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The Effects of T-588, a Novel Cognitive Enhancer, on Noradrenaline Uptake and Release in Rat Cerebral Cortical Slices

Mutsuko Maekawa[1,2], Toshihiko Murayama[1], Satoshi Ono[2], Hirokazu Narita[2] and Yasuyuki Nomura[1,*]

[1]*Department of Pharmacology, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060–0812, Japan*
[2]*Research Laboratories, Toyama Chemical Co., Ltd., Toyama 930–8508, Japan*

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ABSTRACT—Previously, we reported that (*R*)-(−)-1-(benzo[*b*]thiophen-5-yl)-2-[2-(*N*,*N*-diethylamino)-ethoxy]ethanol hydrochloride (T-588), a novel cognitive enhancer, stimulated noradrenaline (NA) release from rat cerebral cortical slices. In this study, we investigated the effects of T-588 on NA uptake and release, compared to the effects of desipramine, a blocker of the NA carrier on the plasma membrane. Both T-588 and desipramine caused dose-dependent inhibition of [^{3}H]NA uptake into the slices. Addition of 3 mM T-588 stimulated [^{3}H]NA release from the prelabeled slices even in the presence of 10 μM desipramine, which inhibited NA uptake completely. Tyramine, which accelerates NA carrier-mediated release, also stimulated [^{3}H]NA release, and tyramine-stimulated release was inhibited by desipramine. These findings indicated that T-588-stimulated NA release was not mediated by 1) inhibition of reuptake or 2) reverse transport mediated by NA carriers. Reserpine, which interacts with the intracellular vesicular transport system, increased [^{3}H]NA efflux from slices. High K^+-, not T-588-, stimulated [^{3}H]NA release was shifted upward by reserpine. These findings suggest that T-588 evokes NA release by a mechanism similar to that induced by reserpine. T-588 might act as a cognitive enhancer via neurotransmitter release in the brain.

Keywords: T-588, Noradrenaline uptake and release, Cerebral cortical slice, Desipramine, Reserpine

It was reported that cholinergic mechanisms may play an important role in learning and memory (1). The memory deficits produced by scopolamine are reinstated by cholinomimetic drugs such as oxotremorine and physostigmine (2). On the other hand, it was also reported that some adrenergic drugs can attenuate scopolamine-induced deficits (2, 3). Desipramine, a blocker of the noradrenaline (NA) carrier on the plasma membrane, has also been shown to attenuate the scopolamine-induced deficit in radial maze performance (4). Moreover, a relationship between the central noradrenergic and cholinergic systems has been reported to be involved in learning and memory processes (5). Recently, it was suggested that NA may exert a regulatory influence on the activity of hippocampal cholinergic neurons (6) and that the noradrenergic neurotransmitter system mediates favorable effects on various forms of amnesia (7). Therefore, NA is believed to play an important role in the enhancement of cognitive function.

T-588, (*R*)-(−)-1-(benzo[*b*]thiophen-5-yl)-2-[2-(*N*,*N*-diethylamino)ethoxy]ethanol hydrochloride, is a compound that is currently being assessed for its value in reversing the dementia associated with Alzheimer's disease and cerebrovascular disease. Previously, we reported that T-588 ameliorated memory and learning impairments in rats (8–11). The memory and learning deficits induced by injection of carbon-microspheres into the internal carotid artery were significantly improved by T-588, as determined by an active avoidance response assay (10). T-588 significantly improved the working memory deficit using a three-panel runway task (9) and the spatial memory deficit (11), following transient forebrain ischemia in rats, by daily administration at the chronic phase. We also reported that T-588 significantly prolonged the survival time in experimental cerebral anoxia in mice (8, 12). The anti-hypoxic effect of T-588 was completely inhibited by scopolamine, while the anti-anoxic effect was partially inhibited. Furthermore, the anti-ischemic effect of T-588 was not affected by scopolamine. Several investigations have reported relationships between catecholamines and hypoxia, anoxia, ischemia, and cognition (13–15). Accordingly, it is assumed that T-588 might en-

* To whom correspondence should be addressed.

hance not only cholinergic function but also other systems such as the noradrenergic system in the brain. In an in vivo microdialysis study, oral administration of 3 or 30 mg/kg T-588 to rats significantly increased acetylcholine and NA overflow in cortical and hippocampal dialysates (16). We also reported that T-588 increased the release of catecholamines such as NA, dopamine, and 5-hydroxytryptamine from rat brain slices (16), and the release of NA from rat cerebral cortical slices in a Ca^{2+}- and calmodulin-independent manner (17).

