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# Inhibition by Tetanus Toxin of Sodium-Dependent, High-Affinity [$^3$H]5-Hydroxytryptamine Uptake in Rat Synaptosomes

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**ABSTRACT.** Tetanus toxin (TeTx) is a powerful clostridial neurotoxin that inhibits $Ca^{2+}$-dependent neurotransmitter secretion as do the botulinum neurotoxins (BoNTs). We found that TeTx (but not BoNT/A) produced a specific time- and dose-dependent inhibition of $Na^+$-dependent [$^3$H]5-hydroxytryptamine (serotonin, 5-HT) uptake in rat CNS synaptosomes. This effect was found in all CNS tryptaminergic areas, being maximal in the hippocampus and occipital cortex. TeTx produced the maximum reduction in [$^3$H]5-HT uptake after 30 min of preincubation, being significant also at lower doses ($10^{-12}$ M) or shorter incubation times (10 min). Serotonin transport inhibitors such as fenfluramine ($IC_{50}$, 11.0 ± 0.9 μM), paroxetine ($IC_{50}$, 33.5 ± 0.1 μM), and imipramine ($IC_{50}$, 89.9 ± 5.7 μM) were 3 or 4 orders of magnitude less potent than TeTx ($IC_{50}$, 8.7 ± 1.0 nM). Of the two fragments of TeTx, (the C-terminal portion of the neurotoxin heavy chain, which is responsible for the binding to the nerve tissue) was consistently more effective than the L-$H_N$ fragment (the light neurotoxin chain disulfide linked to the N-terminal portion of the heavy chain, which is responsible for the toxic metalloprotease action) as inhibitor of [$^3$H]5-HT uptake in synaptosomal preparations (56 ± 5% and 95 ± 3% with respect to control, respectively). Antagonism of the toxin-induced [$^3$H]5-HT uptake blockade could not be reversed by zinc chelators but did have the ability to antagonize the TeTx inhibition of basal and $K^+$-evoked [$^3$H]5-HT release in rat synaptosomes. The reduction in serotonin accumulation induced by TeTx could be responsible for some tetanic symptoms that have been related to the serotonergic system. BIOCHEM PHARMACOL **57**;1:111–120, 1999. © 1998 Elsevier Science Inc.

**KEY WORDS.** tetanus toxin; 5-hydroxytryptamine (serotonin); uptake; release; synaptosomes

TeTx† is a high molecular weight (150 kDa) polypeptide produced by the Gram-positive spore-forming bacilli *Clostridium tetani* that produces a serious illness characterized by muscular contractions and recurring spasms, causing death in a high percentage of cases as a result of respiratory failure [1, 2]. TeTx is synthesized as a single polypeptide chain (*sc*-TeTx). Following lysis of the bacteria, a proteolytic activation occurs in which bacterial or host proteases cleave the *sc*-TeTx in a hinge region exposed by an intermolecular disulfide bond. The reduction of the disulfide bond gives rise to the L(light)-chain (50 kDa) with its toxigenic activity and the H(heavy)-chain (100 kDa), which binds specifically to the target tissue. The hydrolysis products obtained from TeTx using papain protease, termed fragments B and C, correspond to fragments L-$H_N$ and $H_C$, with a molecular mass of 100 and 50 kDa, respectively [3, 4].

*In vivo*, the main target of TeTx is the central nervous system, where it is believed to block synaptic release by some mechanisms that probably work synergisticaly. The first mechanism emerged with the discovery of the action of TeTx as a $Zn^{2+}$-metalloprotease [5], this being supported by the finding of the selective proteolysis of synaptobrevin II (vesicle-associated membrane protein, VAMP), an integral membrane protein of synaptic vesicles [6, 7] or cellubrevin, a synaptobrevin homologue [8, 9]. A second mechanism suggests the activation of transglutaminases by TeTx, a family of $Ca^{2+}$-dependent, GTP-modulated transglutaminases which catalyze the formation of covalent bonds between glutamine residues and primary amino groups [10]. These transglutaminases are present in nerve endings where they cross-link synapsin I, an abundant synaptic vesicle phosphoprotein involved in neurotransmission [11, 12]. TeTx inhibits neuroexocytosis even when its zinc-dependent protease activity is removed [13]. A third mechanism suggests that TeTx has the ability to abolish synapsin I and II phosphorylation after depolarizing rat brain synaptosomes [14]. This phosphorylation may be related to the

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† *Abbreviations:* BoNT/A, botulinum neurotoxin type A; CNT, clostridium neurotoxin; 5-HT, 5-hydroxytryptamine, serotonin; OP, 1,10-phenanthroline; SERT, serotonin transporter; TeTx, tetanus toxin; *sc*-TeTx, single chain tetanus toxin; $H_C$, C-terminal portion of the neurotoxin heavy chain; L-$H_N$, light neurotoxin chain disulfide linked to the N-terminal portion of the homologous heavy chain; and FRR, fractional release rate.

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activation of protein kinase C (PKC; EC 2.7.1.37) followed by down-regulation found after intracerebral injection of TeTx on adult and neonatal rat brain [15–17]. These data indicate that TeTx is a powerful toxin that acts by different mechanisms inducing an alteration of relevant processes in the CNS. None of these mechanisms might be related to the death of animals but could elucidate the mechanisms of action of the clostridial neurotoxins.

Despite these observations, the molecular events responsible for the toxic action of TeTx are poorly understood. In the present work, serotonergic transport in synaptosomes was evaluated under treatment with the neurotoxin. TeTx, *sc*-TeTx or some TeTx fragments were able to produce inhibition of the $Na^+$-dependent transport of [$^3$H]5-HT under different conditions.

## MATERIALS AND METHODS

### *Tetanus Toxins and Fragments*

TeTx (4 mg/mL), *sc*-TeTx (1.5 mg/mL), and L-$H_N$ (0.5 mg/mL) were kindly provided by Prof. Ephraim Yavin (Weizmann Institute of Science, Rehovot, Israel) and $H_C$ was purchased from List Biological Laboratories, Inc. BoNT/A was kindly provided by Juan Blasi (Dept. Biologia Cellular i A. Patol., Facultat de Medicina, Universitat de Barcelona). In all cases, purity was measured by SDS-PAGE. A toxicity test was performed for TeTx, giving a toxicity of $6.7 \times 10^6$ mouse minimum lethal doses (MLD) per milligram of protein. One MLD is the minimal dose required for killing an albino mouse, weighing about 20 g, within 96 hr of intraperitoneal injection of the toxin [18]. Dilutions of TeTx, *sc*-TeTx, and fragments L-$H_N$ and $H_C$ were carried out in bicarbonate buffered Krebs–Ringer containing 0.05% BSA. In some experiments, TeTx (4 mg/mL) was dialyzed overnight at 4° against a 200-fold volume of 20 mM captopril or against a 200-fold volume of 2 mM of OP in PBS followed by three dialysis steps (4 hr) against PBS without the corresponding chelator [19].

