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Effect of prolonged exposure to milnacipran on norepinephrine transporter in cultured bovine adrenal medullary cells

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Abstract

The antidepressants milnacipran and paroxetine are used clinically worldwide. In the present study, we report here the effects of treatment with milnacipran and paroxetine on the functional activity, binding sites, and mRNA of the norepinephrine (NE) transporter (NET) in cultured bovine adrenal medullary cells. In acute treatment with antidepressants for 20 min, both milnacipran and paroxetine competitively inhibited NET function in cultured adrenal medullary cells. Prolonged treatment of adrenal medullary cells with milnacipran produced time (48–96 h)- and concentration (35–355 nM)-dependent increases in [^{3}H]NE uptake and [^{3}H]DMI binding without any increase in NET mRNA. At a high concentration (800 nM, 72 h), paroxetine suppressed [^{3}H]NE uptake. To examine whether milnacipran-induced [^{3}H]NE uptake is mediated by newly synthesized mRNAs or proteins, we used actinomycin D, an inhibitor of DNA-dependent RNA polymerase, and cycloheximide, an inhibitor of ribosomal protein synthesis. Cycloheximide (1 μM, 72 h) abolished the effect of milnacipran on [^{3}H]NE uptake, while the stimulatory effect of milnacipran was observed in actinomycin D-treated cells. The present findings suggest that prolonged exposure to milnacipran up-regulates the NET function, probably through a post-transcriptional process of NET or other proteins.

Keywords: Adrenal medullary cells; Milnacipran; Norepinephrine transporter; Serotonin norepinephrine reuptake inhibitor; Up-regulation

1. Introduction

The norepinephrine transporter (NET) belongs to the family of Na^+/Cl^--dependent neurotransmitter transporters [see reviews, 1,2]. NETs located on plasma membranes of noradrenergic nerve terminals remove NE from the extracellular milieu [3,4] and terminate the action of NE in synapses [2]. NET is known to be a target for antidepressants. Enhanced noradrenergic transmission occurs as a direct consequence of the antidepressant-induced inhibition of NET, which has been speculated to be the first process of antidepressant action. However, since prolonged treatment with antidepressants is necessary to achieve clinical efficacy for depression, molecular adaptations of the NET may also be involved in adaptive responses to antidepressants.

Milnacipran is one of the serotonin norepinephrine reuptake inhibitors (SNRIs), selected from a series of cyclopropane derivatives for its biochemical and pharmacological profile, which is known for its equipotent inhibition of NE and serotonin uptake and its lack of effect at any post-synaptic receptors [5,6]. In light of these observations, SNRIs such as milnacipran are regarded as having a mode of action that produces maximal efficacy and early onset with minimal side effects. Paroxetine is a well-known antidepressant that belongs to the group of selective serotonin reuptake inhibitors (SSRIs) prescribed worldwide [7]. Therapeutic concentrations of paroxetine, however, also inhibit [^{3}H]NE uptake and [^{3}H]nisoxetine binding in the rat brain [8].

Abbreviations: KRH, Krebs-Ringer HEPES; DMI, desipramine; NET, norepinephrine transporter; SNRI, serotonin norepinephrine reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor; RT-PCR, reverse transcriptase-polymerase chain reaction

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Adrenal medullary cells derived from the neural crest share many physiological and pharmacological properties with noradrenergic neurons and abundantly express the NET [9]. Lingen et al. [10] reported the cloning of a bovine adrenal medullary NET. They also showed that the pharmacological properties of the NET in bovine adrenal medullary cells were similar to those of central and peripheral noradrenergic neurons [11]. Therefore, bovine NET in adrenal medullary cells has been used as a convenient model system to analyze the actions of antipsychotic drugs or other agents on NET functions [12–15].

Desipramine (DMI), which is known to be a selective NET inhibitor, has been shown to down-regulate [16–19] or up-regulate [20,21] binding sites or the mRNA levels of the NET. Therefore, the regulation of NET expression or function in response to treatment with this antidepressant still remains controversial. In the present study, we examined the effects of prolonged exposure to milnacipran on the NET in cultured bovine adrenal medullary cells. To compare with milnacipran, we chose paroxetine as an inhibitor for the NET [8]. Moreover, to gain knowledge of the alteration of the NET expression after exposure to milnacipran, we measured the level of NET mRNA and used inhibitors of mRNA and protein synthesis such as actinomycin D and cycloheximide, respectively. We found that milnacipran up-regulates the NET function without any increase in its mRNA, while paroxetine suppresses it.

2. Materials and methods

2.1. Materials

Drugs and reagents were obtained from the following sources: l-NE (Nacalai Tesque, Kyoto, Japan); DMI hydrochloride, actinomycin D, and cycloheximide (Sigma, St. Louis, MO, USA); nisoxetine hydrochloride (Research Biochemicals International, Natick, MA, USA), l-[7, 8-^{3}H]NE (34.0 Ci/mmol), and [Benzene ring, 10,11-^{3}H]-DMI (73.0 Ci/mmol) (Perkin-Elmer Life Sciences, Boston, MA, USA). Milnacipran hydrochloride was a generous gift from Asahi Kasei Corporation (Tokyo, Japan). Paroxetine hydrochloride was kindly provided by Smith Kline Beecham (West Sussex, UK).