Neurotransmitter release is in general a strongly Ca^{2+}-dependent, exocytotic-like release (18). On the other hand, the existence of carrier-mediated, Na^{+}-dependent release of monoamines was confirmed (19, 20). This carrier-mediated release is blocked by transport inhibitors. Desipramine preferentially blocks the transport of NA. The effect of tyramine, which accelerates NA release via NA carrier-mediated transport (21), is blocked by desipramine. In addition, the NA re-uptake system, which is also sensitive to desipramine, contributes to NA release. It is also possible that T-588 inhibits the re-uptake of NA and/or enhance the reverse transport activity of the NA carrier, and thus T-588 stimulates NA release from the slices.

In this study, we investigated the effects of T-588 on NA uptake and NA release, compared to the effects of desipramine, in cerebral cortical slices. We also discuss the possible involvement of reserpine-sensitive processes in T-588-stimulated NA release from these slices.

MATERIALS AND METHODS

Materials

T-588 was synthesized in our laboratory. Desipramine was purchased from Sigma (St. Louis, MO, USA). Reserpine was purchased from Funakoshi (Tokyo) and tyramine and other materials were obtained from Wako Pure Chemical (Osaka). l-[7,8-^{3}H]NA (1.44 TBq/mmol) was purchased from Amersham (Buckinghamshire, UK). All chemicals were diluted with assay buffer after first being dissolved in distilled water and added to the assay mixture.

Animals

Male Wistar rats (Japan Laboratory Animals Inc., Tokyo) weighing 200–400 g were used in all the experiments. They were housed under conditions of constant temperature and controlled illumination (light on between 7:30 and 19:30). Food and water were available ad libitum.

Preparation of rat cerebral cortical slices

Rats were decapitated and the brains were rapidly removed. Cerebral cortical slices were prepared essentially according to the method described by Glowinski and Iversen (22). Briefly, after dissection of the cerebral cortex, crosschopped slices (400×400 μm) were made by hand, followed by filtration through a nylon mesh (300×300 μm). The slices were washed twice with a Tyrode's HEPES buffer (composition: 134 mM NaCl, 3 mM KCl, 1 mM Na_2HPO_4, 12 mM $NaHCO_3$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose and 20 mM HEPES, pH 7.0) followed by centrifugation at 4℃ ($100 \times g$, 15 sec). The Tyrode's buffer was oxygenated by bubbling with O_2 gas before use.

Measurement of [^{3}H]NA uptake

The slice suspensions (100–200 μg protein/tube) were incubated with 20 nM [^{3}H]NA for 8 min at 37℃ in Tyrode's buffer (pH 7.0) containing 10 μM nialamide (monoamine oxidase inhibitor), 20 units/ml aprotinine (protease inhibitor), 0.3 mM phenylmethylsulfonyl fluoride (serine protease inhibitor) and 0.1 mM ascorbic acid. The pH of the buffer used for the labeling reaction was lowered to avoid degradation of NA. The total volume was 0.3 ml. [^{3}H]NA uptake was terminated by the addition of 2 ml of ice-cold Tyrode's buffer containing choline chloride, substituted for NaCl, followed by rapid filtration through GF/C glass filters presoaked for 2 hr in 0.03% (v/v) polyethyleneimine to reduce nonspecific binding. This was followed by 3 washes with 2 ml of ice-cold choline-containing buffer. The bound radioactivity was determined with a liquid scintillation counter.

Measurement of [^{3}H]NA release

The slice suspensions were labeled with 50 nM [^{3}H]NA for 30 min at 37℃ in the Tyrode's buffer (pH 7.0) containing 10 μM nialamide, 20 units/ml of aprotinine, 0.3 mM phenylmethylsulfonyl fluoride and 0.1 mM ascorbic acid. The labeled slices were washed 3 times with ice-cold Tyrode's buffer (pH 7.4) by centrifugation at 4℃ ($100 \times g$, 15 sec) to remove extracellular [^{3}H]NA. The labeled and washed slices (100–200 μg protein/tube) were incubated for 8 min at 37℃ in modified Tyrode's buffer (in total volume, 0.3 ml) containing 0.2% bovine serum albumin with or without test compounds. The release reaction was terminated by addition of 0.5 ml of ice-cold HEPES buffer (137 mM NaCl, 20 mM HEPES, 5 mM EGTA and 5 mM EDTA, pH 7.4) followed by centrifugation at 4℃ ($1000 \times g$, 15 sec). The radioactivity in the supernatant was measured in a liquid scintillation counter. Data are presented as percentages of the total amount of [^{3}H]NA incorporated.