### *Other Substances*

5-[1,2-$^3$H]Hydroxytryptamine creatinine sulfate ([$^3$H]5-HT), specific activity 17.8 Ci/mmol; I-[7,8-$^3$H]noradrenaline ([$^3$H]NA), specific activity 34 Ci/mmol, and [7,8-$^3$H]dopamine ([$^3$H]DA), specific activity 48.0 Ci/mmol were purchased from Amersham International. [$^3$H]5-HT, captopril, OP, B-NADH, BSA, and pyruvic acid were from Sigma Chemical Co. Pavoxetin is a gift of Smith Kline Beecham. All other reagents were from Fisher Scientific Co.

### *Rat Brain Dissection*

Male Sprague–Dawley rats weighing 200-250 g were killed by cervical dislocation and immediately decapitated with a guillotine. The brain was quickly removed (never taking more than 30 sec) and placed on a cold surface. The nervous tissue was dissected according to Glowinski and Iversen [20].

### *Preparation of Synaptosomes from Rat CNS*

All experiments were performed with a crude synaptosomal fraction ($P_2$) prepared from rat brain according to Gray and Whittaker [21], with slight modifications. Each individually dissected area was weighed and homogenized in 40 volumes (wt/vol) of 0.32 M sucrose adjusted to pH 7.4 with 5 mM Tris–HCl. The homogenization was performed with 12 strokes (900 rpm) using a Potter homogenizer with Teflon pestle (0.1–0.15 mm clearance). The homogenate was centrifuged at 4° for 5 min at 1000 *g*. The resultant supernatant was centrifuged at 12,000 *g* for 20 min. The crude synaptosomal pellet obtained was gently resuspended in 50 volumes of Krebs–Ringer bicarbonate buffer.

### *Krebs–Ringer Bicarbonate Buffer*

The standard medium used for incubation of synaptosomes was a Krebs–Ringer bicarbonate buffer containing 125 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 22 mM $NaHCO_3$, 1 mM $NaH_2PO_4$, and 10 mM glucose. Before use, it was gassed with a mixture of 95% $O_2$ and 5% $CO_2$ for 20 min and adjusted to pH 7.4. In some experiments, modified Krebs–Ringer bicarbonate buffers were used: a sodium-free buffer in which NaCl was substituted by LiCl or choline, and a buffer with an altered divalent cation composition by omission of $CaCl_2$ and/or $MgSO_4$ without replacement of either of the constituents.

### *[$^3$H]5-Hydroxytryptamine Uptake*

In the standard assay to measure the synaptosomal 5-HT uptake, 100 μL of synaptosomal fraction diluted with oxygenated Krebs–Ringer bicarbonate buffer, to achieve a final protein concentration of 0.4–0.8 mg/mL, was mixed in a polypropylene tube with 50 μL of toxin (TeTx, *sc*-TeTx, fragment L-$H_N$ or fragment $H_C$) diluted in Krebs–Ringer bicarbonate buffer containing 0.05% BSA, or with 50 μL of the same medium without toxin as a control. For TeTx-inactivated samples, TeTx was preboiled for 15 min. After a preincubation period of 30 min at 37° in a shaking water bath, uptake was started by addition of 50 μL of [$^3$H]5-HT. For saturation experiments, the tritiated amine concentration range was from 5 to 160 nM and for the rest of the experiments a concentration of 60 nM was used. After an incubation of 5 min at 37°, samples were filtered under vacuum over Whatman GF/C (Whatman International Ltd.) filters in a vacuum filtration manifold (Brandel Inc.) and washed three times with 5 mL of ice-cold Krebs–Ringer bicarbonate buffer. Filters were then dried, placed in vials with 5 mL of Biodegradable Counting Scintillant (Amersham Int. plc) and counted in a Wallac-1409 liquid scintillation counter, with a counting efficiency of 45%. Radioactivity accumulated by synaptosomes at

0–4° for 5 min was routinely subtracted, and temperature-dependent uptake was defined as the difference between uptake carried out at 37° and at 0–4°. Protein was determined according to the method of Bradford [22], using immunoglobulin G as standard (BioRad).

### *[$^3$H]5-Hydroxytryptamine Release*

The synaptosomes were resuspended in Krebs–Ringer bicarbonate buffer. TeTx previously dialyzed overnight with captopril followed by three washing dialysis steps was incubated for 30 min at 37° with and without captopril, and the mixture was then added to synaptosomes at a final concentration of 2 mM captopril and $10^{-9}$ M TeTx for 2 hr at 37°. [$^3$H]5-HT at a final concentration of 60 nM was added to the synaptosomes, preincubated with toxin or not. After 30 min of incubation at 37°, the solution was distributed on 0.56 μm cellulose nitrate filters [23] in a 20-chamber susperfusion apparatus held thermostatically at 37°. The same experiments were performed with TeTx previously dialyzed overnight with OP and then eliminated by further dialysis for eight hours with the same buffer without OP to avoid synaptosome alteration. The synaptosomes were layered onto the filters by aspiration from the bottom under moderate vacuum. Susperfusion was started (t = 0 min) at a rate of 1.0 mL/min with standard medium; after an equilibrium period of 30 min, fractions were collected every minute until time = 60 min. The radioactivity present in the fractions was counted with 5 mL of Biodegradable Counting Scintillant (Amersham Int.). The FRR was calculated as the amount of radioactivity released into each minute of fraction as a percentage of the total radioactivity present on the filter at the start of that fraction. The FRR before the stimulus of 15 mM of $K^+$ and after 7 min of the stimulus (time = 30–32 and time = 39–41) are reported as basal release. The overflow, i.e. the stimulus-induced release (15 mM $K^+$), was calculated as the difference between the FRR in the presence and absence of the blocking agent (time = 34–38). The effect of the $K^+$ added at t = 32 was only detectable 1 min later, since the fluid takes about 1.5 min to flow from the filters to the collecting vials [23].

### *Data and Statistical Analysis*

Uptake was calculated as fmols of [$^3$H]5-HT per minute and mg of synaptosomal protein. The kinetic constants, apparent $K_M$ and $V_{max}$ of synaptosomal amine uptake, were analyzed by means of the Michaelis–Menten curves, and in some case the Linewaver–Burk plot (reciprocal rate of transport against reciprocal [$^3$H]5-HT concentration) was necessary to correct optical interpretation. Correlation coefficients were in all cases higher than 0.98. All data are presented as means with standard deviation (mean ± SD) or as means with standard error of mean (mean ± SEM) and differences among groups were compared using the student's *t*-test. $P < 0.01$ was considered statistically significant.

