# Effects of Systemic Administration of Saxitoxin on Serotonin Levels in Some Discrete Rat Brain Regions

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**Abstract:** The present study is related with the toxicity of Saxitoxin (STX), a neurotoxic compound, produced by certain dinoflagellates. Its main toxicological activity is observed through the blockage of the sodium channels. It might originate a reduction of the amplitude and speed of conduction of the action potentials by the peripheral and central nerves, as well as weakening of the skeletal muscular contraction. The aim of this study was to analyze the effect of STX on serotonin (5-HT) levels in some discrete rat brain regions after acute intraperitoneal (i.p.) administration of 5 and 10 μg $Kg^{-1}$ STX body weight. 5-HT levels were analyzed at 30, 60 and 120 min after the administration of 5 μg $Kg^{-1}$ of STX, and 30 min after administration of 10 μg $K^{-1}$ of the toxin. Animals were sacrificed by cervical dislocation and the brains were removed and dissected in seven regions. Tissue samples were analyzed by using a chromatographic technique with electrochemical detection (HPLC/ED). Our results suggest that systemic administration of the STX reaches the brain producing alterations in neurotransmission increasing the levels of 5-HT in all the brain regions studied. With respect to the serotonin metabolite, 5-hidroxiindoleacetic acid (5-HIAA), we observed an increase in its levels in all the brain regions studied with the high dose of toxin, whereas different alterations were observed with the low dose of toxin.

**Key Words:** Saxitoxin, rat brain regions, serotonin, 5-HIAA, HPLC/EC, intraperitoneal.

## 1. INTRODUCTION

Saxitoxin (STX) is a neurotoxic compound produced by dynoflagellates (e.g. *Alexandrium catenella, Alexandrium tamarense, Pyrodinium bahamense, Gymnodinium catenatum*) [1-4]. It was the first known and the most studied toxic component of the paralytic shellfish poisoning (PSP) [5-6]. The paralyzing poisoning is produced by consumption of shellfish, and it is a neurotoxic syndrome associated to the STX presence in the marine seafood [7-8]. STX can exert its neurotoxicity through the disruption of the neurotransmitter function: it binds to the neuronal sodium channels preventing the passage of sodium ions through the cell membrane and disrupting the nerve impulse. Humans exposed to this toxin usually exhibit neurotoxic shellfish-poisoning symptoms, including difficulties in movement, nausea, and breathe arrest. Although STX mechanism of action is well known at molecular level, there are not many studies about its distribution in the organism. The lack of this information is principally about its effects on neurotransmitters in different brain regions. Thus, the aim of this study is to determine the STX effects on neurotransmitter serotonin (5-HT) and its metabolite 5-hidroxiindoleacetic acid (5-HIAA) after its systemic administration in discrete rat brain regions.

Previously, the STX distribution in some discrete rat brain regions after its acute administration of different doses has been studied. Several evidences have been found that after the i.p. injection of STX in male rats (doses of 5 and 10 μg $Kg^{-1}$) the toxin crossing the blood-brain barrier reaches ppm levels in different rat brain regions [9]. In parallel way, STX effects on neuroactive amino acids [10] and DA levels (submitted data)[1] have also been srudied. In the case of neuroactive amino acids, it has been reported that the two STX doses evoked differential changes in the different brain regions. With respect to DA, significant differences on brain DA levels with 5 and 10 μg $Kg^{-1}$ of STX[1] have been detected.

## 2. RESULTS

All the results obtained with the two STX doses and the different experimental periods were compared with control groups.

### 2.1. Effect of 5 μg $kg^{-1}$ of STX on Serotonin Levels

Fig. (**1**) shows the effects of STX on 5-HT levels, after the administration of 5 μg $Kg^{-1}$ body weight (b.w.) of STX. The STX administration produces 5-HT increases in all the brain regions investigated. In control group, 5-HT levels ranged from 360.4 ± 21.8 ng/g in the right hemisphere and 748.3 ± 48.5 ng/g in the midbrain. In most of the regions, the maximum effect of STX on 5-HT levels seems to appear after 60 min of the treatment.

