a developing country in the South Pacific. Several studies have been conducted in Fiji intermittently over the past 20 years, analyzing environmental levels of a number of hazardous metals. Gangaiya et al. [2] reported in 2001 that copper, lead and zinc concentrations in sediments of certain sites within the Lami estuary were higher than other areas along Suva Harbor, previously considered to be highly contaminated. Maata and Singh [3] documented 7 years later of levels of lead, copper, zinc and iron in sediments of the Suva Harbor to be 6.2, 3.9, 3.3, and 2.1 times more than the accepted background reference levels, respectively. Morrison et al. [4] reported lagoon sediments and shellfish showing high levels of mercury. Chand et al. [5] reported levels of the metalloid arsenic in surficial sediments to be 334 mg/kg in dry weight, almost 30 times more than the USA's Environmental Protection Agency (EPA) cleanup guideline level for sediments of 12 mg/kg, and expected to pose ecological risks.

The history of Fiji shows that its people have primarily made their livelihood by utilizing marine resources within the vicinity of their dwelling areas. However, globalization and economic development over the years has also increased populations and industrialization of urban areas, resulting in over-utilization of marine resources, destruction of habitats, incorrect methods of waste management and depleted marine life [3]. The 2006 Fiji National Liquid Waste Management Strategic Plan reports these changes being significant in the industrial zone of Walu Bay due to high levels of tributyl tin from marine antifouling paints, petroleum pollutants, leached hazardous metals from a battery factory and effluents from nearby food processing factories [6].

Two prime industrial zones are located in the Suva Harbor comprising food-processing factories, a shipyard and large oil storage tanks. Within 3 km away on the coast lie the stinking remains of an open-style, non-segregated rubbish dump that used to accommodate 50,000 tons of all types of waste annually [3]. The dump was decommissioned in 2005 when waste had reached 15 m above ground level. Years of non-segregated, improper waste disposal has caused pesticides, oil and other hazardous pollutants and metals to leach into adjoining waters that flush freshwater into the harbor. Within a km west of this site, is an area of natural beauty that hosts a resort complex and a marina for visiting yachts. People living in the area also use the coastal stretch around this area for recreational purposes and for subsistence fishing [7].

All studies on environmental hazardous metal levels conducted in Fiji to date has been in various areas of the Suva Harbor, located on the south east of the main island. This area serves as a major commercial center with more than 150,000 people, one quarter of the population of the entire main island. Economic growth has resulted in progressive migration of the population from rural to urban centers, associated with a corresponding escalation of environmental problems [3].

Despite all the available scientific data for Fiji confirming elevated levels of several hazardous metals in surficial sediments for the past 20 years, there have been no attempts to establish any effects in human populations, at least in those dwelling in the vicinities of the Suva Harbor. The regular Fijian still lacks a sense of responsibility towards the environment as is evident in the habit of casual trashing and spitting in public spaces, so it may not be overly presumptuous to assume that there is much less awareness of environmental pollution with hazardous metals. It would therefore also be unlikely that there is awareness of neither the bioaccumulation of hazardous metals nor the health effects of human exposure–an area that needs proactive attention and advocacy. Hazardous metal pollution data similar to Fiji's is provided by the South Pacific Regional Environment Program for the neighboring countries of the Solomon Islands, Tahiti, Papua New Guinea and Vanuatu, showing elevated surficial sediment and shellfish concentrations of hazardous metals [8]. Similar to the situation in Fiji, it is also probably unlikely that the general population is aware or informed.

Exposure to hazardous metals is associated with various conditions such as developmental retardation, cancers, kidney failure, autoimmunity, and even death if exposed to very high concentrations [1]. Autoimmunity has been associated with development of diseases of the joint, renal, circulatory and central nervous systems. Lead primarily has neurotoxic effects to which children are more susceptible than adults because they absorb more and excrete less of the lead that their bodies have been exposed to. Relatively more of the retained lead is deposited in the brain, and it causes more damage to the developing brains of children than to mature brains [1].

In a similar manner, the most significant health effect of methylmercury in fetuses, infants, and children is impaired development of the central nervous system. Consumption of methylmercury-contaminated fish and shellfish by a pregnant woman has deleterious effects on the developing fetal neurological system that results in impaired memory, cognition, concentration span, language and fine motor and visuo-spatial skills [1].

There is indeed room for argument in the premises of this paper because it makes reference to hazardous metal levels in surface sediment as the basis for extrapolating a hypothesis of probable human effects. O'Connor and Paulb [9] compared US EPA Environmental Monitoring and Assessment Program Estuaries Program data with bio-effects data on sediment chemistry and toxicity reported by the National Oceanic and Atmospheric Agency, and they could not find any one indicator that accurately predicted toxicity. This finding questions the appropriateness

and reliability of using sediment data as a monitoring tool for contamination and probably deserves more investigation. Eliciting levels of the metabolites and breakdown products of these contaminants may be a valid alternative, as they become the main source of toxicity once the parent compound is no longer detectable. Nevertheless, until an accurate, reliable alternative is developed, surface sediment will continue to provide relevant data on marine environmental health.

It is evident that globalization is pressuring countries into economic development, but industrialization in developing nations like Fiji commonly occurs at the expense of the environment; the release of hazardous metals into the environment should therefore not be expected to reach a plateau anytime soon. Existing environmental data that conclusively prove considerable contamination of the Suva Harbor with various hazardous metals warrant the need for thorough assessment of the waste practices of industries located upstream from the estuaries, but it is also timely that metal contamination of the human food chain and consequent biological effects were investigated. Analyses for hazardous metals in soil, water, dust and where indicated, root crops and vegetables, of proximal residential dwellings need to be considered. Government, non-government and academic institutions with mandates or interest to investigate hazardous metal pollution of the environment should be encouraged to collaborate with the ministry of health and its affiliates in linking this aspect of environmental health to human health. With the effects of climate change already evident in Fiji and its smaller neighboring states, this collaborative proposal provides the added opportunity to address the knowledge gap by raising awareness in the general public about the less-known but vital connections between the environment and human health.

The same proposal is relevant and can be applied to neighboring countries in the Pacific region as well, but for now, the need to conduct exposure and health impact assessments in regards hazardous metals in Fiji, especially in women and children, is pertinent and pressing.

Conflict of Interest

The authors have no conflicts of interest with the material presented in this paper.