## RESULTS

TeTx, but not BoNT/A, inhibited [$^3$H]5-HT uptake into a synaptosomal enriched fraction ($P_2$). [$^3$H]5-HT uptake inhibition was time- and dose-dependent, as shown in Fig. 1. Panel A shows the effect of TeTx (1 nM, which corresponds to approximately 1000 DLM) on hippocampal synaptosomes preincubated at the different times given on the abscissa (min). Serotonin transport activity was measured in the presence of 60 nM of [$^3$H]5-HT for 5 min at 37° and plotted as a percentage with respect to control values (426.2 ± 21.0 fmol/min/mg protein). The control values were obtained from synaptosomes preincubated without TeTx and corrected by the nonspecific serotonin transport determined at 0°. After 5 and 15 min of TeTx preincubation, [$^3$H]5-HT uptake reduction was evident (15% and 50%, respectively) and was maximum after 60 and 120 min.

In Fig. 1B, we fixed the time exposure to the toxin (60 min) and changed the dose of TeTx from 0.01 to 10 nM (abscissa). The [$^3$H]5-HT uptake reduction was significant at 0.1 nM (21%) and arrived at its maximum at 1 and 10 nM (67% and 73%, respectively).

A similar protocol to that represented in Fig. 1B was performed with BoNT/A (from 0.1 to 10 nM). BoNT/A produced no statistically significant changes in [$^3$H]5-HT uptake, nor even a moderate positive increase (30%) (data not shown).

Accumulation of [$^3$H]5-HT in synaptosomal-enriched fractions obtained from rat hippocampus was a temperature-dependent process. At the physiological temperature (37°), [$^3$H]5-HT accumulation was linear from 1 to 10 min. Therefore, the incubation time selected was 5 min for all the experiments in the present work. Serotonin accumulation was also linear in the range of protein concentrations used (between 0.2 and 0.4 mg/mL). We observed that TeTx (1 nM) did not affect the viability of the rat brain synaptosomes incubated from 5 to 120 min at 37° using the lactate dehydrogenase release assay [24].

Previous studies have shown that serotonin uptake in the brain is proportional to external $Na^+$ concentration [25–27]. We determined, as seen in Fig. 2, whether $Li^+$ or choline replacement of $Na^+$ ions (as indicated in Materials and Methods) could affect the reduction produced by TeTx (1 nM) for 30 min at 37°. The results indicated that a [$^3$H]5-HT uptake reduction due to $Na^+$ ion substitution was greater than the reduction produced by TeTx. This could indicate that TeTx inhibition is in the range of $Na^+$-dependent uptake.

Elimination of $Ca^{2+}$ and/or $Mg^{2+}$ cations from the Krebs–Ringer bicarbonate buffer (as indicated in Materials and Methods) produced the effects observed in Fig. 3. First, when $Ca^{2+}$ was omitted in control synaptosomes, [$^3$H]5-HT uptake was similar to the reduction produced by TeTx

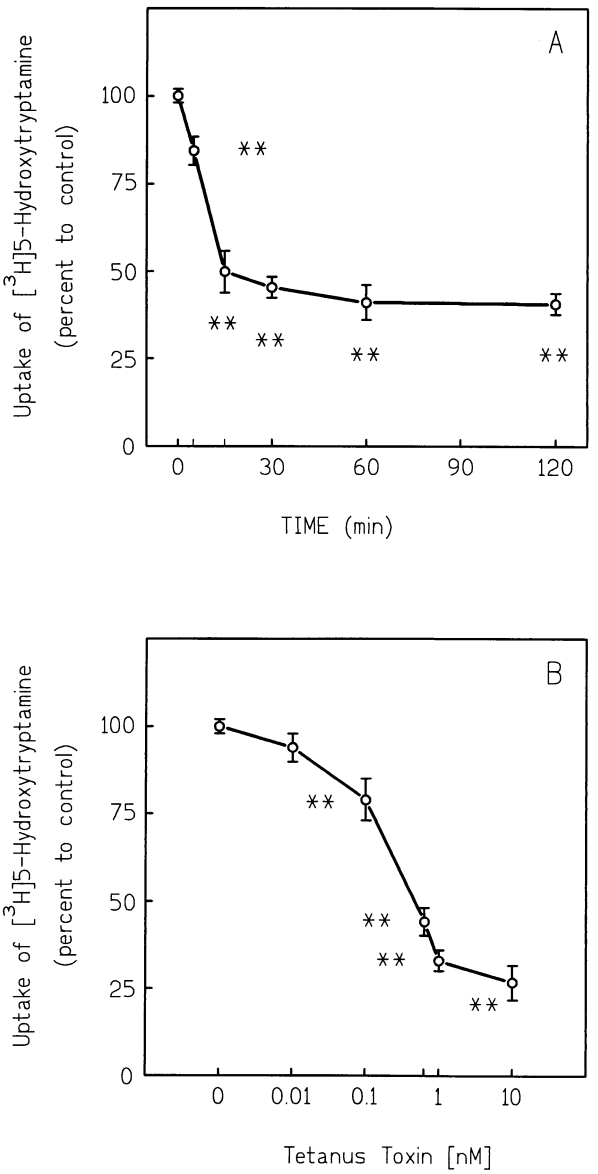



**FIG. 1. Effect of tetanus toxin on $Na^+$-dependent [$^3$H]5-hydroxytryptamine uptake in rat hippocampal synaptosomes. (A) Synaptosomes were preincubated in the presence of 1.0 nM of TeTx at the indicated times. Transport activity was measured in the presence of 60 nM of [$^3$H]5-HT for 5 min at 37°, and the reaction was stopped by adding 3 mL of ice-cold incubation buffer followed by rapid filtration. (B) Synaptosomes were preincubated with TeTx at indicated concentrations for 60 min. Transport activity was measured under the same kinetic conditions as in A. Data are expressed as percentage of control values obtained from synaptosomes preincubated with or without TeTx, corrected by the nonspecific transport determined at 0° (control = 426.2 ± 21.0 fmol/min/mg protein). Each point represents the mean ± SEM of three experiments performed in triplicate. **Student's *t*-test, $P < 0.001$.**

(1 nM). However, no further reduction in uptake was evident in TeTx-treated $Ca^{2+}$-omitted synaptosomes. Second, when $Mg^{2+}$ was omitted, [$^3$H]5-HT uptake was not significantly different from the control and TeTx-treated samples. And third, when $Ca^{2+}$ and $Mg^{2+}$ were simultaneously omitted, a total inhibition of $Na^+$/temperature-dependent serotonin uptake was produced in both control and TeTx-treated samples. These results indicate that the toxin inhibition correlated with the $Ca^{2+}$-dependent uptake.