2.2. Isolation of bovine adrenal medullary cells and primary culture

Bovine adrenal medullary cells were isolated by collagenase digestion of slices of the adrenal medulla, and were suspended in Eagle's minimum essential medium (MEM) containing 10% calf serum and several antibiotics [22,23]. These cells were maintained in a monolayer culture at a density of 4×10^6 cells per dish (Falcon 35 mm, Becton Dickson Labware, Franklin Lakes, NJ, USA) in 5% CO_2/95% air.

2.3. [^{3}H]NE uptake by the cells after prolonged exposure to antidepressants

Bovine adrenal medullary cells (4×10^6/dish) were treated with or without antidepressants for 24–96 h in a CO_2 incubator at 37 °C. After treatment, cells were washed and incubated in drug-free culture medium for another 3 h to remove antidepressants from cells. Then, the cells were used for assay of [^{3}H]NE uptake. Cells were incubated with Krebs-Ringer HEPES (KRH) buffer containing 100 μM pargyline to inhibit metabolism of NE by monoamine oxidases, 1 mM ascorbic acid and [^{3}H]NE (500 nM, 0.1 μCi) at 37 °C for 20 min in the presence or absence of DMI (10 μM) [24]. 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. In some experiments, [^{3}H]NE (1–30 μM, 0.1 μCi) was used for the kinetic analysis. After washing the cells, the radioactivity in the cells was counted. DMI-sensitive uptake was calculated by subtracting the value obtained in the presence of 10 μM DMI from that obtained in the absence of DMI.

2.4. [^{3}H]NE uptake by the cells after acute exposure of cells to antidepressants

Cultured adrenal medullary cells were incubated with [^{3}H]NE (500 nM or 1–30 μM, 0.1 μCi) at 37 °C for 20 min in the presence or absence of antidepressants. After washing, DMI-sensitive [^{3}H]NE uptake by the cells was measured as described above.

2.5. [^{3}H]DMI binding to crude plasma membranes from cultured cells after prolonged exposure to milnacipran

Plasma membranes were prepared from cultured bovine adrenal medullary cells according to the method of Jayanthi et al. [25] with a slight modification [24]. In brief, the cells were cultured in 100-mm diameter Petri dishes at a density of 1×10^8 cells per dish in the presence or absence of milnacipran. After treatment with milnacipran for the indicated period, cells were washed and incubated in milnacipran-free medium for 3 h. Cells were lysed with ice-cold 10 mM Tris–HCl buffer (pH 7.4) containing 2 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride, homogenized for 30 s in an Ultra-Turrax, and centrifuged at $3000 \times g$ for 15 min. The supernatant was centrifuged at $60{,}000 \times g$ for 30 min, and the pellet containing plasma membranes was suspended in a binding buffer. The binding buffer was 10 mM Tris–HCl buffer (pH 7.4) containing 135 mM NaCl, 5 mM KCl, and 1 mM $MgSO_4$. The crude plasma membranes (100 μg of proteins/250 μL/tube) were used for [^{3}H]DMI binding assay. The equilibrium binding of [^{3}H]DMI to the plasma membranes was assayed by

incubating the membranes with different concentrations of the ligand (10 nM or 1–24 nM) at 25 °C for 30 min. Specific binding of [^{3}H]DMI was defined as a binding that was inhibited by the selective NET inhibitor nisoxetine (10 μM). The amount of membrane protein was determined by the method of Lowry et al. [26].

2.6. Preparation of plasma membranes of bovine adrenal medulla in acute experiments of antidepressants

Plasma membranes were partially purified from fresh bovine adrenal medulla as described previously [27] with a slight modification [12]. The plasma membranes (20 μg of proteins/250 μL/tube) of the adrenal medulla were incubated with [^{3}H]DMI (10 nM or 1–24 nM, 0.1 μCi) in the presence or absence of antidepressants at 25 °C for 30 min. Nisoxetine-sensitive binding of [^{3}H]DMI was measured as described above.

2.7. Reverse transcriptase-polymerase chain reaction (RT-PCR) of NET mRNA

Total RNA was extracted from bovine adrenal medullary cells (4×10^6/dish) with a Midi kit (Qiagen, Hilden, Germany), and reverse transcriptase-polymerase chain reaction (RT-PCR) was performed with a thermal cycler (Perkin-Elmer, Norwalk, CT) with use of a kit (Toyobo, Osaka, Japan). The sense and antisense primers for NET were 5′-GCACATCGACTTCTACCGCCTAA-3′ and 5′-CCAAAGGAGACAGCAAACGTGAA-3′, and those for cyclophilin B were 5′-GGTGTACTTTGACCTGCGAATTG-3′ and 5′-GCTCTCTACCTTCCGTACTACATC-3′, respectively. The PCR was performed under the following conditions: 24 cycles at 94 °C for 30 s, 58 °C for 60 s, and 72 °C for 90 s. Linear increases of the PCR products were observed between 22 and 26 cycles for NET and between 20 and 26 cycles for cyclophilin B (unpublished observations). Cyclophilin B was used as a housekeeping gene [28]. The sizes of PCR products expected on the basis of each primer pair are 471 bps for NET and 417 bps for cyclophilin B, respectively. The resultant PCR product was subjected to gel electrophoresis (2% agarose), stained with GelStar Nucleic Acid Gel Stain (Takara, Otsu, Japan), and analyzed using FLA-2000 Fluoroimage Analyzer (Fujifilm, Tokyo, Japan).