Statistics

Values are reported as the means±S.E.M. In the case

of multiple comparisons, significance of differences was determined by ANOVA followed by Dunnett's or the Tukey test. For pairwise comparisons, the Student's 2-tailed *t*-test was used. A probability value of $P<0.05$ was considered significant.

RESULTS

We investigated the effects of T-588 on the uptake of [^{3}H]NA into cerebral cortical slices (Fig. 1). Addition of T-588 caused the dose-dependent inhibition of [^{3}H]NA uptake. The IC_{50} value was 52.0±10.4 μM (n=4). Addition of desipramine, a blocker of NA carriers on the plasma membrane, also inhibited [^{3}H]NA uptake in a dose-dependent manner. The IC_{50} value of desipramine was 44.3±24.5 nM (n=4).

The uptake of neurotransmitters such as NA by the membrane carrier(s) is independent of extracellular Ca^{2+} (21). To confirm the Ca^{2+}-independency in our slice preparations, we studied the effects of 1 mM extracellular $CaCl_2$ on [^{3}H]NA uptake into rat cerebral cortical slices. The uptake of [^{3}H]NA into the slices in the presence and absence of extracellular $CaCl_2$ were 4.9±0.8 (% of total [^{3}H]NA in assay mixture, n=4) and 5.2±0.6 (% of total [^{3}H]NA in assay mixture, n=4), respectively. Addition of T-588 inhibited uptake in a similar dose-dependent manner in both the presence and absence of extracellular $CaCl_2$. The IC_{50} value of T-588 in the absence of $CaCl_2$ was 57.4±2.3 μM (n=4).

We measured [^{3}H]NA release from prelabeled slices. The procedure for measuring [^{3}H]NA release is well-established in neuronal tissues (23, 24), and the increase in tritium was composed predominantly of intact NA, not its metabolites (25). There were no differences in T-588-stimulated or KCl-evoked NA release in the presence or absence of the monoamine oxidase inhibitor nialamide in the assay mixtures (data not shown). Therefore, we studied NA release in assay mixtures without nialamide, except during labeling with [^{3}H]NA.

The addition of desipramine to the assay mixture for [^{3}H]NA release stimulated [^{3}H]NA release slightly; the net increase was 3.9±1.0% by 10 μM (Fig. 2). However, the addition of 3 mM T-588, a concentration that inhibited [^{3}H]NA uptake almost completely, stimulated [^{3}H]NA release markedly (net increase was 16.0±1.4%), as previously reported (17). Tyramine, which accelerates NA release via NA carrier-mediated transport (21), also stimulated [^{3}H]NA release. Desipramine inhibited the stimulatory effect of tyramine in a dose-dependent manner. However, T-588-stimulated [^{3}H]NA release was not affected by desipramine. These findings indicated that the stimulatory effect of T-588 may not be mediated by NA carrier-mediated transport on the plasma membrane.