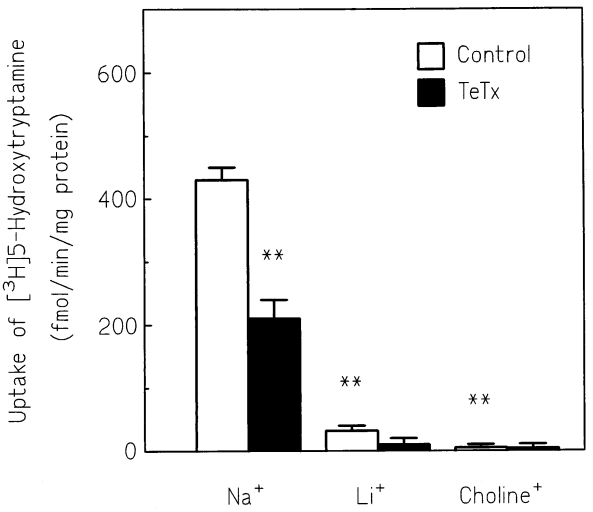



**FIG. 2. Effect of $Na^+$ replacement on temperature-dependent [$^3$H]5-HT uptake. Synaptosomes from hippocampus rat brain were incubated with or without 1 nM of TeTx for 30 min at 37° in unmodified Krebs–Ringer buffer or in Krebs–Ringer buffer, in which $Na^+$ was substituted for in an equimolar manner by $Li^+$ or choline. Uptake was carried out with 60 mM of [$^3$H]5-HT as described in Materials and Methods. Vertical bars represent mean values ± SEM from three individual experiments, each one performed in triplicate. **Student's *t*-test, $P < 0.001$.**

The central nervous system was dissected according to

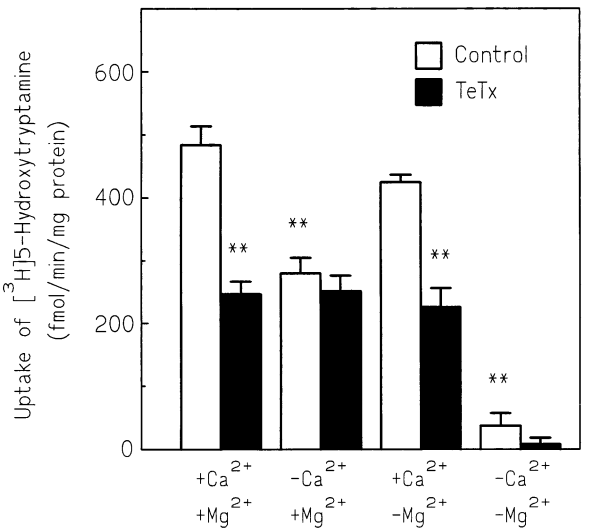



**FIG. 3. Effect of the divalent cations $Ca^{2+}$ and $Mg^{2+}$ on temperature-dependent [$^3$H]5-HT uptake. Synaptosomes from rat brain hippocampus were incubated with or without 1 nM of TeTx for 30 min at 37° in unmodified Krebs–Ringer buffer or in Krebs–Ringer buffer in which $Ca^{2+}$ and/or $Mg^{2+}$ was omitted. Uptake was carried out with 60 mM of [$^3$H]5-HT as described in Materials and Methods. Vertical bars represent mean values ± SEM from two separate experiments, each one performed in triplicate. **Student's *t*-test, $P < 0.001$.**

**TABLE 1. Kinetic parameters of [$^3$H]5-HT uptake in synaptosomes from rat central nervous system areas**

| | Control | | Tetanus toxin | |
|---|---|---|---|---|
| | $K_M$ (nM) | $V_{max}$ (pmol/min$^{-1}$/mg$^{-1}$) | $K_M$ (nM) | $V_{max}$ (pmol/min$^{-1}$/mg$^{-1}$) |
| Cerebellum | 70.1 ± 22.0 | 0.45 ± 0.04 | 127.1 ± 58.0 | 0.52 ± 0.14 |
| Midbrain | 55.9 ± 7.4 | 4.35 ± 0.26 | 46.0 ± 1.3 | 2.78 ± 0.08** |
| Frontal cortex | 51.6 ± 19.3 | 2.26 ± 0.14 | 40.4 ± 17.3 | 1.51 ± 0.13** |
| Occipital cortex | 38.1 ± 11.5 | 1.69 ± 0.02 | 32.1 ± 11.7 | 1.09 ± 0.03** |
| Striatum | 32.0 ± 5.0 | 1.75 ± 0.10 | 26.0 ± 32.0 | 1.11 ± 0.05** |
| Hippocampus | 59.3 ± 2.8 | 1.99 ± 0.05 | 48.3 ± 24.2 | 1.44 ± 0.03** |
| Hypothalamus | 30.9 ± 7.3 | 2.43 ± 0.18 | 31.3 ± 15.0 | 1.70 ± 0.07** |
| Thalamus | 34.0 ± 3.6 | 2.49 ± 0.10 | 38.0 ± 47.0 | 1.93 ± 0.12** |
| Pons-medulla | 25.9 ± 7.4 | 1.80 ± 0.09 | 24.5 ± 7.8 | 1.25 ± 0.07** |
| Spinal cord | 119.9 ± 58.8 | 3.43 ± 0.01 | 116.2 ± 61.4 | 2.41 ± 0.01** |

The CNS was dissected as described under Materials and Methods. Synaptosomes from each region were incubated with or without 1 nM TeTx for 30 min at 37°. Uptake was carried out by adding [$^3$H]5-HT at a concentration range of 5–160 nM. Apparent $K_M$ (nM) and $V_{max}$ (fmol/min/mg protein) were calculated by a nonparametric analysis of saturation curves. Values represent the means ± SEM of three to four separate experiments, each one performed in triplicate.

Glowinski and Iversen [20], and synaptosome-enriched fractions obtained from each individual regions were preincubated with or without tetanus toxin (1 nM) for 30 min at 37°. The [$^3$H]5-HT was used in concentrations between 5 and 160 nM and, as has been previously demonstrated [27], the serotonin accumulation was saturable in all CNS areas. TeTx (1 nM) changed the kinetic parameters, with the reductions in the apparent $V_{max}$ found to be statistically significant (Table 1). The changes in $V_{max}$, not found in $K_M$, could demonstrate a competitive inhibitory action of TeTx on the SERT. Of the ten CNS areas studied, only in cerebellum was [$^3$H]5-HT accumulation not found significantly altered by TeTx.