#### *2.1.1. Striatum*

Significant increases in 5-HT brain levels were observed at 30, 60, and 120 min after treatment. At 30 min the increase ($P \leq 0.001$) was about 192 % (430.4 ± 56.5 ng/g in control group *vs* 1,259.8 ± 187.1 ng/g in treated group), at 60

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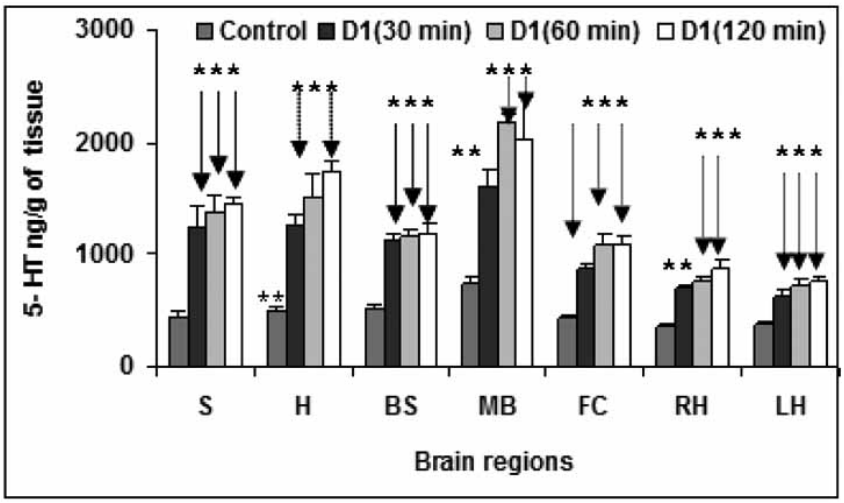


**Fig. (1).** 5-HT concentration in the different rat brain regions investigated, at 30, 60, and 120 min after i.p. administration of 5 μg $Kg^{-1}$ STX dose. The values represent the MEAN ± SEM of 8 determinations for control group and 5 determinations for the treated group.

** $P \leq 0.01$, *** $P \leq 0.001$ significant differences with respect to the control group.

Striatum (S), Hypothalamus (H), Brain Stem (BS), Midbrain (MB), Frontal Cortex (FC), Right Hemispheres (RH), Left hemispheres (LH).

min the increase ($P \leq 0.001$) was 221 % (1,380.7 ± 156 ng/g), and at 120 min the increase ($P \leq 0.001$) was 238.7 % (1,457.7 ± 70.1 ng/g). There are no significant differences between 30, 60, and 120 min.

### *2.1.2. Hypothalamus*

Control group presents 500.7 ± 22.1 ng/g of 5-HT. The STX administration produced increases at 30 min ($P \leq 0.01$), 60, and 120 min ($P \leq 0.001$) of 156 % (1,279.1 ± 83.7 ng/g), 203 % (1,517.4 ± 205.5 ng/g), and 249 % (1,747.7 ± 98 ng/g), respectively.

### *2.1.3. Brain Stem*

Brain stem presented significant increases in all experimental periods compared with control group (522.8 ± 50.1 ng/g). At 30 min the increase was about 113.5 % (1,115.9 ± 88.3 ng/g). With respect to 60 min the increase was about 120.2 % (1,159 ± 79.8 ng/g). At 120 min the increase was 128.3% (1,193.5 ± 103.4 ng/g). The significance levels were $P \leq 0.001$ for all the experimental periods.

### *2.1.4. Midbrain*

In this region, the changes with respect to control group (748.3 ± 48.5 ng/g) in 5-HT levels were 116.3 % (1,618.7 ± 140.6 ng/g) at 30 min ($P \leq 0.01$), at 60 min the increase was about 191.9 % (2,148.3 ± 71.4 ng/g), and at 120 min the increase was 171 % (2,028.9 ± 170.2 ng/g). The significance levels were $P \leq 0.001$ in both cases.

