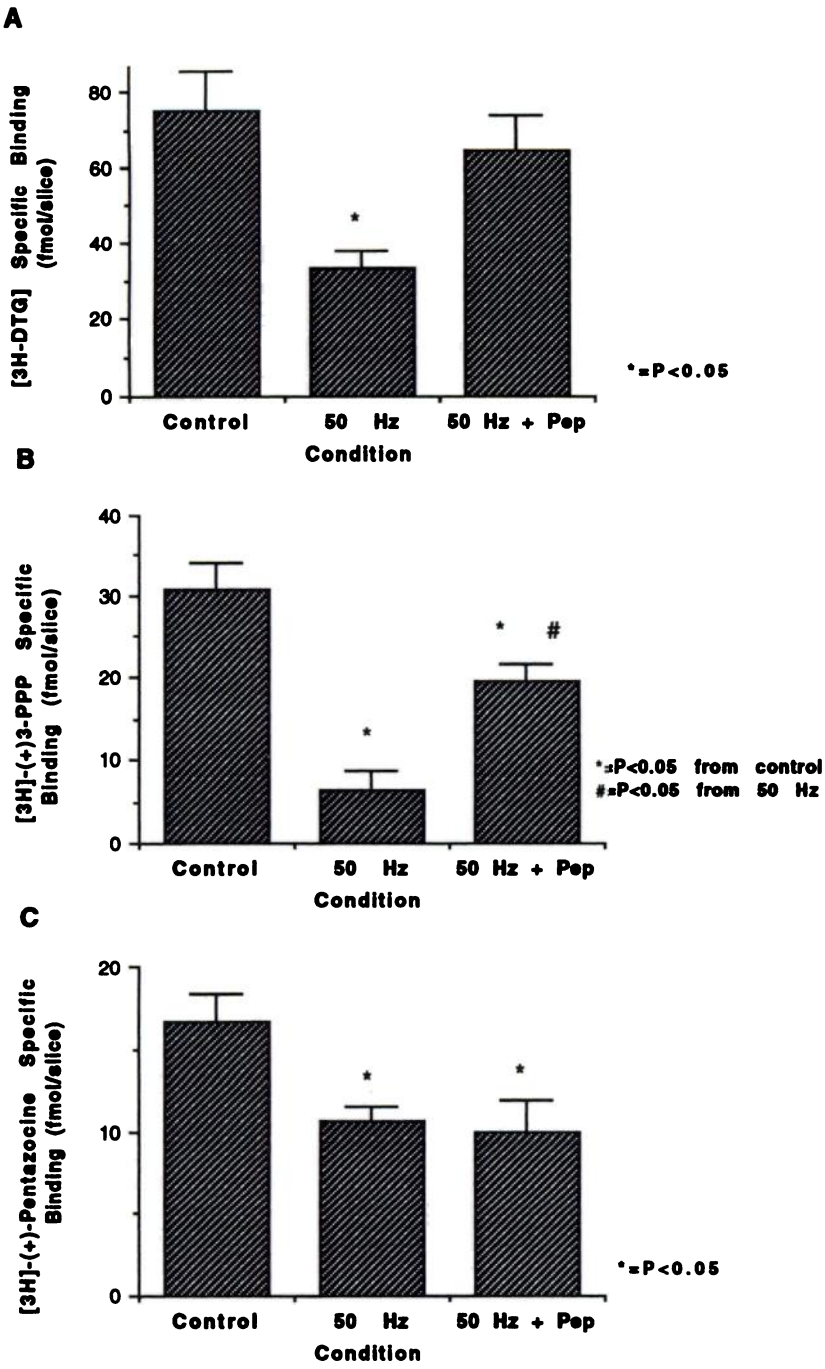


Fig. 5. Mouse metallothionein peptide inhibits electrically stimulated displacement of [^{3}H]DTG and (+)-[^{3}H]3-PPP but not (+)-[^{3}H]pentazocine in the hippocampal slice. Assays were performed as described in Materials and Methods, and metallothionein peptide (*Pep*) (100 μM) was added to the slices 15 min before stimulation commenced. The hilar region of the slice was stimulated with 300 μA pulses of 0.3 msec duration, in 50 Hz trains, for 1 sec every 10 sec, for a total of 30 or 35 min. *Bar values* represent the mean ± standard error of *n* independent determinations. A, Stimulation of the hilus causes a decrease in the specific binding of [^{3}H]DTG to the hippocampal slice, to 43 ± 6% of control (n = 11, $p < 0.05$). After preincubation with 100 μM metallothionein peptide, high frequency stimulation decreases [^{3}H]DTG binding to 87 ± 12% of control (n = 11). B, Stimulation of the hilus causes a decrease in the specific binding of (+)-[^{3}H]3-PPP to 19 ± 6% of control (n = 17, $p < 0.001$); this decrease is partially reversed by preincubation with the metallothionein peptide. Specific binding of (+)-[^{3}H]3-PPP is 65 ± 7% of control after focal stimulation in the presence of the peptide. This is significantly different from both control specific binding and specific binding after focal stimulation in the absence of peptide. C, High frequency stimulation of the hilus causes a decrease in the specific binding of (+)-[^{3}H]pentazocine to 65 ± 6% of control (n = 14, $p < 0.05$). Preincubation with the metallothionein peptide does not block the decrease; specific binding is 59 ± 12% of control after focal stimulation in the presence of the peptide (n = 11, $p < 0.05$).

most effective site at causing σ transmitter release and that the greatest decrease in [^{3}H]DTG binding occurred in the dentate region of the slice (8). The potency of Zn^{2+} at displacing [^{3}H]DTG from σ_2 sites and its localization in the dentate region of the hippocampus led us to hypothesize that released Zn^{2+} was responsible for at least part of the observed decrease in [^{3}H]DTG binding after focal electrical stimulation. We used a fragment of the mouse metallothionein peptide that specifically chelates Zn^{2+}, to test this hypothesis. The peptide we used was a synthetic fragment of mouse liver thionein 1 that has been demonstrated to bind Zn^{2+} and Cd^{2+} (25). The six-amino acid peptide (Lys-Cys-Thr-Cys-Cys-Ala) (peptide 1) binds a single molecule of Cd^{2+}, with a K_d of <1.5 μM at pH 7; although its affinity for Zn^{2+} was not reported, related peptide fragments were shown to have similar affinities for Cd^{2+} and Zn^{2+}. The metallothionein peptide and related thionein fragments have been shown to antagonize specifically the toxicity induced by Cd^{2+} in both whole mice and cultured kidney cells, apparently without any effects of their own (25).