In order to verify if TeTx produces a competitive or noncompetitive inhibitory action, the serotonin uptake of $P_2$ fraction from rat hippocampus was determined at [$^3$H]5-HT concentrations between 5 and 160 nM, and the kinetic parameters were compared using the control versus the TeTx-treated synaptosomes (control, $10^3$, $5 \times 10^3$, and $10^4$ DLM of TeTx). We can observe, in Fig. 4A, the Michaelis–Menten curves from which we could calculate the apparent $V_{max}$ and $K_M$, with which we obtained the TeTx inhibition constant ($K_i$ = 8.87 nM) with the selected conditions (5 min at 37°, with 30 min of preincubation with TeTx) graphically indicated in Fig. 4C. In Fig. 4B, we can observe that with 1 and 5 nM of TeTx, the apparent $K_M$ (31.7 ± 2.6 nM and 30.5 ± 7.7 nM, respectively) were similar to the control $K_M$, (40.4 ± 3.4 nM). With 10 nM TeTx, there were not significant changes in $K_M$ (28.9 ± 2.4 nM), but the $V_{max}$ suffered a progressive loss of activity (115.9 ± 3.3 fmol/min/mg with 10 nM of TeTx with respect to the control $V_{max}$, 404.6 ± 11.7 fmol/min/mg).

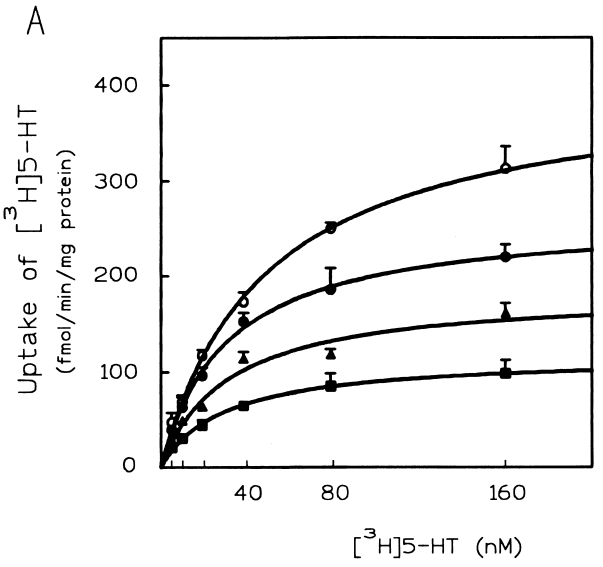
Since transmitter transport is a temperature-dependent process, we demonstrate in Fig. 5 the same inhibitory effect on [$^3$H]5-HT uptake in both preparations preincubated at 0° and 37° for 30 min, compared with its respective controls, also preincubated with Krebs–Ringer bicarbonate buffer at the indicated temperatures. (The reduction in [$^3$H]5-HT uptake in synaptosomes treated with TeTx with respect to its respective control was: 57.5% and 52.9% at 0° and 37° respectively). To avoid differences in [$^3$H]5-HT uptake between the samples at 0° or at 37°, we incubated all samples at 37° for 1 min before the indicated incubation with the [$^3$H]5-HT to arrive at the same temperature and obtain exactly the same conditions.

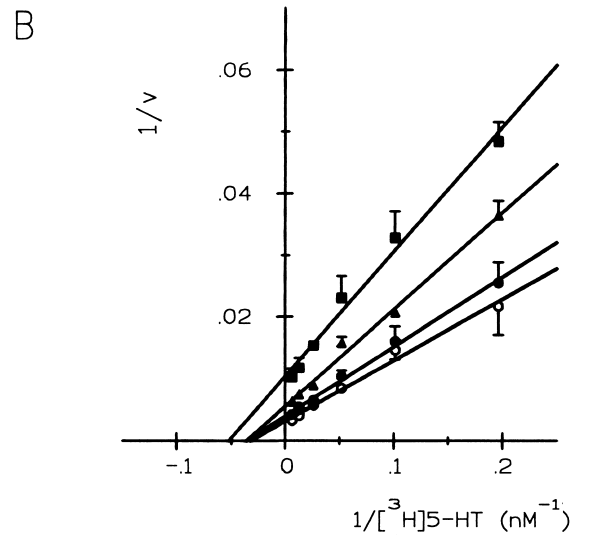
Synaptosomal enriched fractions obtained from cortical rat brain were treated with or without dialyzed TeTx (1 nM) and in the absence or presence of the zinc chelators captopril (2 mM) or OP (2 mM) 30 min before the uptake. Figure 6 demonstrates that the serotonin uptake blocking produced by TeTx was independent of the zinc–endopeptidase action. Nevertheless, both chelators, captopril (1 mM) and OP (1 mM), were effective in preventing the basal and evoked serotonin release inhibition exhibited by TeTx (Table 2).

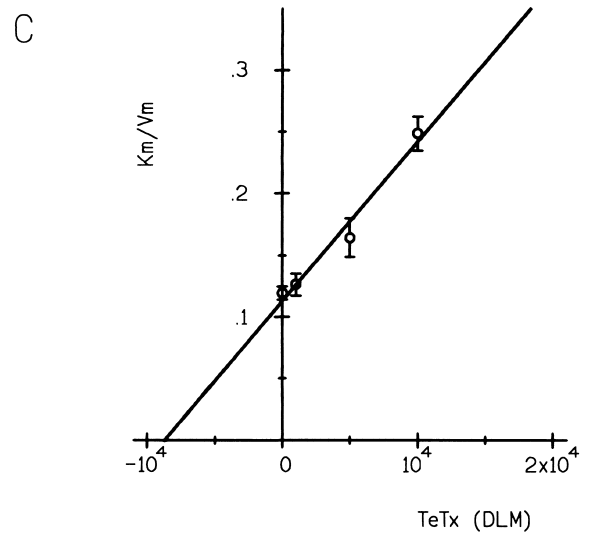
The cortical rat brain synaptosomes were incubated with [$^3$H]5-HT (60 nM) and preincubated for 30 min with TeTx or with the serotonin uptake blockers imipramine, fenfluramine, and paroxetine at the concentration range indicated in Fig. 7. TeTx ($IC_{50}$, 8.7 ± 1.0 nM) is a potent serotonin uptake inhibitor, as is demonstrated by the fact that it is 3 or 4 orders of magnitude more potent than the known specific uptake inhibitors fenfluramine ($IC_{50}$, 11.0 ± 0.9 μM), paroxetine ($IC_{50}$, 33.5 ± 0.1 μM), and imipramine ($IC_{50}$, 89.9 ± 15.7 μM) (Fig. 7).

The dose-response of fenfluramine to the fixed TeTx dose did not change, as is demonstrated in Fig. 8, but the initial uptake was different as was expected with TeTx. The fenfluramine $IC_{50}$s of the TeTx-treated cortical rat brain synaptosomes were not significantly different from the fenfluramine $IC_{50}$ of the control synaptosomes (in the experiment shown in Fig. 8, the kinetic constants were $IC_{50}^{fen}$ = 10.8 μM, and $IC_{50}^{fen/5\ nM\ TeTx}$ = 6.5 μM).