### *2.1.5. Frontal Cortex*

In this region, changes with respect to control group (429.1 ± 22.2 ng/g) for 5-HT levels were 105 % (877.9 ± 34.6 ng/g) for 30 min ($P \leq 0.01$), at 60 min the increase was about 152 % (1,080.6 ± 94.1 ng/g), and at 120 min the increase was 153.2 % (1,086.7 ± 67.5 ng/g). The significance levels were $P \leq 0.001$ in all experimental times.

### *2.1.6. Right Hemispheres*

In this case, at 30, 60, and 120 min 5-HT levels also presented significant increases ($P \leq 0.001$) compared with control group (360.4 ± 21.8 ng/g). At 30 min the increase was about 93.4 % (697.1 ± 17.8 ng/g). At 60 min the increase was about 108.7 % (751.8 ± 50.7 ng/g) and at 120 min the increase was about 144 % (877.1 ± 63.1 ng/g).

### *2.1.7. Left Hemispheres*

At 30, 60, and 120 min significant increases ($P \leq 0.001$) in 5HT levels compared with control group (348.4 ± 17.1 ng/g) were observed. At 30 min the increase was about 63.4 % (627.9 ± 57.9 ng/g), at 60 min the increase was about 88 % (722.7 ± 53.7 ng/g), and at 120 min the increase was about 96.5 % (755.1 ± 38.6 ng/g).

### 2.2. Effect of 10 μg $kg^{-1}$ of STX on Serotonin Brain Levels

This dose evoked an increase in all rat brain regions studied (Fig. **2**). In striatum, the increase of 5-HT was 290.3 % (1,680.0 ± 65.8 ng/g) with respect to control group ($P \leq 0.001$). In hypothalamus, the STX administration increased ($P \leq 0.001$) in 364.4 %the 5-HT levels (2,325.1 ± 232.3 ng/g). In brain stem, the 5-HT levels increased from 522.8 ± 20.1 ng/g to 1,465.3 ± 75.2 ng/g ($P \leq 0.001$). In midbrain, the levels of 5-HT changed from 748.3 ± 48.5 ng/g to 2,110.4 ± 170.2 ng/g ($P \leq 0.001$). In the right hemisphere, the increase was about 296.1 % (1,067.3 ± 109.9 ng/g) and in the left hemisphere, the increase was about 239.3 % (919.7 ± 28.5 ng/g), ($P \leq 0.001$ in both regions). As it can be appraised, the effect producing this dose at 30 minutes is greater than the effect produced at 30 and 60 min with 5 μ $kg^{-1}$ STX dose and even, in many cases, at 120 min after the treatment.

### 2.3. Effect of 5 μg $kg^{-1}$ of STX on 5-HIAA Levels

Fig. (**3**) shows the effects of STX (5 μg $Kg^{-1}$) on 5-HIAA levels at 30, 60, and 120 min after the administration. In control group, 5-HIAA varies from 117.9 ± 5.7 ng/g of tissue in