2.8. Statistics

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 analysis of variance (ANOVA). If a significant *F*-value was found, Scheffé's test for multiple comparisons was carried out to identify differences among groups. When $p < 0.05$, the differences were considered statistically significant.

3. Results

3.1. Kinetic study of acute inhibitory effect of milnacipran and paroxetine

When cultured adrenal medullary cells were incubated with various concentrations of milnacipran and paroxetine at 37 °C for 20 min, they significantly reduced [^{3}H]NE uptake in a concentration-dependent manner (Fig. 1(A)). Milnacipran inhibited [^{3}H]NE uptake by 27, 51, 75, and

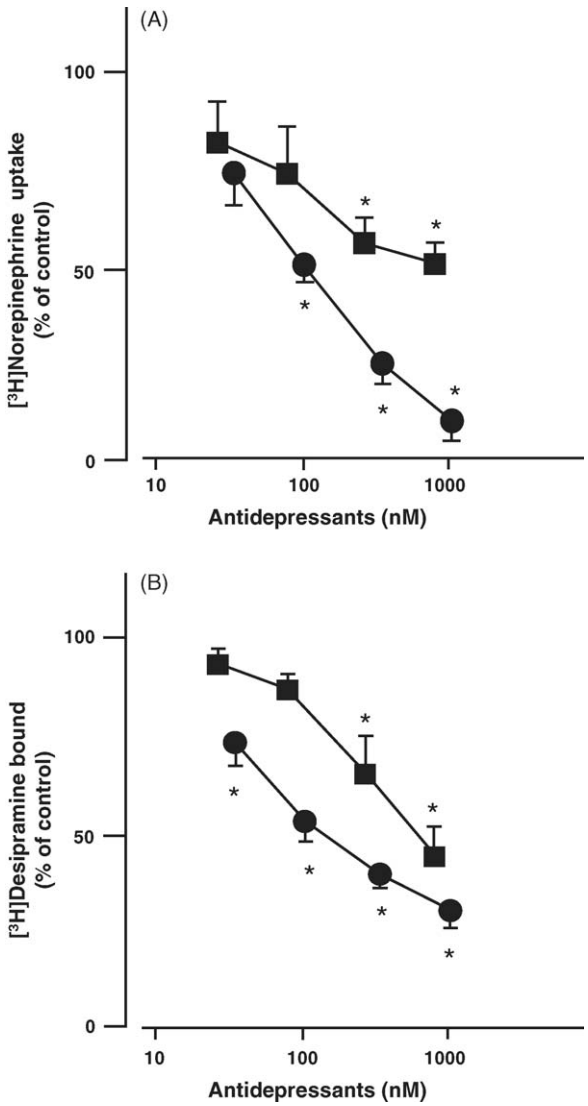


Fig. 1. Acute effects of antidepressants on [^{3}H]NE uptake by cultured adrenal medullary cells (A) and on [^{3}H]DMI binding to the plasma membranes (B). (A) The cells were incubated with milnacipran (●) or paroxetine (■) in the presence of [^{3}H]NE (500 nM, 0.1 μCi) at 37 °C for 20 min. The desipramine-sensitive uptake of [^{3}H]NE by the cells was measured. (B) The plasma membranes partially purified from bovine adrenal medulla were incubated with milnacipran (●) or paroxetine (■) in the presence of [^{3}H]DMI (10 nM, 0.1 μCi) at 25 °C for 30 min. The nisoxetine-sensitive binding of [^{3}H]DMI to the plasma membranes was counted. Data are means (±S.E.M.) of at least three separate experiments carried out in triplicate and expressed as a percentage of the control. Control [^{3}H]NE uptake and [^{3}H]DMI binding were 1.51 ± 0.11 pmol/4×10^6 cells/min and 2.67 ± 0.27 pmol/min/mg protein, respectively. $^*p < 0.05$, compared with control (no drug).