Finally, the synaptosomes were preincubated with or without TeTx, *sc*-TeTx, TeTx-L-$H_N$, TeTx-$H_C$ and heated TeTx, at 1 nM of each indicated compound for 30 min at 37°. The results indicated that *sc*-TeTx had the same effect and potency as TeTx and, of the two fragments of the



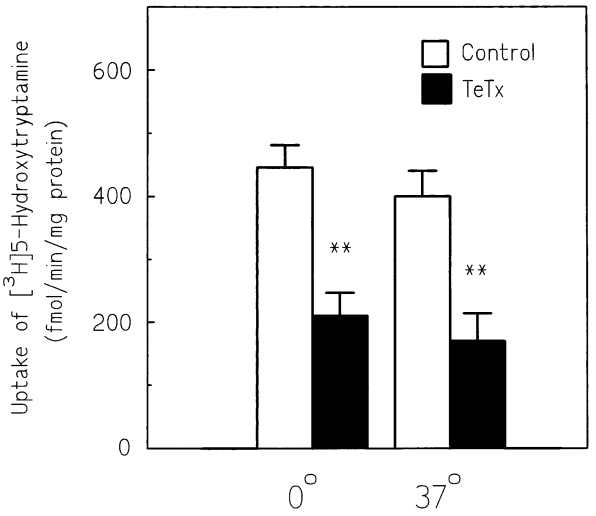




**FIG. 5. Tetanus toxin blocks [$^3$H]5-hydroxytryptamine uptake in a temperature-independent manner. Synaptosomes were incubated with or without 1 nM of TeTx for 30 min at 37° or 0°. Uptake was carried out with 60 nM of [$^3$H]5-HT as described in Materials and Method. Vertical bars represent mean values ± SEM from two separate experiments, each one performed in triplicate. **Student's *t*-test, $P < 0.001$.**

toxin, the C-terminal of the heavy chain (TeTx-$H_C$) had the indicated effect on serotonin uptake. As was expected, heated TeTx lost its specific action on serotonin uptake (Fig. 9).

## DISCUSSION

Studies on the uptake of transmitters, or precursors, were carried out during the 1980s, in different nerve-tissue preparations. However, only slight or no effects of CNTs in the uptake of transmitters have been observed [28]. The Habermann group reported that TeTx, and to a lesser extent BoNT/A, partially and noncompetitively inhibited the uptake of [$^3$H]choline into a crude synaptosomal fraction from rat brain cortex [29] as well as that of [$^3$H] choline and [$^3$H]glycine into spinal cord synaptosomes [30, 31].

**FIG. 4. The effect of tetanus toxin on 5-HT uptake evaluated by Lineweaver-Burk, dose-dependent, and kinetic plots. The transport activity was measured with six different concentrations of [$^3$H]5-HT (5–160 nM) for 5 min at 37°, for each treatment. (A) $Na^+$-dependent 5-HT uptake into rat brain synaptosomes preincubated for 30 min with or without the tetanus toxin. Krebs–Ringer buffer, control (open circles), TeTx $10^3$ DLM (black circles), TeTx $5 \times 10^3$ DLM (black triangles), and TeTx $10^4$ DLM (black squares). The kinetic experiments were analyzed by means of the Michaelis–Menten curves. (B) Lineweaver–Burk plot (double-reciprocal plot) of data presented in A. Where no error bars are shown, they are so small as to be concealed by the symbols. (C) Plot of $K_m/V$ (slopes obtained in B) against TeTx concentration to calculate the inhibition constant ($K_i$). Plots A and B show the temperature-dependent uptake calculated as the difference between total uptake at 37° and temperature-independent uptake (carried out at 0°). Data points are means ± SEM of triplicate determinations from a representative experiment.**

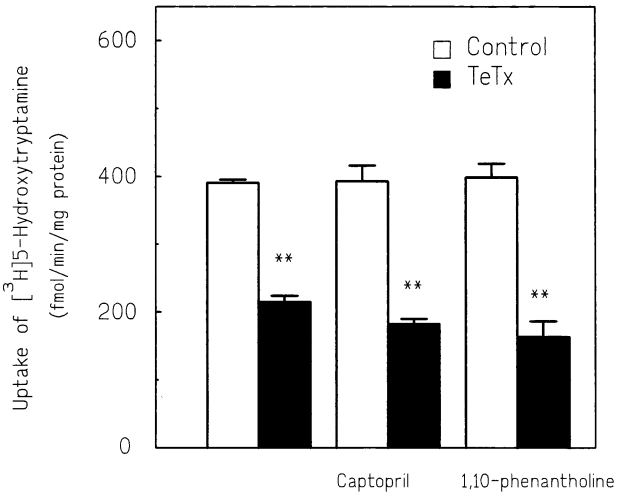



**FIG. 6. Effects of tetanus toxin and $Zn^{2+}$ chelators, captopril, and OP on $Na^+$-dependent [$^3$H]5-HT uptake. Synaptosomes were incubated with or without 1 nM of TeTx for 30 min at 37°. Where indicated, TeTx was previously dialyzed with captopril (2 mM) or with OP (2 mM), and then extensively dialyzed against the same Krebs–Ringer buffer at 4°. Before synaptosome treatment, the chelators were added to the indicated final concentration (2 mM). Uptake was carried out with 60 nM of [$^3$H]5-HT as described in Materials and Methods. Vertical bars represent mean values ± SEM of two separate experiments, each one performed in triplicate. **Student's *t*-test, $P < 0.001$.**

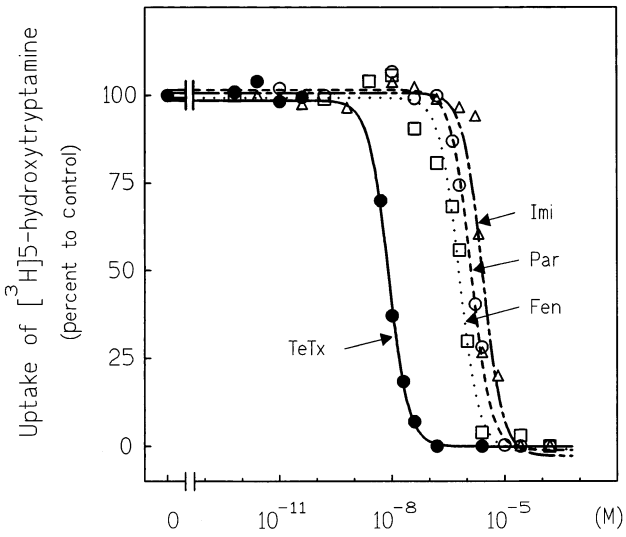



**FIG. 7. Effect of TeTx and 5-HT uptake blockers. Concentration effect of tetanus toxin (TeTx, ●), fenfluramine (Fen, □), paroxetine (Par, ○), and imipramine (Imi, △) on $Na^+$-dependent uptake of [$^3$H]5-HT. Synaptosomes from cortical rat brain were incubated with [$^3$H]5-HT (60 nM) and preincubated for 30 min with the toxin or with the 5-HT uptake blockers. The experimental $IC_{50}$s were: $IC_{50}^{TeTx}$ = 8.7 ± 1.0 nM, $IC_{50}^{Fen}$ = 11.0 ± 0.9 μM, $IC_{50}$ = 33.5 ± 0.1 μM, and $IC_{50}$ = 89.9 ± 5.7 μM. Data points are means of triplicate determinations from a representative experiment. Error bars are avoided but SD were below 5% of corresponding uptake.**