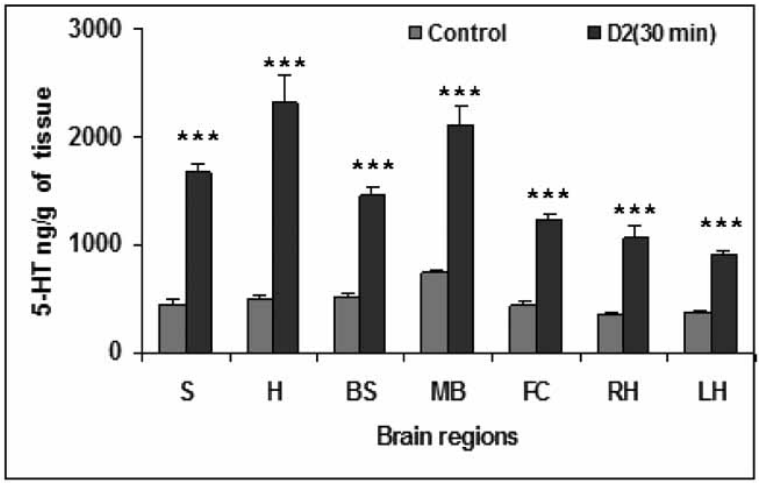


**Fig. (2).** 5-HT concentration in the different rat brain regions investigated, at 30 min after i.p. administration of 10 μg $Kg^{-1}$ STX dose. The values represent the MEAN ± SEM of 8 determinations for control group and 5 determinations for the treated group.

*** $P \leq 0.001$ significant differences with respect to the control group.

Striatum (S), Hypothalamus (H), Brain Stem (BS), Midbrain (MB), Frontal Cortex (FC), Right Hemisphere (RH), Left Hemisphere (LH).

striatum to 415.9 ± 25.5 ng/g in midbrain. It was observed that in both hemispheres, 5-HIAA values were very similar. STX administration produces different changes in this metabolite.

### *2.3.1. Striatum*

In this case, significant increases have been observed at 30 min (368.1 ± 55.1 ng/g), 60 min (382.2 ± 78.3 ng/g) and 120 min (381.0 ± 22.2 ng/g) after STX administration compared with control group (117.9 ± 5.7 ng/g). They were about 212.3 %, 224.3 %, and 223.1 %, respectively.

### *2.3.2. Hypothalamus*

Significant increases were detected at 30 and 120 min after STX administration. The increases were about 95.8 % (470.2 ± 57.6 ng/g) and 83 % (439.2 ± 35.3 ng/g) at 30 min and 120 min compared with control group (240 ± 21.4 ng/g), respectively.

Other brain regions analyzed such as brain stem, midbrain, frontal cortex, right and left hemispheres did not present significant differences in 5-HIAA levels with respect to control group after systemic administration of STX.

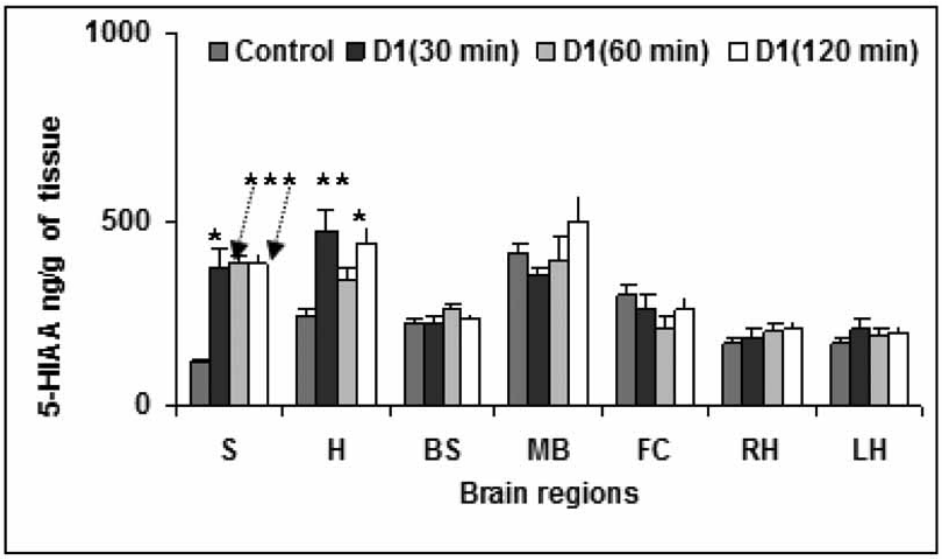


**Fig. (3).** 5-HIAA concentration in the different rat brain regions investigated, at 30, 60, and 120 min after i.p. administration of 5 μg $Kg^{-1}$ STX dose. The values represent the MEAN ± SEM of 8 determinations for control group and 5 determinations for the treated group.