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Full Paper

Pentazocine Inhibits Norepinephrine Transporter Function by Reducing its Surface Expression in Bovine Adrenal Medullary Cells

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Abstract. (±)-Pentazocine (PTZ), a non-narcotic analgesic, is used for the clinical management of moderate to severe pain. To study the effect of PTZ on the descending noradrenergic inhibitory system, in the present study we examined the effect of [^{3}H]norepinephrine (NE) uptake by cultured bovine adrenal medullary cells and human neuroblastoma SK-N-SH cells. (−)-PTZ and (+)-PTZ inhibited [^{3}H]NE uptake by adrenal medullary cells in a concentration-dependent (3 – 100 μM) manner. Eadie-Hofstee analysis of [^{3}H]NE uptake showed that both PTZs caused a significant decrease in the V_{max} with little change in the apparent K_m, suggesting non-competitive inhibition. Nor-Binaltorphimine and BD-1047, κ-opioid and σ-receptor antagonists, respectively, did not affect the inhibition of [^{3}H]NE uptake induced by (−)-PTZ and (+)-PTZ, respectively. PTZs suppressed specific [^{3}H]nisoxetine binding to intact SK-N-SH cells, but not directly to the plasma membranes isolated from the bovine adrenal medulla. Scatchard analysis of [^{3}H]nisoxetine binding to SK-N-SH cells revealed that PTZs reduced the B_{max} without changing the apparent K_d. Western blot analysis showed a decrease in biotinylated cell-surface NE transporter (NET) expression after the treatment with (−)-PTZ. These findings suggest that PTZ inhibits the NET function by reducing the amount of NET in the cell surface membranes through an opioid and σ-receptor–independent pathway.

Keywords: adrenal medullary cell, descending noradrenergic inhibitory system, norepinephrine transporter, pentazocine, SK-N-SH cell

Introduction

The racemic compound (±)-pentazocine (PTZ), a non-narcotic analgesic, is used for the management of moderate to severe pain in humans. (−)-PTZ is known to act as an opioid analgesic, and (+)-PTZ is a σ-receptor agonist without analgesic effects. The antinociceptive effects of (−)-PTZ have been reported to be mediated by its agonist action at the κ-opioid receptor (1). Although opioids remain the standard analgesics, there are clinical situations in which alternative approaches to analgesia are desired. For example, physicians are often reluctant to prescribe opioids for a chronic pain condition because of concerns about the potential for abuse and tolerance development. Additionally, opioids are of questionable effectiveness in treating some pain conditions such as neuropathic pain (2).

Analgesia can alternatively be affected through modulation of monoamine activity with serotonin (5-HT) or norepinephrine (NE) uptake inhibitors such as amitriptyline or desipramine (3, 4). These agents are often more effective than opioid analgesics in treating neuropathic pain (5) and are not usually associated with abuse potential. Furthermore, compounds that modify monoamine levels have been reported to modulate opioid-induced analgesia (6 – 8). In some paradigms, the interactions

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between opioid drugs and monoamine uptake inhibitors have been reported as additive, whereas in other models the interactions are synergistic (9, 10).

The NE transporter (NET) is selectively expressed on NE nerve terminals, where it can exert spatial and temporal control over the action of NE (11 – 13). NET induces the termination of neurotransmission by the reuptake of NE released into the extracellular milieu. Human NET, which belongs to the gene family (SLC6A2) of sodium- and chloride-dependent neurotransmitter transporters (12, 14, 15), was the first monoamine transporter to be cloned, and its mRNA is abundantly localized in the brain stem and adrenal medulla (11). NET is also a critical target for various antidepressant and psychostimulants that interact with NET to increase extracellular NE by inhibiting NE uptake (11 – 13).

Adrenal medullary cells derived from the embryonic neural crest share many physiological and pharmacological properties with postganglionic sympathetic neurons. The cells express functional NET proteins (16 – 18). The pharmacological properties of NET in bovine adrenal medullary cells are similar to those of NET in central and peripheral noradrenergic neurons (13). Therefore, NET in bovine adrenal medullary cells has provided a convenient model for studying the effects of various agents such as anesthetics and antipsychotic drugs on this transporter (19, 20). Some centrally acting analgesic agents such as tramadol have both opioid and monoamine modes of action (21, 22). Tramadol inhibits NET function by blocking desipramine-binding sites as the basis for its antinociceptive effect (19). Another opioid analgesic, PTZ, has preliminarily been reported to inhibit the uptake of NE in the rat brain cortex (23), but the precise mechanism remains unclear. In the present study, we investigated the effect of PTZ on NET activity in cultured bovine adrenal medullary cells and SK-N-SH cells and found that PTZ inhibits NET activity through suppression of its cell surface expression in an opioid receptor– and σ-receptor–independent manner.

Materials and Methods

Drugs and reagents were obtained from the following sources: Eagle's minimum essential medium (Eagle's MEM) (Nissui Pharmaceutical, Tokyo); α-MEM, Dulbecco's Modified Eagle's medium (DMEM), l-NE, pargyline hydrochloride, and ascorbic acid (Nacalai Tesque, Kyoto); collagenase (Nitta Zerachin, Osaka); calf serum (Cell Culture Technologies, Gravesano, Switzerland); fetal bovine serum (SAFC Biosciences, Inc., Lenexa, KS, USA); (−)-PTZ, (+)-PTZ, 5-hydroxytryptamine (5-HT), desipramine hydrochloride, clomipramine hydrochloride, naloxone hydrochloride dehydrate, calphostin C, and chelerythrine (Sigma, St. Louis, MO, USA); nisoxetine hydrochloride (Research Biochemicals International, Natick, MA, USA); nor-Binaltorphimine dihydrochloride and GF109203X (Wako, Osaka); BD-1047 dihydrobromide (Tocris Bioscience, Bristol, JK, USA); biotin sucinimidyl ester (sulfo-NHS-biotin), MagnaBind™ Streptavidin Beads (Thermo Scientific, Barrington, IL, USA); L-[7,8-^{3}H]NE, hydroxytryptamine creatinine sulfate, 5-[1,2-^{3}H(N)], [*N*-methyl-^{3}H]nisoxetine hydrochloride (Perkin-Elmer Life Sciences, Boston, MA, USA); and $^{45}CaCl_2$ (GE Health Care UK Ltd., Little Chalfont, Buckinghamshire, UK).