Table 1
Acute inhibition of [^{3}H]NE uptake and [^{3}H]DMI binding by antidepressants

	^{3}H-NE uptake			^{3}H-DMI binding		
	IC_{50} (nM)	K_m (μM)	V_{max} (pmol/4 × 10^6 cells/min)	IC_{50} (nM)	K_d (nM)	B_{max} (pmol/mg)
Control		2.9 ± 0.3	3.9 ± 0.1		10.5 ± 1.3	3.7 ± 0.6
Milnacipran	106 ± 15	8.6 ± 1.9*	4.2 ± 0.2	169 ± 17	41.6 ± 9.5*	4.0 ± 0.8
Paroxetine	677 ± 131	6.9 ± 0.8*	4.0 ± 0.4	659 ± 118	41.1 ± 7.9*	3.1 ± 0.9

(Left) Acute effects of antidepressants on [^{3}H]NE uptake by cultured adrenal medullary cells. Cells were incubated with or without milnacipran (177 nM) and paroxetine (800 nM) in the presence of [^{3}H]NE (1–30 μM, 0.1 μCi) at 37 °C for 20 min. The DMI-sensitive uptake of [^{3}H]NE by the cells were measured and evaluated by using GraphPad Prism soft ware (San Diego, CA, USA). Data are means (±S.E.M.) of at least three separate experiments carried out in duplicate. *$p < 0.05$, compared with control. (Right) Acute effects of antidepressants on [^{3}H]DMI binding to plasma membranes isolated from bovine adrenal medulla. The plasma membranes were incubated with or without milnacipran (177 nM) or paroxetine (800 nM) in the presence of [^{3}H]DMI (1–24 nM, 0.1 μCi) at 25 °C for 30 min. The nisoxetine-sensitive binding of [^{3}H]DMI to plasma membranes was measured and evaluated. Data are means (±S.E.M.) of at least three separate experiments carried out in duplicate. *$p < 0.05$, compared with control.

90% of control at 35, 106, 355, and 1065 nM (10, 30, 100, and 300 ng/mL), respectively (IC_{50} = 106 nM). Paroxetine inhibited [^{3}H]NE uptake by 20, 27, 45, and 49% of control at 27, 80, 267, and 800 nM (10, 30, 100, and 300 ng/mL), respectively (IC_{50} = 677 nM). Incubation of cells with increasing concentrations of [^{3}H]NE (1–30 μM) showed a saturable process of [^{3}H]NE uptake with increasing concentrations of [^{3}H]NE in control (no drug) and milnacipran (177 nM; 50 ng/mL)- or paroxetine (800 nM; 300 ng/mL)-treated cells (data not presented). Acute treatment with milnacipran and paroxetine increased the apparent Michaelis constant (K_m) of [^{3}H]NE uptake without any significant change of the maximal velocity (V_{max}) (Table 1). Milnacipran and paroxetine also inhibited the specific binding of [^{3}H]DMI, a selective radioligand for NET, in a concentration-dependent manner (Fig. 1(B)). Milnacipran decreased [^{3}H]DMI binding by 28, 48, 62, and 70% of control (no drugs) at 35, 106, 355, and 1065 nM, respectively (IC_{50} = 169 nM). Paroxetine reduced [^{3}H]DMI binding by 8, 15, 36, and 57% of control at 27, 80, 267, and 800 nM, respectively (IC_{50} = 659 nM). Specific binding of [^{3}H]DMI showed a saturable process with increasing concentrations of [^{3}H]DMI in control (no drugs) and milnacipran (177 nM)- or paroxetine (800 nM)-treated cells (data not presented). Milnacipran and paroxetine increased the dissociation constant (K_d) of [^{3}H]DMI binding without changing the maximal binding (B_{max}) (Table 1).

3.2. Effect of prolonged exposure to antidepressants on [^{3}H]NE uptake

Prolonged exposure of cells to milnacipran at 355 nM (100 ng/mL) for 72 h caused a significant increase in [^{3}H]NE uptake by the cells, whereas paroxetine at 267 nM (100 ng/mL) had little effect but suppressed it at 800 nM (300 ng/mL) (Fig. 2). The milnacipran-induced increase was significant at 48 h and reached a plateau at 72 h (64% increase over the control) (Fig. 3(A)). Milnacipran stimulated [^{3}H]NE uptake in a concentration-dependent manner (35–355 nM) (Fig. 3(B)).

After treatment with or without milnacipran (355 nM) for 72 h, cells were incubated with with [^{3}H]NE (1–30 μM). [^{3}H]NE uptake was saturable both in control and milnacipran-treated cells. We observed a significant increase in V_{max} (control, 3.8 ± 0.4 pmol/4 × 10^6 cells per min; milnacipran, 6.5 ± 1.8 pmol/4 × 10^6 cells per min, $p < 0.05$) without any change in K_m (control, 5.4 ± 2.0 μM; milnacipran, 6.2 ± 1.8 μM) (Fig. 4(A)).