Reserpine is known to increase the efflux of NA by

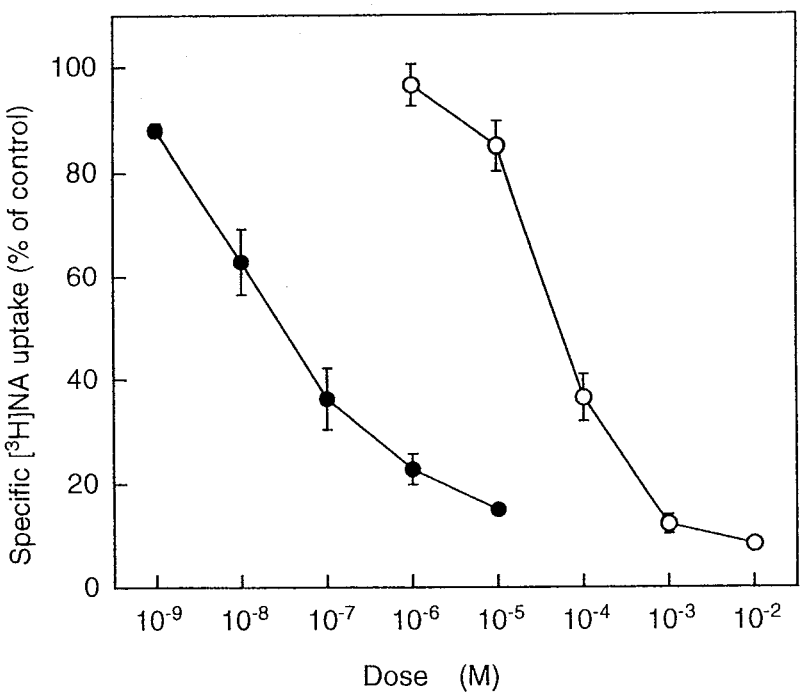


Fig. 1. Effects of T-588 and desipramine on the uptake of [^{3}H]NA into cerebral cortical slices. The slices were incubated with [^{3}H]NA for 8 min at 37℃ in the presence of the indicated concentrations of T-588 (○) or desipramine (●). Data are each the mean±S.E.M. from 4 experiments performed in triplicate.

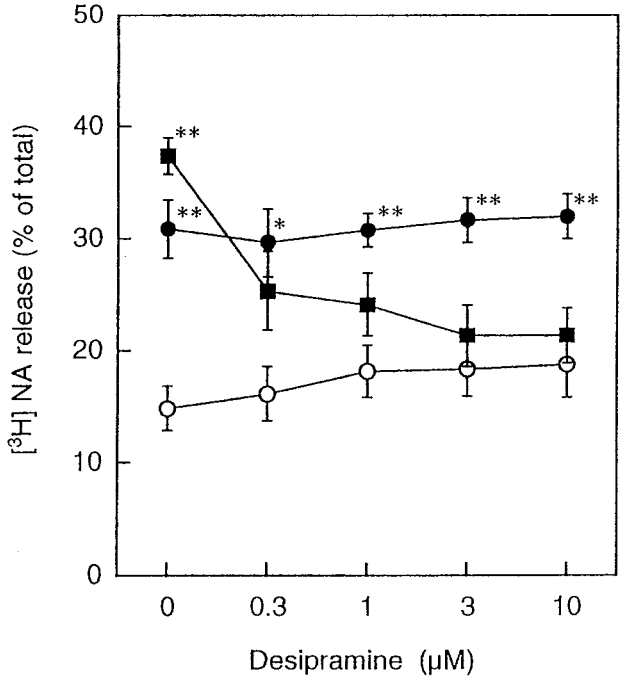
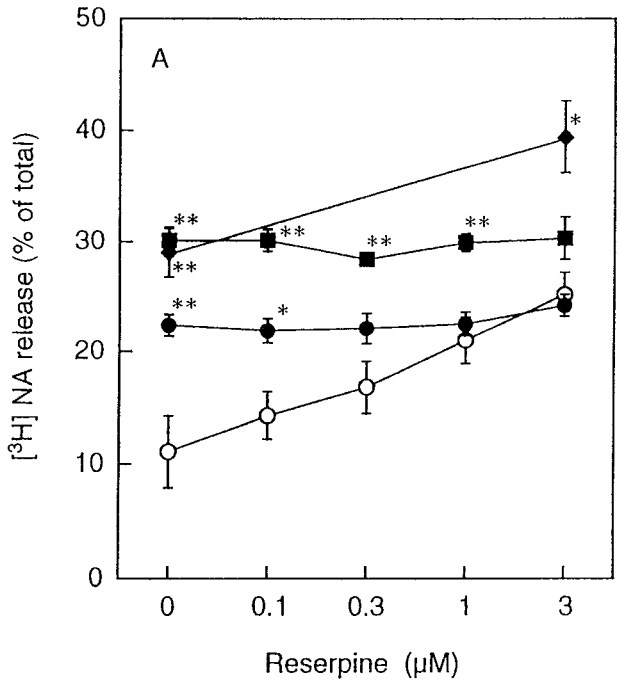


Fig. 2. Effects of desipramine on T-588- or tyramine-stimulated [^{3}H]NA release. The labeled slices were incubated for 8 min at 37℃ with 3 mM T-588 (●), 10 μM tyramine (■) or vehicle (○). The assay mixture was further supplemented with the indicated concentrations of desipramine. Data are each the mean±S.E.M. from 3 or 4 experiments performed in triplicate. *P<0.05, **P<0.01, significantly different from the vehicle alone.