In the present study, metallothionein peptide did block the reduction in [^{3}H]DTG binding caused by stimulation. Important controls document the specificity of this peptide; 1) incubation of the tissue with the peptide did not directly affect radioligand binding, and 2) the peptide did not directly affect transmitter release ([^{3}H]pentazocine displacement was unaffected). We conclude that the metallothionein peptide bound the Zn^{2+} released by tissue stimulation and, thus, sequestered the free Zn^{2+} that would normally be free to compete with [^{3}H]

DTG for binding to σ_2 sites in the slice. This result suggests that Zn^{2+} can act as an endogenous ligand at σ_2 sites in the dentate region of the slice. Although the specific pattern of distribution of σ_1 and σ_2 receptors in the rat brain and their relative abundance in the hippocampus is not yet known, the binding studies presented here indicate that, in physiological buffer, [^{3}H]DTG mostly binds to σ_2-type receptors in membranes prepared from whole rat brain.

(+)-Pentazocine is a selective ligand for σ_1 receptors, which we found were relatively insensitive to Zn^{2+}. Focal electrical stimulation of the dentate region still resulted in a decrease in (+)-[^{3}H]pentazocine binding, but this decrease was insensitive to preincubation with the peptide. This result is important for two reasons; firstly, it provides evidence that there is a second endogenous ligand released by focal electrical stimulation that can interact with σ_1 receptors and, secondly, it indicates that the peptide has no direct effect on the ability of focal electrical stimulation to cause the release of putative neurotransmitter substances. We have recently provided evidence for the existence of a soluble factor, found both in acid extracts of rat brain and in perfusate from brain slices depolarized with KCl, that can displace [^{3}H]DTG from σ receptors in rat brain (9). Preliminary characterization by HPLC indicates that this material is not Zn^{2+}.[1] Additional work will be required to determine whether this material is the same as that competing with (+)-[^{3}H]pentazocine after focal stimulation of the hippocampus.