3.3. Stimulation by milnacipran on [^{3}H]DMI binding to plasma membranes

After treatment of cells with or without milnacipran (355 nM) for 72 h, the plasma membranes isolated from control or milnacipran-treated cells were incubated with

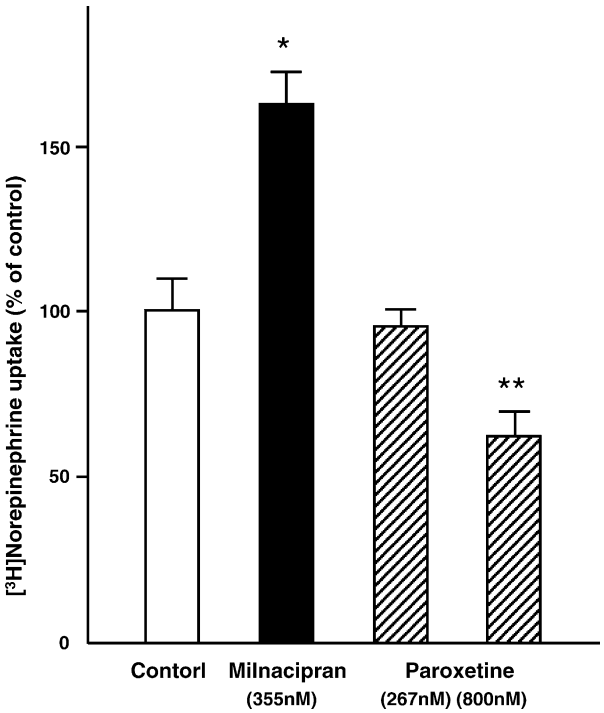


Fig. 2. Effects of 72 h exposure to the antidepressants on [^{3}H]NE uptake. After treatment with milnacipran (355 nM) or paroxetine (267 and 800 nM) for 72 h, the adrenal medullary cells were cultured for another 3 h in drug-free medium, and then incubated at 37 °C for 20 min in the presence of [^{3}H]NE (500 nM, 0.1 μCi). DMI-sensitive[^{3}H]NE uptake was measured. The results are means (±S.E.M.) of at least four separate experiments carried out in triplicate. *$p < 0.05$ and **$p < 0.01$, compared with control.

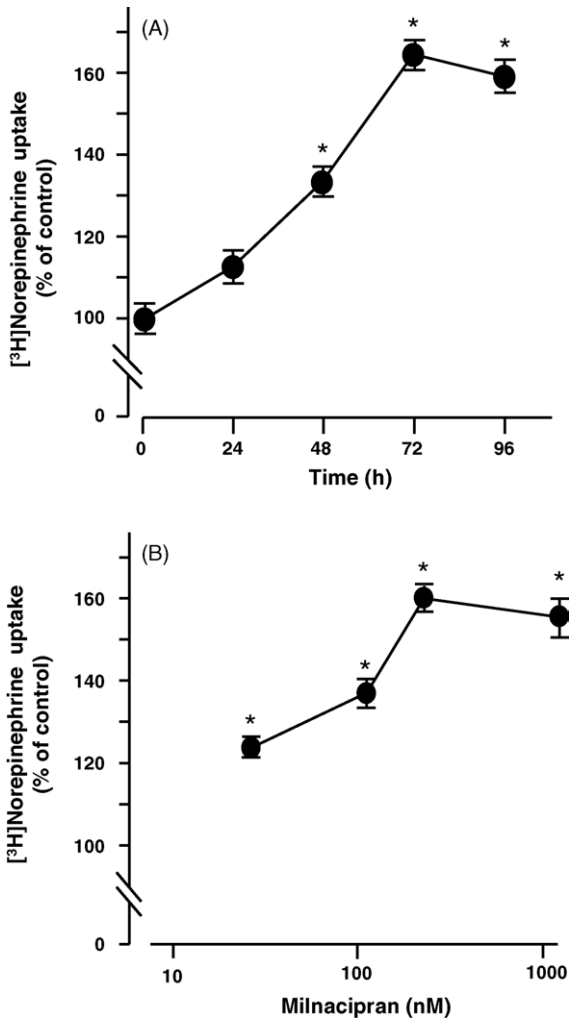


Fig. 3. Effects of long-term treatment with milnacipran on [^{3}H]NE uptake. (A) After preincubation with milnacipran (355 nM) for the period indicated, the cells were cultured for another 3 h in drug-free medium and then incubated at 37 °C for 20 min in the presence of [^{3}H]NE (500 nM, 0.1 μCi). (B) After preincubation with various concentrations (0–1065 nM) of milnacipran for 72 h, cells were cultured for another 3 h in milnacipran-free medium and then incubated at 37 °C for 20 min in the presence of [^{3}H]NE (500 nM, 0.1 μCi). The results are means (±S.E.M.) of at least four separate experiments carried out in triplicate. $^*p < 0.05$, compared with control.

[^{3}H]DMI (1–24 nM). Prolonged treatment with milnacipran caused a significant ($p < 0.05$) increase in the B_{max} (control, 95 ± 9 fmol/mg protein; milnacipran, 155 ± 12 fmol/mg protein, $p < 0.05$) without any change in the K_d (control, 10.0 ± 2.0 nM; milnacipran, 11.2 ± 1.9 nM) (Fig. 4(B)).