Adrenal medullary cells were isolated by collagenase digestion of slices of bovine adrenal medulla. The cells were maintained in a monolayer culture at a density of 1×10^6 cells per well (24-well plate; Corning Life Sciences, Lowell, MA, USA) in culture medium (Eagle's MEM) with 10% calf serum and several antibiotics in 5% CO_2 / 95% air (24) . In some experiments, the human noradrenergic neuroblastoma cell line, SK-N-SH (RCB0424), provided by the RIKEN Cell Bank (Tsukuba), was used. SK-N-SH cells were maintained in culture medium containing α-MEM supplemented with 10% fetal bovine serum, and several antibiotics. Cells were plated on poly-L-lysine–coated plates at a density of 0.3×10^6 cells per well in 5% CO_2 / 95% air.

[^{3}H]NE uptake by the cells was performed as follows: Cultured bovine adrenal medullary cells (1×10^6 / well) or SK-N-SH cells (0.3×10^6 / well) were preincubated with or without PTZ for the indicated times and further incubated at 37°C for another 12 min in KRH buffer containing 100 μM pargyline, 1 mM ascorbic acid, and [^{3}H]NE (500 or 100 nM, respectively, 0.1 μCi) in the presence or absence of desipramine and PTZ. KRH buffer was composed of 154 mM NaCl, 5.6 mM KCl, 1.1 mM $MgSO_4$, 2.2 mM $CaCl_2$, 10 mM HEPES-Tris, and 10 mM glucose, adjusted to pH 7.4. After incubation, the cells were rapidly washed three times with 250 μl of ice-cold KRH buffer and solubilized in 500 μl of 10% Triton X-100. The radioactivity in the solubilized cells was counted with a liquid scintillation counter (Trib-Carb 2900TR; Packard BioScience, Meriden, CT, USA). Desipramine-sensitive uptake was calculated by subtracting the value obtained in the presence of 10 μM desipramine from that obtained in the absence of desipramine (25). In some experiments to determine kinetic parameters, cells were preincubated with or without PTZ (30 μM) for 20 min and then further incubated in the presence of [^{3}H]NE (1 – 30 μM) with or without PTZ for 12 min. The apparent Michaelis constant (K_m) and the maximal velocity (V_{max}) for initial rates of [^{3}H]NE uptake were determined by the Eadie-Hofstee

analysis and calculated by non-linear regression analysis of the data for each individual experiment, using GraphPad Prism 5 software (San Diego, CA, USA).

Specific [^{3}H]nisoxetine binding was proceeded by the following 2 protocols: i) Plasma membranes were prepared from bovine adrenal medulla as described previously (20). The specific binding of [^{3}H]nisoxetine, a selective radioligand for NET, was determined by incubation of membranes (20 μg protein) suspended in a binding buffer (300 mM NaCl, 5 mM KCl, 50 mM Tris-HCl, pH 7.4) for 2 h at 4°C in the presence or absence of 10 μM of nisoxetine. The incubation buffer contained [^{3}H]nisoxetine (2 – 32 nM), and in some experiments additionally PTZ (30 μM). After incubation, binding was terminated by the rapid filtration of the membrane suspension under vacuum through Whatman GF/C glass fiber filters. Specific binding of [^{3}H]nisoxetine was defined as the binding inhibited by the selective NET inhibitor nisoxetine (10 μM). ii) For [^{3}H]nisoxetine binding assays in intact cells, SK-N-SH cells were treated with or without PTZ at 37°C for 30 min and were incubated in 0.3 ml of ice-cold binding buffer (100 mM NaCl, 50 mM Tris, 100 μM ascorbic acid, pH 8.0) containing PTZ and [^{3}H]nisoxetine (2 – 64 nM) at 4°C for 2 h. After washing the cells, the radioactivity in the solubilized cells was counted. Non-specific binding was determined in the presence of 10 μM nisoxetine and the specific binding was obtained by subtracting non-specific binding from the total binding.

Cell surface biotinylation assay was proceeded as described previously (26) with a slight modification. After incubation of SK-N-SH cells with or without (−)-PTZ (100 μM) for 30 min at 37°C, biotinylation was carried out by incubating the cells for 1 h at 4°C in 250 μl of phosphate-buffered saline (PBS)/Ca^{2+}/Mg^{2+} containing 1.5 mg/ml of sulfo-NHS-biotin. The biotinylation reagent was removed, and cells were washed twice with PBS/Ca^{2+}/Mg^{2+} containing 100 mM glycine, followed by quenching for 30 min with the same reagent and three times washed with PBS/Ca^{2+}/Mg^{2+}. Cells in each well were solubilized by gentle shaking for 1 h in 200 μl of radioimmunoprecipitation (RIPA) buffer containing protease inhibitors (Nacalai Tesque). Cell lysates were centrifuged at 20,000 × g for 30 min, and an aliquot of each sample was used for the isolation of biotinylated proteins with streptavidin beads by incubating for 1 h at room temperature with gentle shaking. The biotinylated proteins were then washed five times with RIPA buffer and then eluted by heating the beads in sample buffer at 95°C for 5 min.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were performed as follows: The NET proteins in total, nonbiotinylated and biotinylated fractions were separated by SDS-PAGE (10%), and were electroblotted onto polyvinylidene difluoride (PVDF) membrane (Immobilon-P) with transfer buffer (39 mM glycine, 48 mM Tris, 0.0375% SDS, 20% methanol, pH 8.5). After blocking with a blocking buffer (PVDF Blocking Reagent for Can Get Signal; Toyobo, Osaka) for 1 h at room temperature, the membranes were incubated with a primary antibody against NET (1:1,000; Santa Cruz Biotechnology, Calfornia, USA) or β-actin (1:10,000; Cell Signaling Technology, Beverly, MA, USA) in Can Get Signal Solution-1 (Toyobo) for 1 h at room temperature and then washed with Tris-buffered saline-Tween (10 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1% Tween 20) (TBS-T). The immunoreactive bands were reacted in a solution (Can Get Signal Solution-2; Toyobo) with a polyclonal goat anti-rabbit antibody conjugated to horseradish peroxidase (1:10,000; Cell Signaling Technology) for 1 h at room temperature, and washed repeatedly as above. The immunoreactive bands were visualized by Immobilon Western (Millipore Corporation, Billerica, MA, USA) and quantified by Light-Capture with the CS Analyzer (ATTO Corporation, Tokyo).