** $P \leq 0.01$, *** $P \leq 0.001$ significant differences with respect to the control group.

Striatum (S), Hypothalamus (H), Brain Stem (BS), Midbrain (MB), Frontal Cortex (FC), Right Hemispheres (RH), Left hemispheres (LH).

### 2.4. Effect of 10 μg $kg^{-1}$ of STX on 5-HIAA Brain Levels

Fig. (**4**) shows the effects of STX (10 μg $Kg^{-1}$) on 5-HIAA levels at 30 min after the administration. For striatum, the increase ($P \leq 0.001$) was of 353.1% (534.2 ± 35.2 ng/g). For hypothalamus, the increase ($P \leq 0.001$) was of 152.6% (606.3 ± 72.7 ng/g); for brain stem, the administration of the STX produced an increase ($P \leq 0.001$) of 62.3% (356.2 ± 24.7 ng/g); in midbrain, an increase ($P \leq 0.001$) of 54.3% was observed (641.9 ± 37.2 ng/g); in the right hemisphere, the toxin induced increase ($P \leq 0.01$) of 63.6% (270 ± 5.2 ng/g); and in the left hemisphere, the increase ($P \leq 0.001$) was of 65.5% (277 ± 6.5 ng/g) with respect to control group. In the frontal cortex, significant differences were not observed.

### 2.5. Effect of STX Treatment on the 5-HIAA/5-HT Ratio in the Different Brain Regions Investigated

Table **1** presents the effect of the i.p. administration of 5 μg $Kg^{-1}$ STX dose in the groups of 30, 60, and 120 min, as well as the administration of 10 μg $Kg^{-1}$ STX dose in the group of 30 min, on the 5-HIAA/5-HT ratio. It achieved an ANOVA [11] and the Duncan test (as a *pos hoc* test) for the respective analysis.

A significant decrease at 49 % for 30 and 60 min was observed in the 5 μg $Kg^{-1}$ STX dose for hypothalamus ($P \leq 0.05$) with respect to control group. In the case of the brain stem, 5 μg $Kg^{-1}$ STX dose induces significant decreases of 52.4 %, 47.6 %, and 54.8 % ($P \leq 0.001$) at 30, 60, and 120 min with respect to control group. Also in the case of frontal cortex, significant decreases of 57 %, 71 %, and 64.3 % were induced ($P \leq 0.001$) for the groups of 30, 60, and 120 min, respectively. In midbrain, a significant decreases of 53.5 % ($P \leq 0.05$) for 30 min group, and 54.2% with 60 min group ($P \leq 0.05$) were induced. With respect to both hemispheres, significant decreases were of 44.7 % ($P \leq 0.01$) for 30 and 60 min groups, and 49 % ($P \leq 0.001$) for 120 min in right hemisphere and for left hemisphere was 46 % for 60 min and 49 % for 120 min.

The 10 μg $Kg^{-1}$ STX dose caused significant decreases in the 5-HIAA/5-HT ratio in the hypothalamus, brain stem, frontal cortex and the hemispheres with respect to control group, without significant changes in the other studied regions.

In the hypothalamus, the decrease was 47% ($P \leq 0.05$); for brain stem, it was 42.9 % ($P \leq 0.001$); for the frontal cortex, it was 60% ($P \leq 0.001$); for the left hemisphere, it was 37 %; and for the right hemisphere, it was 42.6 % ($P \leq 0.01$).

## 3. DISCUSSION

The mechanism of action of STX is well known at molecular level. Nevertheless, there are few studies about its distribution in the organism and its effects on neurotransmitters. On the other hand, there is limited information about its properties of union to the sodium channels in the rat brain [12]. The present study investigated the effect of the systemic administration of different STX doses on the levels of 5-HT and its metabolite 5-HIAA in some discrete brain regions.