3.4. Effects of actinomycin D and cycloheximide on milnacipran-induced increase in [^{3}H]NE uptake

To examine whether milnacipran-induced [^{3}H]NE uptake was mediated by newly synthesized mRNAs and proteins, we used actinomycin D, an inhibitor of DNA-dependent RNA polymerase, and cycloheximide, an inhibitor of ribosomal protein synthesis. Treatment with actinomycin D and cycloheximide by themselves suppressed the basal [^{3}H]NE uptake to 50 and 42% of control, respectively. Cycloheximide (1 μM, 72 h) abolished the milnacipran-induced effect on [^{3}H]NE uptake, while the stimulatory effect of milnacipran was still observed in actinomycin D-treated cells (Fig. 5).

3.5. No significant change in NET mRNA expression induced by milnacipran

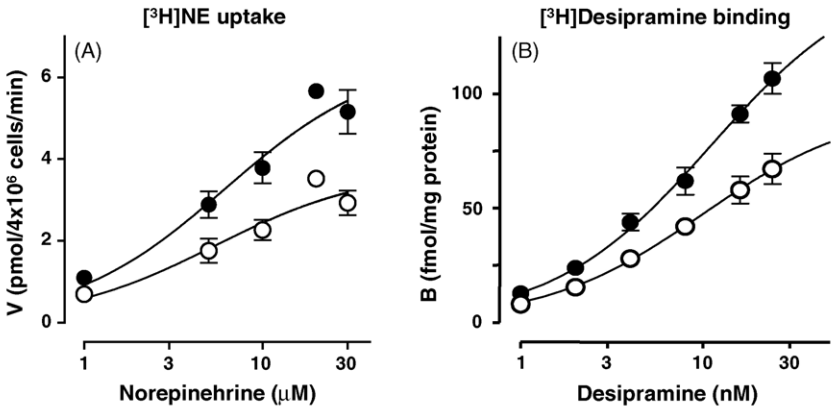
In the assay of mRNA expression, RT-PCR with NET and cyclophilin B primers yielded a single band corresponding to approximately 471 and 417 bps fragments, respectively (Fig. 6). The band for NET mRNA was sequenced and found that its sequence analyzed was identical to that of the reported bovine NET [10] (data not presented). The ratio of NET/cyclophilin B mRNA was decreased rather than increased after treatment with milnacipran (355 nM, 72 h), although the change in its ratio did not reach a statistical significance ($p = 0.08$).

4. Discussion

4.1. Acute competitive inhibition of [^{3}H]NE uptake and [^{3}H]DMI binding by milnacipran and paroxetine

In the present study, we demonstrated that milnacipran acutely inhibits both [^{3}H]NE uptake and [^{3}H]DMI binding in a concentration-dependent manner (the IC_{50} of 106 and 169 nM, respectively) (Fig. 1). Paroxetine also showed the IC_{50} of 677 and 659 nM in [^{3}H]NE uptake and [^{3}H]DMI binding, respectively. There was a good relation between [^{3}H]NE uptake and [^{3}H]DMI binding in the inhibition induced by these antidepressants. From the IC_{50} of milnacipran for [^{3}H]NE uptake, the inhibition constant (K_i) was calculated to be 100 nM, which is similar to that (the K_i of 68 nM) reported by Vaishnavi et al.[29]. On the other hand, the K_i of paroxetine (631 nM) was higher than that of the human NET (the K_i of 156 nM) reported by Owens et al. [8]. Although the reason for this difference is not clear, it may be due to the species difference of NET or different assay conditions. In fact, in the present study, the control K_m (2.9 μM) for [^{3}H]NE uptake and the K_d (10.5 nM) for [^{3}H]DMI binding of the bovine adrenal medullary NET seemed to be somewhat higher (by a factor 2.3–5.4) than the K_m (0.54 μM) for [^{3}H]NE uptake in human NET cells [30] and than the K_d (4.5 nM) for [^{3}H]DMI binding in rat pheochromocytoma cells (PC12-cells) reported by Bönisch and Harder [3].

In the acute treatment, both milnacipran and paroxetine significantly increased the K_m of [^{3}H]NE uptake without changing V_{max} (Table 1), indicating a competitive inhibition. These results suggest that both milnacipran and paroxetine inhibit the NET by interacting with the recognition site for NE. To further study the site of action of milnacipran and paroxetine on the NET, the effects of



	K_m (μM)	V_{max} (pmol/4x10⁶ cells/min)
Control	5.4 ± 2.0	3.8 ± 0.4
Milnacipran	6.2 ± 1.8	6.5 ± 1.8*

	K_d (nM)	B_{max} (fmol/mg protein)
Control	10.0 ± 2.0	95 ± 9
Milnacipran	11.2 ± 1.9	155 ± 12*