COS-7 cells (RCB0539; RIKEN Cell Bank) were maintained in culture medium containing DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Rat serotonin transporter (rSERT) cDNA was used as previously reported (27). Transfection of rSERT cDNA was performed using Effectene Transfection Reagent (Qiagen, Hilden, Germany) at 10:1 (reagent:cDNA) ratios. Cells were incubated after transfection for 24 to 48 h before use in the experiments. Cells (1×10^6 / well) were preincubated at 37°C for 20 min in KRH buffer in the presence or absence of PTZ. The cells were further incubated with KRH buffer containing 10 μM pargyline, 100 μM ascorbic acid, and [^{3}H]5-HT (50 nM, 0.1 μCi) at 37°C for 12 min in the presence or absence of PTZ. Nonspecific uptake was determined in the presence of 10 μM clomipramine.

The influx of $^{45}Ca^{2+}$ was measured in cultured bovine adrenal medullary cells as reported previously (24). Cells (4×10^6 per dish) were incubated with 1.5 μCi of $^{45}CaCl_2$ at 37°C for 5 min with or without 56 mM K^+ and PTZ in KRH buffer. After incubation, the cells were washed 3 times with ice-cold KRH buffer, solubilized in 10% Triton X-100, and the radioactivity counted.

All experiments were performed in duplicate or triplicate, and each experiment was repeated at least three times. All values are given as means ± S.E.M. Data were statistically evaluated by Student's t-test or one-way analysis of valiance (ANOVA). If a significant F value was found, Dunnett's test for multiple compari-

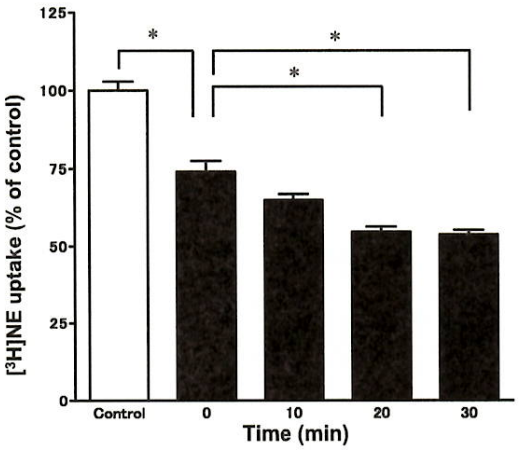


Fig. 1. Time course of effect of (−)-PTZ on [^{3}H]NE uptake in cultured bovine adrenal medullary cells. After preincubation with or without (−)-PTZ (30 μM) for the indicated period, the cells were incubated with or without (−)-PTZ (30 μM) at 37°C for another 12 min in the presence of [^{3}H]NE (500 nM). The desipramine-sensitive [^{3}H]NE uptake by the cells was measured. Data are means ± S.E.M. of three separate experiments carried out in triplicate and expressed as % of the control in which cells were not treated with (−)-PTZ (30 μM) during all procedures. The values of the control [^{3}H]NE uptake was 0.23 ± 0.02 pmol / 10^6 cells per min. *$P < 0.05$, compared with the control or 0 min.

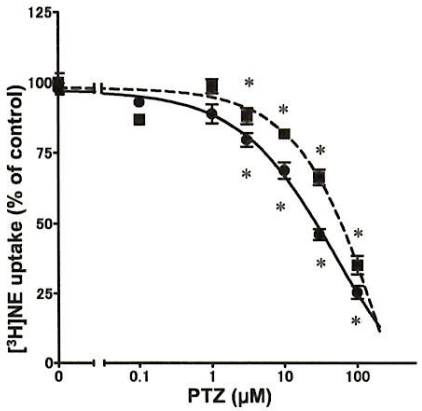


Fig. 2. Concentration–response curves of effects of (−)- and (+)-PTZ on [^{3}H]NE uptake by cultured bovine adrenal medullary cells. After preincubation with (−)-PTZ (closed circles) or (+)-PTZ (closed squares) for 20 min, cells were incubated with 500 nM [^{3}H]NE and various concentrations (0.1 – 100 μM) of PTZ for 12 min at 37°C. The desipramine- sensitive [^{3}H]NE uptake by the cells was measured. Data were expressed as a percentage of the control (0.38 ± 0.08 pmol / 10^6 cells per min). *$P < 0.05$, compared with 0 μM PTZ.

sons was carried out to identify differences among groups. When $P < 0.05$, the differences were considered statistically significant.

Results

(−)-PTZ (30 μM) inhibited [^{3}H]NE uptake by cultured bovine adrenal medullary cells in a time-dependent manner (Fig. 1). Preincubation of cells with (−)-PTZ (30 μM) caused a decrease in [^{3}H]NE uptake by the cells for up to 30 min, with a continuously maximal reduced level occurring at 20 min. Therefore, evaluation of PTZ's effect on [^{3}H]NE uptake was performed using cells pretreated with PTZ for 20 min. Treatment with (−)- or (+)-PTZ (3 – 100 μM) significantly inhibited [^{3}H]NE uptake in a concentration-dependent manner (Fig. 2). The half-maximal inhibitory concentrations (IC_{50}) for inhibition of [^{3}H]NE uptake by (−)- and (+)- PTZ were calculated as 54.59 ± 2.89 μM and 72.38 ± 2.96 μM, respectively. Incubation of cells with increasing concentrations of [^{3}H]NE (1 – 30 μM) showed that [^{3}H]NE uptake was saturable in both control and PTZ-treated cells (Fig. 3). Eadie-Hofstee analysis showed that both (−)- and (+)-PTZ caused a significant decrease in the maximal velocity (V_{max}) of [^{3}H]NE uptake with little change in the apparent Michaelis constant (K_m).

To investigate the involvement of opioid receptors and σ-receptors, we used naloxone, nor-Binaltorphimine, and BD-1047 as a non-selective opioid receptor antagonist, selective κ-opioid receptor antagonist, and selective σ-receptor antagonist, respectively. No antagonists, however, altered the effects of either (−)-PTZ, a κ-opioid receptor agonist, or (+)-PTZ, a σ-receptor agonist, on [^{3}H]NE uptake (Fig. 4), suggesting an opioid receptor– and σ-receptor–independent pathway. Since it is well-known that activation of protein kinase C down-regulates NET function, we examined the involvement of protein kinases in [^{3}H]NE uptake reduced by PTZ. Inhibitors of protein kinase C (calphostin C, chelerythrine, and GF109203X) did not affect the PTZ-induced inhibition of [^{3}H]NE uptake (Fig. 5). Furthermore, H-89, an inhibitor of cAMP-dependent protein kinase, and wortmannin, a phosphoinositide 3-kinase inhibitor, also had little effect (data not shown).