However, no change was found on [$^3$H]noradrenaline in rat brain particles [32] or on [$^3$H] γ-aminobutyric acid in both synaptosomes and brain slices [33, 34]. Nevertheless, it is an open question whether the reduction in transmitter (or precursor) uptake seen in some experiments represents a direct action of the CNTs or only reflects a regulatory answer of the neurons to the impairment of transmitter release by the CNTs. These authors point out that transmitter release is more sensitive to neurotoxins than is transmitter uptake, and that the inhibition of release elicited by CNTs is universal while the uptake inhibition is not. However, we found a specific and strong serotonin uptake inhibition with TeTx, an effect not found with BoNT/A. This inhibition was time- and dose-dependent and was observed even at a low dose of TeTx ($10^{12}$ M) or with a short preincubation time (10 min, Fig. 1). These results could be related to the data found in the literature showing that TeTx acts in a special way in the serotonergic system. It has been reported that intraventricular or intraperitoneal injection of TeTx produces a marked rise in the levels of spinal cord and brain serotonin [35–37]. On the other hand, an increase in tryptophan hydroxylase activity after tetanus intoxication has been observed, although the direct cause of such an activation has not been defined [35]. Finally, the serotonergic system seems to be partially implicated in the classical symptomatology that appears in tetanus disease [38].

The serotonergic cell bodies are present in the raphe nuclei and innervate practically all regions of the brain and spinal cord. The neurotransmitter serotonin is involved in

**TABLE 2. Effect of tetanus toxin on basal and $K^+$-evoked 5-HT release in brain synaptosomes**

| | [$^3$H]5-Hydroxytryptamine release | | | |
|---|---|---|---|---|
| | Captopril | 1,10-Phenanthroline | Basal | Evoked (15 mM) |
| Control | − | − | 100.0 ± 7.8 | 100.0 ± 10.4 |
| TeTx (1 nM) | − | − | 63.9 ± 1.8** | 47.5 ± 5.1** |
| Control | + | − | 119.5 ± 6.4$^{n.s.}$ | 101.3 ± 7.9$^{n.s.}$ |
| TeTx (1 nM) | + | − | 89.1 ± 5.9$^{n.s.}$ | 93.6 ± 8.6$^{n.s.}$ |
| Control | − | + | 103.4 ± 12.1$^{n.s.}$ | 104.1 ± 7.6$^{n.s.}$ |
| TeTx (1 nM) | − | + | 99.7 ± 8.3$^{n.s.}$ | 97.6 ± 5.6$^{n.s.}$ |

Synaptosomes were incubated with or without 1 nM TeTx for 2 hr at 37° and treated simultaneously with or without 1 mM captopril or 1 mM 1,10-phenanthroline. Points represent the means ± SD; N ≥ 4.

** $P < 0.01$, *** $P < 0.001$ and n.s. = not significant, all as determined by Student's *t*-test.

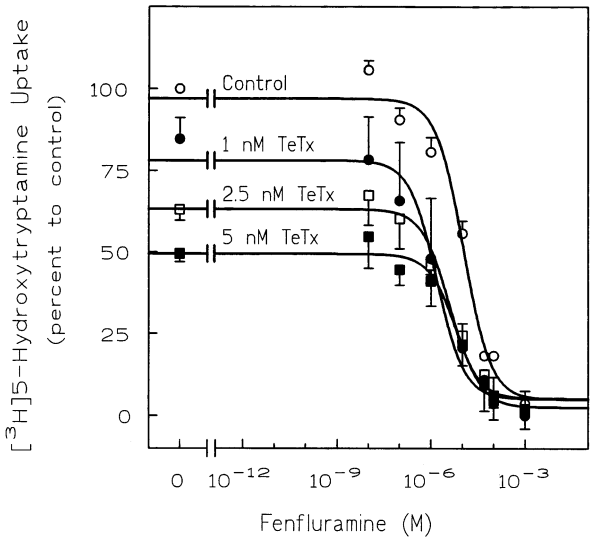



**FIG. 8. Dose-response of TeTx and fenfluramine on cortical synaptosomes and calculation of $IC_{50}$. Concentration effect of fenfluramine on dependent uptake of [$^3$H]5-HT (60 nM) in crude synaptosomes from cortical rat brain, modified by different concentrations of TeTx. Control (○), TeTx 1 nM (●), TeTx 2.5 nM (□), and TeTx 5 nM (■). The fenfluramine $IC_{50}$s of the TeTx-treated synaptosomes were not significantly different from the fenfluramine $IC_{50}$ of the control.**

various processes including sleep control, circadian rhythmicity, mood disorders, eating behavior, etc. Due to the fact that the SERT participates in many behavioral processes, kinetic parameters in ten areas from rat CNS were determined. As is demonstrated in Table 1 and throughout the article, the inhibitory action is found in all CNS tryptaminergic areas, being maximal in hippocampus and in occipital cortex, areas strongly innervated by the serotonergic end-terminal. Cerebellum was the only area where serotonin accumulation was not found to be altered by preincubation with TeTx, which in addition to being coincident with the fact that in cerebellum the serotonergic system is quantitatively minor [37], could also indicate a special difficulty in working with this area.

The different apparent $V_{max}$ and the coincidence of all apparent $K_M$ (all lines of the Lineweaver–Burk plots arrive at the same point in abscissa) indicates that the effect produced by TeTx compared to 5-HT transport is noncompetitive.

Serotonin accumulation in intact synaptosomes is a temperature-dependent process. It has also been demonstrated that after binding, TeTx is internalized inside the cell in a temperature- and energy-dependent process [39, 40]. Nevertheless, preincubating rat synaptosomes with TeTx to produce the inhibitory effect on 5-HT uptake has been demonstrated to be temperature-independent. Figure 5 shows the same action of both TeTx treatment preincubation at 0° and 37° for 30 min.