Both Zn^{2+} and Cu^{2+} had complex effects on the binding of σ radioligands under standard σ receptor binding conditions, i.e., Tris buffer at pH 7.8. Both Zn^{2+} and Cu^{2+} caused an irreversible decrease in the specific binding of [^{3}H]DTG to σ_2 receptors but, whereas Zn^{2+} seemed to decrease total binding without affecting nonspecific [^{3}H]DTG binding, Cu^{2+} not only decreased total binding but also increased the haloperidol-insensitive component of [^{3}H]DTG binding. Additionally, and in contrast to Zn^{2+}, Cu^{2+} seemed to increase both the total and nonspecific binding of the σ_1-selective radioligand (+)-[^{3}H]pentazocine to rat brain membranes, without affecting the specific binding. Although it is clear that Zn^{2+} affects [^{3}H]DTG binding to σ_2 receptors in Tris at pH 7.8, the effects of Cu^{2+} on the nonspecific binding of [^{3}H]DTG make it difficult to assess whether the ion is affecting [^{3}H]DTG binding to σ_1 or σ_2 sites. How Zn^{2+} and Cu^{2+} irreversibly affect the binding of [^{3}H]DTG is not known. The two ions may share a common mechanism of action for inhibition of [^{3}H]DTG binding at σ_2 receptors, but the complex effects of Cu^{2+} on binding to both σ_1 and σ_2 receptors suggest that Cu^{2+} affects σ radioligand binding by additional mechanisms. Cu^{2+} could affect [^{3}H]DTG and (+)-[^{3}H]pentazocine binding by unmasking a haloperidol-insensitive [^{3}H]DTG/(+)-[^{3}H]pentazocine binding site or converting either the σ_1 or σ_2 sites into a haloperidol-insensitive form, possibly by oxidizing a crucial residue at the binding site of the drugs.

It is intriguing that the irreversible effects of Zn^{2+} at σ_2 receptors in Tris buffer at pH 7.8 are not apparent under the more physiological buffer conditions of ACSF at pH 7.2. Why this is so is not known, but there are clearly important differences in the way σ receptors and their ligands interact in the two buffer systems. The most striking change is in the way [^{3}H]DTG behaves. In Tris buffer at pH 7.8, [^{3}H]DTG is a relatively nonselective σ receptor ligand but it shows a 3-fold preference for σ_1 receptors over the σ_2 subtype. In contrast, in ACSF at pH 7.2, [^{3}H]DTG has a markedly decreased affinity for both subtypes of receptor and, although it is still nonselective, its preference is reversed; it now has a 3-fold higher affinity for σ_2 receptors. This decrease in affinity in physiological buffer may explain why it has been difficult thus far to correlate the binding potency of [^{3}H]DTG in Tris at 7.8 with its potency in various physiological assays. These results underscore the importance of performing binding studies under conditions that approximate those of physiological assays as closely as possible.

In summary, this is the first report of selective cation effects on σ binding site subtypes, and these findings suggest the possibility of endogenous regulation of σ_2 sites by ionic zinc. The study further emphasizes the fundamental difference between σ_1 and σ_2 binding sites. The functional consequence of Zn^{2+} binding to σ_2 receptors is unknown, but the results outlined above suggest that Zn^{2+} may act as an endogenous modulator at the σ_2 receptor. Similar hypotheses have been presented for Zn^{2+} modulation of *N*-methyl-D-aspartate receptor (32), γ-aminobutyric acid receptor (36), and opioid receptor function (31). Additional studies are required to define the physiological effects of ionic zinc on σ_2 receptor function.

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