To determine the site of PTZ's action on NET, we examined the effects of PTZ on the specific binding of [^{3}H]nisoxetine, an specific inhibitor of NET, to plasma membranes isolated from bovine adrenal medulla. The specific binding of [^{3}H]nisoxetine to plasma membranes was saturable with an increasing concentration of 2 – 32 nM [^{3}H]nisoxetine, although (−)- and (+)-PTZ (30 μM) did not inhibit [^{3}H]nisoxetine binding (Fig. 6). Scatchard plot analysis showed that (−)- and (+)-PTZ (30 μM) had little effect on the maximal binding (B_{max}) or the dissociation constant (K_d) in comparison with that of the control. Since we could not observe the saturation curve of [^{3}H] nisoxetine specific binding to intact bovine adrenal

(A)

	K_m (μM)	V_{max} (pmol / 10^6 cells per min)
Control	4.88 ± 2.00	3.57 ± 0.21
(-)-PTZ	4.10 ± 1.71	2.28 ± 0.42*

(B)

	K_m (μM)	V_{max} (pmol / 10^6 cells per min)
Control	4.73 ± 0.95	3.26 ± 0.42
(+)-PTZ	4.53 ± 0.56	2.14 ± 0.09*

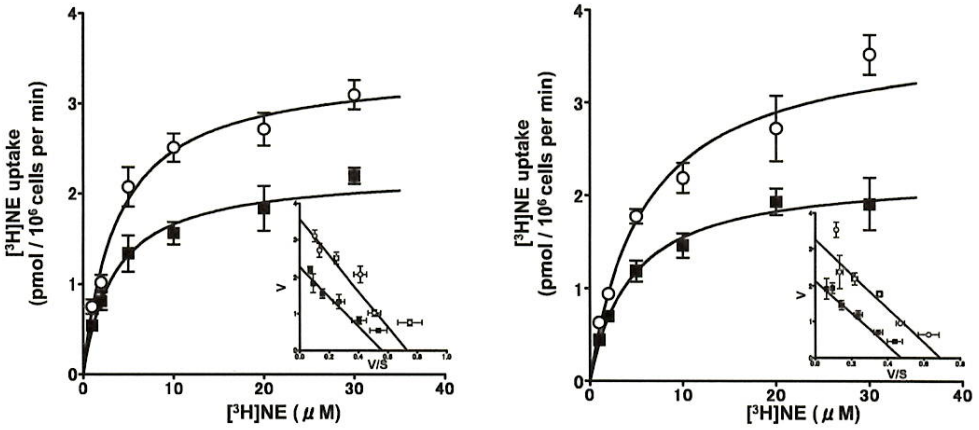


Fig. 3. Saturation curve of [^{3}H]NE uptake and its Eadie-Hofstee analysis. After preincubation with (closed squares) or without (open circles) (−)-PTZ (A) and (+)-PTZ (B) (30 μM) for 20 min, cells were incubated with various concentrations (1 – 30 μM) of [^{3}H]NE in the presence (closed squares) or absence (open circles) of (−)-PTZ (A) and (+)-PTZ (B) (30 μM) for 12 min at 37°C. [^{3}H]NE uptake was measured. Lower inset: Eadie-Hofstee analysis of [^{3}H]NE uptake. Data are means ± S.E.M. of three separate experiments carried out in duplicate. V, velocity (pmol / 10^6 cells per min); V/S, velocity / substrate concentration (pmol/10^6 cells·min·μM). *$P < 0.05$, compared with the control.

medullary cells, we used SK-N-SH cells, a human noradrenergic neuroblastoma cell line, instead of cultured adrenal medullary cells. Both (−)- and (+)-PTZ suppressed the specific binding of [^{3}H]nisoxetine (2 – 64 nM) to SK-N-SH cells and reduced the B_{max} of [^{3}H] nisoxetine binding without any change in the K_d (Fig. 7). (−)-PTZ (1.0 – 100 μM) suppressed the specific binding of [^{3}H]nisoxetine to SK-N-SH cells in a concentration-dependent manner (Fig. 8A) similar to that of [^{3}H]NE uptake by the cells (Fig. 8B). To examine whether (−)-PTZ–induced inhibition of [^{3}H]NE uptake or [^{3}H] nisoxetine binding occurs as a result of changes in surface expression of NETs, we determined the effect of (−)-PTZ on the population of NET proteins accessible to the membrane impermeant biotinylation reagent in human neuroblastoma SK-N-SH cells (Fig. 9). (−)-PTZ caused a significant decrease in the ratio of density of NET band in biotinylated fractions to that of total fraction to 52.2% of the control (Fig. 9B) and to β-actin to 61.7% of the control (Fig. 9C).

To investigate whether the inhibitory effect of PTZ on the transport function is specific for NET or not, we examined the effect of PTZ on another transporter, serotonin transporter (SERT), in rSERT cDNA transfected COS-7 cells. As shown in Fig. 10, (−)- and (+)-PTZ caused a significant reduction in [^{3}H]5-HT uptake in a concentration-dependent manner. We further checked the effect of PTZ on another membrane protein, voltage-dependent Ca^{2+} channels, by measuring $^{45}Ca^{2+}$ influx after treatment of cells with PTZ. (−)- and (+)-PTZ (30 μM), however, had little effect on 56 mM K^+–induced $^{45}Ca^{2+}$ influx (data not shown), suggesting that PTZ preferentially inhibits the function of monoamine transporters.

Discussion

PTZ, a non-narcotic analgesic with weak narcotic antagonistic activity, is advocated for the relief of moderate to severe pain. (−)-PTZ is a κ-opioid receptor agonist that induces analgesic effects, whereas (+)-PTZ is a σ-receptor agonist without analgesic effects. In addition to these receptors, we examined the effect of PTZ on NET function in adrenal medullary cells and SK-N-SH cells, a noradrenergic neuroblastoma cell line, to search for the active site of PTZ's effect on the descending noradrenergic pain modulatory pathways. In the present study, (−)-PTZ and (+)-PTZ significantly inhibited [^{3}H] NE uptake in a concentration (3.0 – 100 μM)-dependent manner. The inhibitory effect of (+)-PTZ (IC_{50} = 72.4 μM) was slightly less potent than that of (−)-PTZ