Fig. 4. Kinetic analysis of [^{3}H]NE uptake (A) and [^{3}H]DMI binding (B) after long-term treatment with milnacipran. (A) After treatment of cells with (●) or without (○) milnacipran (355 nM) for 72 h and preincubation for another 3 h in milnacipran-free medium, cells were incubated with various concentrations of [^{3}H]NE (1–30 μM, 0.1 μCi) at 37 °C for 20 min. (B) After treatment of cells for 72 h with (●) or without (○) milnacipran (355 nM) and incubation for 3 h in milnacipran-free medium, plasma membranes (100 μg of proteins) isolated from these cells were incubated at 25 °C for 30 min in the presence of increasing concentrations of [^{3}H]DMI (1–24 nM, 0.1 μCi). Curve fitting and estimation of K_m and V_{max} values for [^{3}H]NE uptake, and K_d and B_{max} for [^{3}H]DMI binding, respectively, were performed using GraphPad Prism Software (San Diego, CA, USA). These parameters are shown at the bottom. The results are means (±S.E.M.) of four separate experiments carried out in triplicate. $^*p < 0.05$, compared with control.

antidepressants on the specific binding of [^{3}H]DMI to the plasma membranes of adrenal medullary cells were examined. Both drugs significantly increased K_d without altering B_{max} (Table 1), showing a competitive inhibition. These results indicate that milnacipran and paroxetine inhibit the NET by interacting with the binding site for DMI. Although we could not identify the exact site of action of milnacipran or paroxetine on the NET, the competitive inhibition of [^{3}H]NE uptake and [^{3}H]DMI binding by these antidepressants suggests that they bind to a region that overlaps the sites responsible for NE recognition and antidepressant binding. Further studies using various NET mutants, in which the amino acids within the assumed binding area of the antidepressants are changed, would provide more information on their precise sites on the NET.

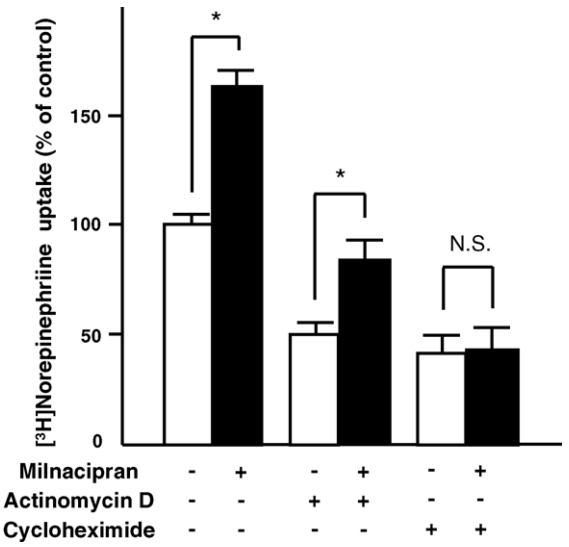


Fig. 5. Effects of actinomycin D and cycloheximide on [^{3}H]NE uptake in milnacipran-treated cells. Cells were treated with or without milnacipran (355 nM), actinomycin D (2 ng/mL), and cycloheximide (1 μM) for 72 h, preincubated for 3 h in drug-free medium and incubated for 20 min at 37 °C with [^{3}H]NE (500 nM, 0.1 μCi). Data are means (±S.E.M.) of four separate experiments carried out in duplicate. $^*p < 0.05$, compared with each milnacipran (−).

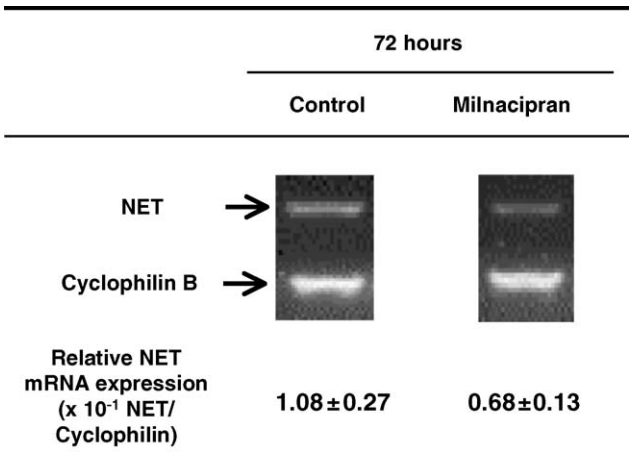


Fig. 6. Effect of long-term treatment with milnacipran on NET mRNA expression. RT-PCR was performed. The figure shows single bands of the PCR products for NET and cyclophilin B mRNA after separation by gel electrophoresis (2% agarose). Relative level of NET mRNA expression was determined by quantifying the fluorescence intensity of the bands. The results are means (±S.E.M.) of separate three experiments carried out in triplicate.