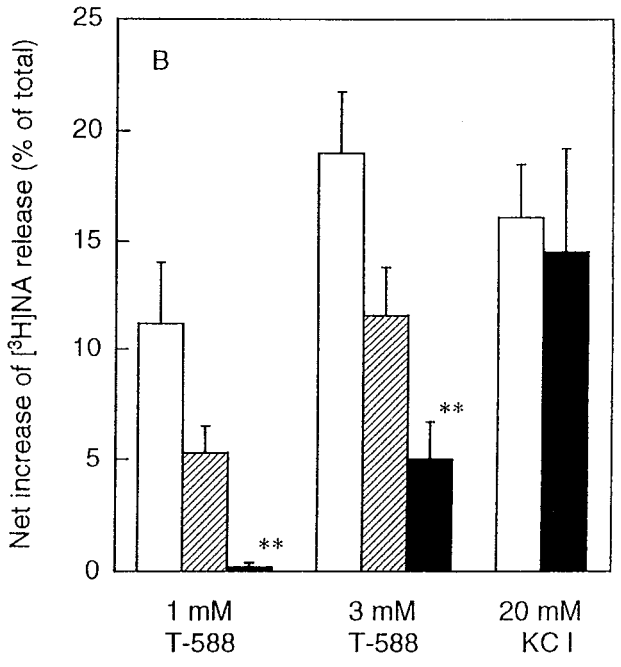


Fig. 3. Effects of reserpine on T-588- or KCl-evoked [^{3}H]NA release. In panel A, the labeled slices were incubated for 8 min at 37℃ with 1 mM (●), 3 mM (■) T-588, 20 mM KCl (◆) or vehicle alone (○). The assay mixture was further supplemented with the indicated concentrations of reserpine. Data are each the mean±S.E.M. from 3 or 4 experiments performed in triplicate. *P<0.05, **P<0.01, significantly different from the vehicle (reserpine) alone. In panel B, bars (without reserpine, open bars; with 0.3 μM reserpine, hatched bars; with 3 μM reserpine, closed bars) show the net increase percentage of [^{3}H]NA release induced by T-588 or KCl, calculated from panel A. Data are each the mean±S.E.M. value from 3 or 4 experiments performed in triplicate. **P<0.01, significantly different from the vehicle alone (without reserpine).

hindering amine uptake into synaptic vehicles in the intracellular space. Reserpine induced [^{3}H]NA efflux in a dose-dependent manner (Fig. 3A). The addition of high K^+ (20 mM) stimulated [^{3}H]NA release 29.0±2.2% (n=3) by itself, and the effect of high K^+ was shifted upward by 3 μM reserpine (Fig. 3A). Conversely, the effect of 3 mM T-588, which stimulated [^{3}H]NA release to the same degree as high K^+, was not shifted upward by reserpine. The sub-maximal effect induced by 1 mM T-588 was also not shifted upward by reserpine. Figure 3B shows the net increases induced by T-588 and by KCl in the absence and presence of reserpine. The net increases induced by 1 mM and 3 mM T-588 were inhibited by co-addition of reserpine in a dose-dependent manner, while 3 μM reserpine did not inhibit the stimulatory effects of high K^+. These findings indicated that T-588 may evoke NA release via a process that is regulated by reserpine.

DISCUSSION

Neurotransmitter release is generally mediated by a Ca^{2+}- and exocytotic vesicular-dependent process. In the case of monoamine release, however, it was also

confirmed that the uptake systems of NA and NA carrier-mediated release regulate NA release in a Ca^{2+}-independent manner. Previously, we reported that the oral administration of T-588 to rats significantly increased NA in a mycrodialysis study (16) and that T-588 increased the release of NA from rat brain slices in an in vitro study (16, 17). The effect of T-588 did not seem to be due to cytotoxicity or an increase in permeability of cell membranes because no stimulatory effect was observed in slices that were preincubated with 1 mM or 3 mM T-588 and then washed by centrifugation (17). Additionally, NA release induced by T-588 was not dependent on extracellular $CaCl_2$ or calmodulin (17). These findings raise two possibilities: 1) T-588 may stimulate NA release by inhibition of NA re-uptake, and 2) T-588 may stimulate NA release via NA carriers on the membrane reverse transport systems. In this study, we investigated the effects of T-588 on NA uptake and the release mediated by NA carriers on the membrane.