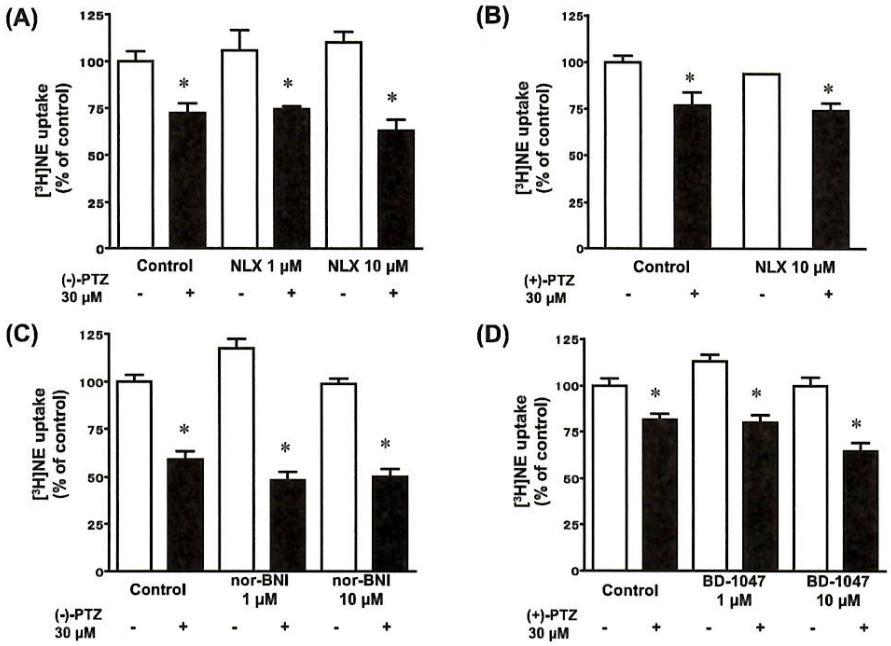


Fig. 4. Effect of naloxone (A and B), nor-Binaltorphimine (C), and BD-1047 (D) on (−)- or (+)-PTZ–induced inhibition of [^{3}H]NE uptake. A and B) After preincubation with or without the nonselective opioid receptor antagonist naloxone (NLX, 1 or 10 μM) for 20 min, cells were incubated for 12 min with [^{3}H]NE in the presence (black column) or absence (white column) of (−)-PTZ (A) or (+)-PTZ (B) (30 μM) and naloxone (NLX). C and D) After preincubation with or without the κ-opioid receptor antagonist nor-Binaltorphimine (nor-BNI, 1 or 10 μM) (C) or the σ-receptor antagonist BD-1047 (1 or 10 μM) (D) for 20 min, cells were incubated for 12 min with [^{3}H]NE in the presence (black column) or absence (white column) of (−)-PTZ (C) or (+)-PTZ (D) (30 μM) and each antagonist. The desipramine-sensitive [^{3}H]NE uptake by the cells was measured. Data are means ± S.E.M. of three separate experiments carried out in triplicate and expressed as % of the control. *$P < 0.05$, compared with each 0 μM PTZ.

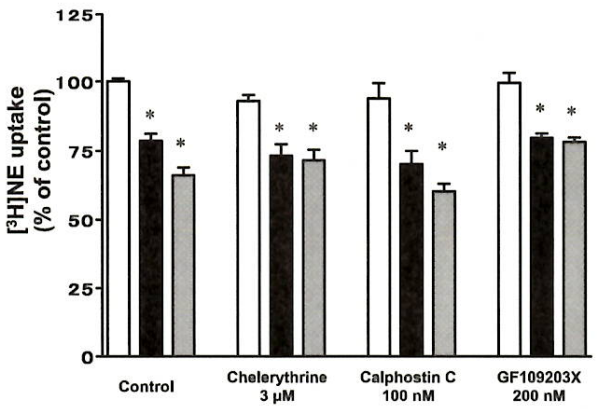
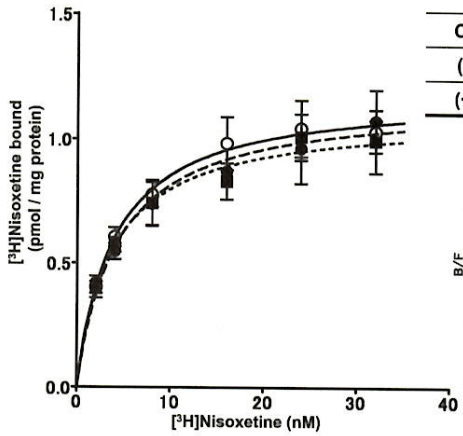


Fig. 5. Effect of various inhibitors of protein kinase C on PTZ-induced inhibition of [^{3}H]NE uptake. The cells were pretreated for 20 min with or without various inhibitors of protein kinase C and then incubated for 12 min with [^{3}H]NE in the presence (black column or gray column) or absence (white column) of (−)-PTZ (black column) or (+)-PTZ (gray column) (30 μM) and various inhibitors of protein kinase C. The desipramine-sensitive [^{3}H]NE uptake by the cells were measured. Data are means ± S.E.M. of three separate experiments carried out in triplicate and expressed as % of the control. *$P < 0.05$, compared with each 0 μM PTZ.

(IC_{50} = 54.6 μM). As much as 80% – 90% of NE released from presynaptic terminals is believed to be physiologically taken up again by the presynaptic neurons, thereby terminating neurotransmission (12). Therefore, even a slight inhibition of NET activity induced by PTZ may enhance noradrenergic neurotransmission.

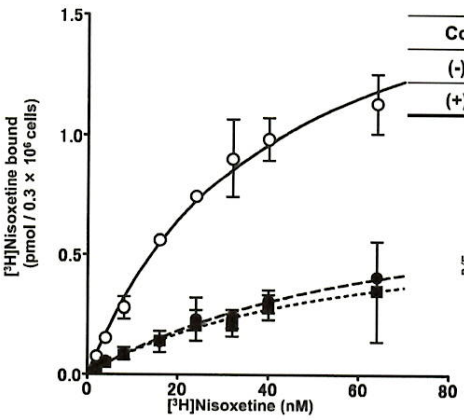
Down-regulation of NET function by PTZ

To study the site(s) of action of PTZ on NET, we examined the effects of PTZ on kinetic parameters for [^{3}H]NE uptake by the cells. The Eadie-Hofstee analysis of [^{3}H]NE uptake revealed that PTZ induces a decrease in the V_{max} of [^{3}H]NE uptake without any change in the K_m. These results suggest that PTZ inhibits the NET function by interacting with a site or sites other than the recognition site for NE. To investigate the possible involvement of opioid receptors, we used naloxone, a nonselective opioid receptor antagonist; nor-Binaltorphimine, a selective κ-opioid receptor antagonist; and BD-1047, a selective σ-receptor antagonist. None of these opioid and σ-receptor antagonists reversed the suppression of [^{3}H]NE uptake induced by (−)- and (+)-PTZ, suggesting that these effects are independent of opioid and σ receptors.