4.2. Up-regulation of NET by prolonged treatment with milnacipran

In the present study, we found that prolonged exposure to milnacipran increased [^{3}H]NE uptake in time (48–96 h)- and concentration (35–355 nM)-dependent manners (Fig. 3). As shown in Fig. 4, long-term treatment with milnacipran stimulated the functional activity of NET and [^{3}H]DMI binding to the plasma membranes, suggesting that the increased function is related to the increased plasma transporter expression, with the caveat that the surface expression of NET, however, was not determined in the study. The increase in [^{3}H]NE uptake induced by milnacipran was diminished by cycloheximide, an inhibitor of ribosomal protein synthesis, while actinomycin D was found to have little effect on the stimulatory effects of milnacipran (Fig. 5). Although these inhibitors can lead to many non-specific effects and the interpretation of results using these inhibitors would be viewed with caution, milnacipran might increase the NET function, probably through a post-transcriptional process of NET or other proteins. In the former case, it is compatible with the present result that treatment with milnacipran (355 nM) for 72 h did not affect a significant change in the level of NET mRNA (Fig. 6). In the latter case, recent studies have shown that an interaction of NET with a number of intracellular proteins such as syntaxin 1A, protein phosphatase 2A, or 14-3-3 proteins [31,32] influence NET activity, trafficking, and response to drugs, although the definition of interactions and relating those interactions to NET regulation remains to be clarified. To determine the precise mechanism of milnacipran on NET function, further experiments including membrane trafficking, internalization or degradation of NET would be required. Nonetheless, to our knowledge, this is the first direct evidence to show that prolonged treatment with milnacipran up-regulates the NET function.

Several compounds structurally unrelated to selective NET inhibitors are known to up-regulate NE uptake. For example, we previously reported the up-regulation of NET function and mRNA levels by prolonged treatment with clozapine, a prototype of atypical antipsychotic agents [15], and ketamine, an intravenous anesthetic [13] in cultured bovine adrenal medullary cells. Both of them acutely inhibit NET functions in the cells. However, as shown in Fig. 2, treatment with paroxetine at 800 nM for 72 h suppressed [^{3}H]NE uptake by the cells. A number of studies have reported that chronic treatment with typical NET inhibitors, DMI [33] and nisoxetine [18] and amphetamine, a psychostimulant [30] down-regulate the functional activity or binding sites of NET. For example, the repeated administration of DMI caused significant decreases in the binding of [^{3}H]nisoxetine in the rat brain in vivo [16]. Furthermore, long-term treatment with DMI reduced the B_{max} but not the K_d of [^{3}H]nisoxetine binding to NET in PC12 cells [18] and the NET protein levels in 293-human NET cells [19].

However, as has been demonstrated by others, reports on the NET mRNA levels remain controversial. In the present study, milnacipran had little effect on the level of NET mRNA. This result is in agreement with several previous reports showing that the 3-day exposure of 293-human NET cells to DMI did not alter NET mRNA [19], and that no change in the NET mRNA in the locus coeruleus of the rats was observed by treatment with DMI [33]. In contrast, NET mRNA was increased in the rat locus coeruleus following short- and long-term treatment with DMI [34], while Zavosh et al. [17] reported that 72-h DMI treatment decreased NET mRNA in a human neuroblastoma cell line.

In the present study, it is interesting to note that the up-regulation of NET function was induced by milnacipran, but not paroxetine. As described above, both milnacipran and paroxetine are competitive NET inhibitors which bind to the DMI binding site. However, as reported here, together with earlier reports, it is possible that the change in NET expression may not be due to the blockade of the transporter by NET inhibitors. The relationship between the functional inhibition and expression (or regulation) of NET still remains to be clarified.

4.3. Clinical relevance of NET up-regulation induced by milnacipran

In the present study, milnacipran, one of the SNRIs, and paroxetine, a well-known SSRI, acutely inhibited [^{3}H]NE uptake by the cells. After oral administration of the therapeutic dose, the plasma concentrations of milnacipran and paroxetine in patients with a major depressive disorder reached approximately 10–250 ng/mL (35–887 nM) and 30–310 ng/mL (80–827 nM), respectively (our unpublished observations). Furthermore, previous studies have found that the plasma concentrations of milnacipran rapidly peak at around 2.0 h post-dose with a mean maximal plasma concentration (C_{max}) of 135 ng/mL (479 nM) (single dose of 50 mg, oral administration)[35] or that steady-state plasma concentrations of paroxetine in nonelderly depressed patients receiving a chronic oral administration of a daily dose of 40 mg paroxetine are in the range of 1.7–407 ng/mL (3.7–1086 nM) [36]. These reports are in agreement with our unpublished observations. Therefore, the concentrations of antidepressants used in the present study would be clinically relevant.

Readers assessing the significance of these results should bear in mind the limitations of this study. First, the cellular mechanism by which milnacipran modulates membrane NET concentrations has not been elucidated. Although we found that milnacipran up-regulates the NET function without any increase in NET mRNA, further works such as NET internalization or degradation are needed to elucidate its molecular mechanisms after prolonged exposure to milnacipran. Second, cultured adrenal medullary cells may not be suitable for the study of long-

term (over 1 week) treatment with milnacipran. Much longer treatment to analyze the clinical effects of antidepressants on NET functions would be ideal because almost all antidepressants achieve clinical efficacy on the depressive state after at least 2 weeks.

In conclusion, our study suggests that prolonged exposure to milnacipran, but not paroxetine, up-regulates NET function, probably through a post-transcriptional process of NET or other proteins. The present findings add the new pharmacological action of milnacipran to our understanding of the treatment of patients with major depressive disorders.

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