These results demonstrate both the independence of uptake inhibition from temperature and the rapid apparition of the serotonin uptake blockade and the fact that the inhibition is produced by the TeTx interaction with an acceptor localized in the plasma membrane.

TeTx, as well as BoNTs, is a zinc endopeptidase acting specifically on the same protein substrates of the exocytotic vesicle: synaptobrevin II, SNAP-25, or syntaxin [41,42]. Treatment with or without dialyzed TeTx (1 nM) and in the absence or presence of captopril (2 mM), 30 min before uptake, did not produce significant changes in serotonin accumulation in either control or TeTx-treated synaptosomes (Fig. 6). The lack of effect of captopril, the specific metalloprotease inhibitor, reinforces the idea that uptake inhibition is not only an independent action of TeTx translocation inside the cytosol, but is also independent of TeTx metalloprotease activity. TeTx pretreated with the same chelator (captopril 2 mM) was unable to produce the classical inhibition in either the basal or evoked [$^3$H]5-HT release, as is shown in Table 2. These results reinforce the data presented above, demonstrating the independence of the TeTx blockade of serotonin uptake from its inhibitory action of neurotransmitter release. Similarly, OP (2 mM) was unable to abolish the serotonin uptake inhibition (Fig. 6), but was effective in preventing the basal and evoked effect of TeTx on [$^3$H]5-HT release (Table 2).

The effect of TeTx on the serotonergic system is selective because we did not find the present effect on [$^3$H]norepinephrine or [$^3$H]dopamine uptake. The monoamine transporters are members of a large family of $Na^+/Cl^-$-dependent transporters [43–45]. Nevertheless, SERT has

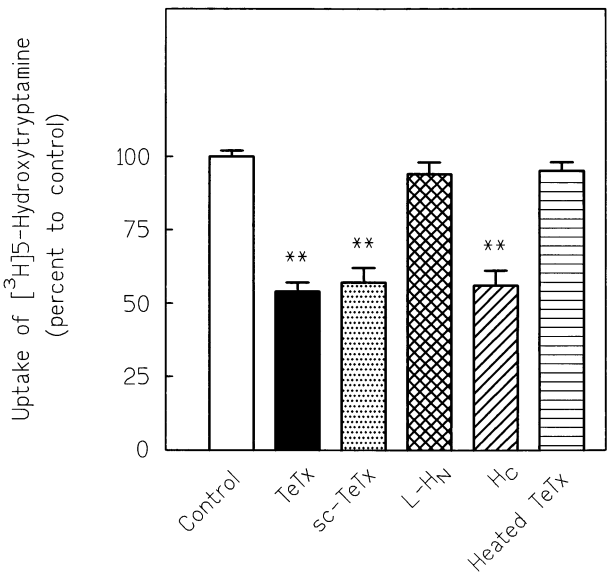



**FIG. 9. Effect of TeTx, sc-TeTx, toxin fragments, and inactivated TeTx on temperature-dependent [$^3$H]5-HT uptake. Synaptosomes were incubated with 1 nM of each indicated compound for 30 min at 37°. Control (open bar), tetanus toxin (black bar), single-chain TeTx (speckled bar), L-$H_N$ fragment (criss-cross bar), $H_C$ fragment (diagonal bar), and TeTx heated to 90° for 15 min (horizontal bar). Uptake was carried out with 60 nM of [$^3$H]5-HT as described in Materials and Methods. Vertical bars represent mean values ± SEM of 2–4 separate experiments, each one performed in triplicate. **Student's *t*-test, $P < 0.001$.**

shown a high selectivity for 5-HT and does not efficiently transport either norepinephrine or dopamine [46]. Other differences between these monoamine transporters are that SERT requires recycling $K^+$, but there is no evidence that the other two transporters of dopamine and noradrenaline have such a requirement [47].

The SERT is inhibited by many antidepressant drugs such as paroxetine, fluoxetine, and citalopram [48], or by tertiary amine tricyclic antidepressants such as imipramine and amytriptyline, which have been found to have higher affinities for SERT than other transporters. In the present work, we compare the potency of some of these inhibitors with the potency of TeTx. TeTx is three orders of magnitude more potent than even the specific serotonergic blocker fenfluramine (nM against μM concentration, respectively). Since fenfluramine works *bis a bis* with the serotonin transporter [49] to produce the same inhibitory action, TeTx must produce its effect by an indirect mechanism that produces the observed block by an amplification of the signal. We suggest that TeTx affects serotonin uptake by altering a component involving inositol phospholipid hydrolisis, which is associated with protein kinase C activity translocation as was recently demonstrated [17]. This notion is consistent with the inhibitory action produced in serotonin uptake by 12-*O*-tetradecanoylphorbol 13-acetate, a protein kinase C activator, that decreased imipramine-sensitive serotonin uptake with a reduction in $V_{max}$ without affecting $K_M$ [50], in the same way as TeTx.

Previous studies showing the inhibition of [$^3$H]paroxetine binding by tricyclic and nontricyclic 5-HT uptake inhibitors indicate a common binding site on SERT for substrates and many, but not all, inhibitors of SERT (e.g., imipramine and paroxetine). TeTx seems to act on a different site, as does fenfluramine [49, 51]. Nevertheless, treatment of TeTx and fenfluramine demonstrates than TeTx affects [$^3$H]5-HT transport by binding to a different site on SERT or by affecting serotonin transport indirectly. In Fig. 8, we can observe nonsignificant changes in fenfluramine $IC_{50}$ after progressively increasing amounts of TeTx concentration.

Of the two papain-split TeTx fragments, the $H_C$ fragment (which is responsible for the binding to the nerve tissue) is consistently more effective than the L-$H_N$ fragment (which is responsible for the toxic metalloprotease action) as an inhibitor of [$^3$H]5-HT uptake in synaptosomal preparations (56 ± 5% and 95 ± 3%, respectively). *sc*-TeTx (single chain of tetanus toxin, before being split by the bacterial endopeptidase) has the same potency as TeTx to produce the inhibition of serotonin accumulation. The inhibition is abolished when TeTx is inactivated by heat (Fig. 9). It is known than *sc*-TeTx and the $H_C$ fragment have no effect on transmitter release inhibition, probably because the zinc-metalloprotease domains are occult or do not exist, as is the case on the carboxyl-terminal of the TeTx heavy chain. Nevertheless, *sc*-TeTx and $H_C$ have a significant effect on serotonin transport in our protocols, which is consistent with the idea of a different domain of the toxin being responsable for the known effect on serotonin plasmatic transport.

The results of the present work suggest that inhibition of serotonin transport by tetanus toxin is produced by a specific action located on the plasma membrane and is intimately related to the characteristic differences of SERT with respect to other transmitter transporters.

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