	K_d (nM)	B_{max} (pmol / mg protein)
Control	4.01 ± 0.34	1.19 ± 0.06
(-)-PTZ	4.30 ± 0.42	1.16 ± 0.13
(+)-PTZ	3.84 ± 0.57	1.10 ± 0.10

Fig. 6. Effects of (−)- or (+)-PTZ on specific binding of [^{3}H]nisoxetine to plasma membranes of bovine adrenal medulla and its Scatchard plot analysis. Plasma membranes isolated from bovine adrenal medulla were incubated at 4°C for 2 h with (closed circles or closed squares) or without (open circles) (−)-PTZ (closed circles) or (+)-PTZ (closed squares) (30 μM) in the presence of increasing concentrations (2 – 32 nM) of [^{3}H]nisoxetine. The specific binding of [^{3}H]nisoxetine was measured. Right inset: Scatchard plot analysis data of [^{3}H]nisoxetine binding. Data are means ± S.E.M. of three separate experiments carried out in duplicate. B, bound (pmol / mg protein); B/F, bound/free (pmol / mg protein per nM).



	K_d (nM)	B_{max} (pmol / 0.3 × 10^6 cells)
Control	51.41 ± 5.41	2.25 ± 0.24
(-)-PTZ	59.71 ± 6.85	0.79 ± 0.11*
(+)-PTZ	58.01 ± 8.82	0.70 ± 0.14*

Fig. 7. Effects of (−)- or (+)-PTZ on specific binding of [^{3}H]nisoxetine to intact SK-N-SH cells and its Scatchard plot analysis. After preincubation with (closed circles or closed squares) or without (open circles) (−)-PTZ (closed circles) or (+)-PTZ (closed squares) at 30 μM, SK-N-SH cells (0.3 × 10^6 cells) were incubated in the presence of increasing concentrations (2 – 64 nM) of [^{3}H] nisoxetine with or without (−)- or (+)-PTZ (30 μM) at 4°C for 2 h. The specific binding of [^{3}H]nisoxetine was measured. Right inset: Scatchard plot analysis data of [^{3}H]nisoxetine binding. Data are means ± S.E.M. of three separate experiments carried out in duplicate. B, bound (pmol / 0.3 × 10^6 cells); B/F, bound/free (pmol / 0.3 × 10^6 cells per nM). *$P < 0.05$, compared with the control.

NET is regulated by a number of intracellular signaling pathways. One common pathway is phosphorylation by several protein kinases such as cAMP-dependent protein kinase and protein kinase C (15, 28). Activation of protein kinase C is reported to down-regulate the function of NET in SK-N-SH cells (29) or transfected COS-7 cells (30). In the present study, inhibitors of protein kinase C (calphostin C, chelerythrine, and GF109203X), cAMP-dependent protein kinase (H-89), and phosphoinositide 3-kinase (wortmannin) had little effect on PTZ-induced inhibition of [^{3}H]NE uptake, suggesting that these protein kinases are not involved in the PTZ-

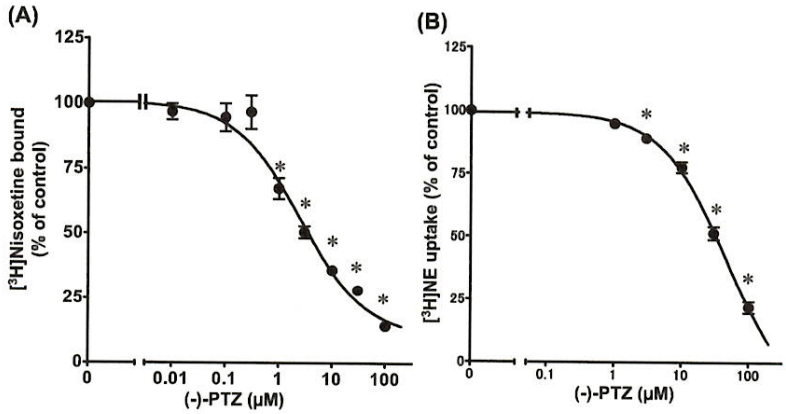


Fig. 8. Effect of various concentrations of (−)-PTZ on [^{3}H]nisoxetine specific binding (A) and [^{3}H]NE uptake (B) by SK-N-SH cells. A) After preincubation with various concentrations of (−)-PTZ for 30 min, the cells were incubated in [^{3}H]nisoxetine at 4°C for 2 h. The specific binding of [^{3}H]nisoxetine (20 nM) was measured. Data are means ± S.E.M. of three separate experiments carried out in duplicate and expressed as % of the control. The values of the control (0 μM PTZ) were 0.52 ± 0.01 pmol / 0.3 × 10^6cells. B) After preincubation with various concentrations of (−)-PTZ for 20 min, cells were incubated in the presence of [^{3}H]NE (100 nM) with various concentrations of (−)-PTZ for 12 min. The desipramine-sensitive [^{3}H]NE uptake by the cells was measured. Data were expressed as a percentage of the control (0.45 ± 0.04 pmol / 10^6 cells per min). *$P < 0.05$, compared with 0 μM PTZ.