We investigated the effects of T-588 and desipramine, a blocker of NA carriers on the plasma membrane, on the uptake of [^{3}H]NA into rat cerebral cortical slices. Both compounds dose-dependently inhibited [^{3}H]NA uptake. The IC_{50} values were 52.0 ± 10.4 μM (n=4) and 44.3 ± 24.5 nM (n=4) for T-588 and desipramine, respectively. Thus, T-588 is at least 1000-fold less potent in inhibiting the uptake of [^{3}H]NA than desipramine. The addition of 3 mM T-588, a concentration that inhibited [^{3}H]NA uptake almost completely, stimulated [^{3}H]NA release markedly. However, desipramine-induced [^{3}H]NA release was very slight even at a maximal inhibitory dose (10 μM) for uptake. Accordingly, the contribution of the uptake mechanism of [^{3}H]NA was minimized in our release experiments. Addition of 3 mM T-588 stimulated [^{3}H]NA release even in the presence of 10 μM desipramine, which inhibited [^{3}H]NA uptake completely (Fig. 1). These findings suggested that T-588 may not stimulate NA release through its inhibitory effect on NA re-uptake.

Next, we investigated the second possibility; i.e., reverse transport by NA carrier. Tyramine accelerates the heteroexchange between extracellular tyramine and intraterminal NA via NA carrier-mediated transport (21). In brain slice preparations, desipramine, a blocker of carriers, reduced reverse carrier-mediated NA release (26). Although desipramine reduced tyramine-stimulated [^{3}H]NA release, T-588-stimulated [^{3}H]NA release was not affected by desipramine. These findings indicated that T-588 may not stimulate NA release from rat cerebral cortical slices via the NA carrier-mediated reverse transport system.

Reserpine is known to stimulate neurotransmitter efflux by inhibition of uptake into synaptic vesicles from cytoplasmic sites. For instance, when synaptosomes prelabeled with [^{3}H]dopamine in the absence of nialamide were superfused with reserpine, the increase in the efflux of radioactivity observed was almost totally accounted for by radioactive metabolites of dopamine (27). Under our conditions, reserpine induced [^{3}H]NA efflux in a dose-dependent manner. Thus, it is likely that T-588, like reserpine, inhibits the uptake into synaptic vesicles and thus stimulates NA release. Although high K^+-stimulated [^{3}H]NA release was shifted upward by reserpine, T-588-stimulated [^{3}H]NA release was not shifted. The net increases induced by 1 mM and 3 mM T-588 were inhibited by co-addition of reserpine in a dose-dependent manner, while 3 μM reserpine did not inhibit the stimulatory effects of high K^+. This suggested that reserpine and T-588 have a similar mechanism of action. Reserpine predominantly stimulated the release of NA metabolites, not intact NA (27). On the other hand, T-588 stimulated endogeneous NA release, not metabolites such as normetanephrine or 3-methoxy-4-hydroxyphenylglycol, from the hippocampus in an in vivo microdialysis study (16), and the NA released by T-588 from the slices was intact (data not shown). In addition, the stimulatory effect of T-588 on NA release was not modified significantly by MAO inhibitors, and T-588 had no effect on MAO activity (data not shown). These results showed that T-588 stimulates intact NA release, not the metabolites. Serum and brain levels of T-588 after single oral administration to rats at 10 mg/kg, which is an effective dose in vivo, were calculated to be about 0.3 μM and 3 μM, respectively (8, 16). Thus the ED_{50} value of T-588 in the present study was much higher than that reported previously in vivo. It is likely that T-588 was not taken up into the slice preparations effectively because T-588 regulated NA release at μM concentrations in rat pheochromocytoma PC12 cells (M. Maekawa et al., unpublished data). Further studies on T-588 are required to clarify these points. Also the effect of T-588 on endogeneous NA release from slices must be studied.

In conclusion, we found that T-588 enhances NA release via a new mechanism of action that is 1) distinct from the inhibition of NA re-uptake by NA carriers on the plasma membrane, 2) also distinct from the reverse transport system, and 3) might be similar to that of reserpine.

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