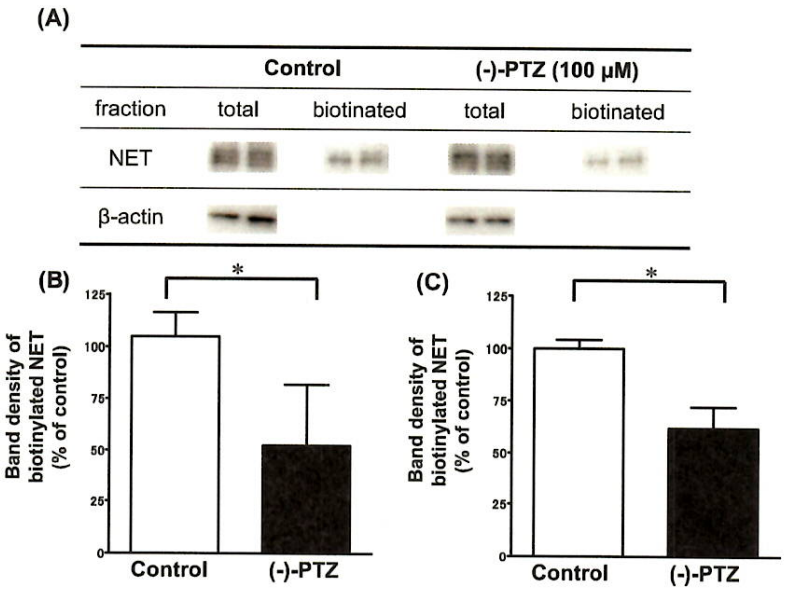


Fig. 9. Effect of (−)-PTZ on cell surface expression of NET proteins in SK-N-SH cells. SK-N-SH cells were pretreated with (black column) or without (white column) (−)-PTZ (100 μM) for 30 min and then biotinylated with sulfo-NHS-biotin. A) Aliquots of total and nonbiotinylated fractions were loaded, whereas the entire eluate from streptavidin beads was loaded as the biotinylated sample and blots were probed with NET antibody as described. B, C) Data are means ± S.E.M. of three separate experiments and are expressed as % of the control NET band [the ratio of the density of biotinylated NET fraction (54 kDa) to that of total NET] (B) and as % of the control (the ratio of the density of biotinylated NET fraction to that of β-actin) (C). *$P < 0.05$, compared with the control.

induced down-regulation of NET function.

Treatment of cells with PTZ for 30 min caused a suppression in the specific binding of [^{3}H]nisoxetine to intact SK-N-SH cells, although PTZ did not directly inhibit the specific binding of [^{3}H]nisoxetine to plasma membranes isolated from bovine adrenal medulla. Scatchard plot analysis showed that PTZ significantly decreased the B_{max} with little change in the K_d in SK-N-SH cells. These findings suggest that treatment of cells with PTZ inhibits the specific binding of [^{3}H]nisoxetine by reducing the binding sites of [^{3}H]nisoxetine on NET. Indeed, in the present study, we observed the decrease in membrane surface expression of NET proteins after treatment with PTZ. At present, however, the intracellular mechanism of PTZ-induced down-regulation of NET remains to be clarified. This is probably due to a reduction of the membrane trafficking of NET to the plasma membrane or an increase in its degradation or endocytosis by the lysozomal degradation system. The latter possibility may partially be excluded by the sub-

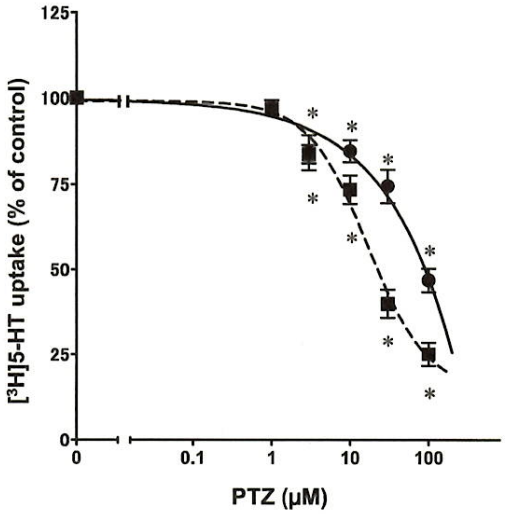


Fig. 10. Effects of (−)- and (+)-PTZ on [^{3}H]5-HT uptake by rat SERT cDNA transfected COS-7 cells. After transfection with cDNA rSERT for 24 – 48 h, COS-7 cells (1×10^6 / well) were preincubated at 37°C for 20 min in KRH buffer in the presence or absence of various concentrations of (−) (closed circles)- or (+) (closed squares)-PTZ. The cells were further incubated with [^{3}H]5-HT (50 nM, 0.1 μCi) at 37°C for 12 min in the presence or absence of (−)- or (+)-PTZ. Data are means ± S.E.M. of three separate experiments carried out in triplicate and expressed as % of the control (0.43 ± 0.01 pmol / 10^6 cells per min). *$P < 0.05$, compared with 0 μM each PTZ.

sequent finding that bafilomycin A1, a lysosomal inhibitor, did not reverse the inhibition of [^{3}H]NE uptake induced by PTZ (data not shown). Further study will be required to determine the mechanism by which PTZ down-regulates NET function.

The pharmacological significance of PTZ-induced down-regulation of NET

After intramuscular administration of 40 or 80 mg of PTZ, mean peaks of PTZ plasma concentration at 15 min were 102 and 227 ng/ml (0.318 and 0.707 μM), respectively (31). In the present study, the [^{3}H]NE uptake in adrenal medullary cells was significantly inhibited by PTZ at 3.0 – 100 μM, and the specific binding of [^{3}H] nisoxetine and [^{3}H]NE uptake of intact SK-N-SH cells were significantly inhibited by PTZ at 1 – 100 μM and 3.0 – 100 μM, respectively. Taken together, the present findings and previous data suggest that near-clinical concentrations of PTZ partly suppress the NET function of cultured bovine adrenal medullary cells.

Several lines of evidence have shown that the descending inhibitory system consists of noradrenergic and/or serotonergic neurons (32, 33). A recent study reported a potential use of 5-HT$_7$ receptor agonists as adjuvants of opioid analgesia because spinal activation of 5-HT$_7$ receptors has a role in the expression of opiate-induced analgesia through activation of descending inhibition (34). Furthermore, the antinociceptive effects of some clinical drugs, such as tricyclic antidepressants, are partially explained by enhanced noradrenergic or serotonergic neurotransmission induced by suppression of the NET or SERT in the descending inhibitory system in the brain and spinal cord (35). Indeed, in the present study, PTZ inhibited not only NET function but also SERT function, suggesting a preferential inhibition by PTZ of monoamine transporter functions. Furthermore, several anesthetics such as ketamine and propofol also inhibited the NET function (36, 37). Taken together with these results, it is intriguing to propose that PTZ induces antinociceptive effects via the down-regulation of NET and/or SERT in addition to the activation of opioid receptors.

Readers assessing the significance of the present findings should bear in mind the limitations of this study. First, the cellular mechanism by which PTZ induces the down-regulation of NET function has not been elucidated. Further investigations, including those on NET membrane trafficking and internalization or degradation, are needed to clarify its molecular mechanism after exposure to PTZ. Second, although cultured bovine adrenal medullary cells or SK-N-SH cells are a good in vitro model system of noradrenergic neurons, in vivo animal studies of PTZ are required to establish the involvement of NET down-regulation by PTZ in its antinociceptive effect.

In conclusion, the present findings suggest that near-clinical concentrations of PTZ induce the down-regulation of NET via suppression of cell surface expression of NET proteins. This may add a new antinociceptive aspect of PTZ to our pharmacological understanding of analgesics.

Acknowledgments

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