The administration of the lower STX dose induced a significant increase of 5-HT levels in all the brain regions analyzed, in the three experimental periods investigated. With the i.p. administration of 10 μg $Kg^{-1}$ STX dose, a significant increase of 5-HT in all the investigated brain regions was also detected.

The levels of 5-HT in control group were very similar in all the regions investigated, with a higher level in the midbrain. By the way. in this region, the greater effect of the STX was also observed, this fact would be observed because in the mammalian brain, the principal source of the serotoninergic innervation of the forebrain is the dorsal raphe nucleus that is located in the midbrain [13-16].

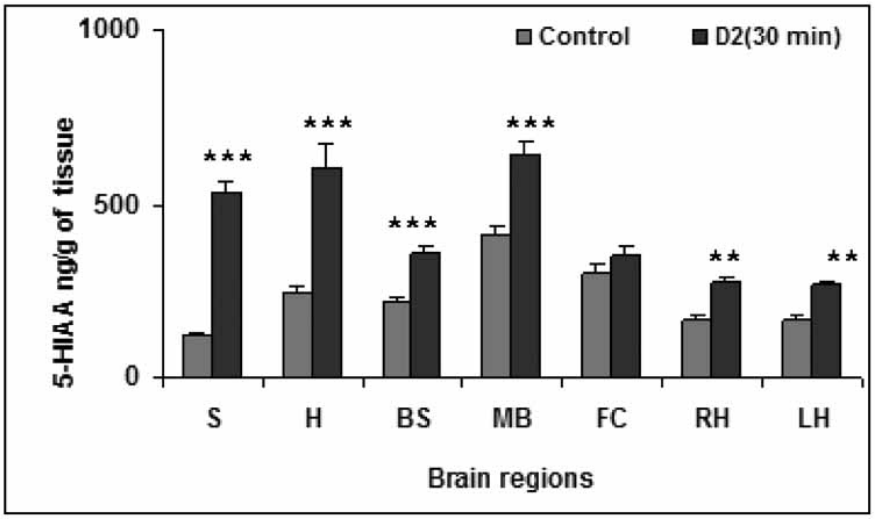


**Fig. (4).** 5-HIAA concentration in the different rat brain regions investigated, at 30 min after i.p. administration of 10 μg $Kg^{-1}$ STX dose. The values represent the MEAN ± SEM of 8 determinations for control group and 5 determinations for the treated group.

**$P \leq 0.01$, ***$P \leq 0.001$ significant differences with respect to the control group.

Striatum (S), Hypothalamus (H), Brain Stem (BS), Midbrain (MB), Frontal Cortex (FC), Right Hemisphere (RH), Left Hemispheres (LH).

**Table 1. Effects of the Treatment of 5 and 10 µg $Kg^{-1}$ STX Dose on the Relation 5-HIAA/5-HT in the Rat Brain Regions Investigated at 30, 60, and 120 min for 5 $Kg^{-1}$ STX Dose and 30 min for 10 $Kg^{-1}$STX Dose. With n = 8 for Control Group and = 5 for Treated Groups**

| Time (min) | STX Dose µg $kg^{-1}$ | Ratio | S | H | BS | MB | FC | LH | RH |
|---|---|---|---|---|---|---|---|---|---|
| Control Group | 5 | 5-HIAA/ 5-HT | 0.3 ± 0.04 | 0.49 ± 0.06 | 0.42 ± 0.02 | 0.43 ± 0.07 | 0.70 ± 0.07 | 0.46 ± 0.06 | 0.47 ± 0.04 |
| 30 | 5 | 5-HIAA/ 5-HT | 0.3 ± 0.03 | 0.38 ± 0.06 | ***0.20 ± 0.02 | *0.20 ± 0.02 | ***0.30 ± 0.06 | 0.35 ± 0.08 | **0.26 ± 0.03 |
| 60 | 5 | 5-HIAA/ 5-HT | 0,29 ± 0.07 | *0.25 ± 0.05 | ***0.22 ± 0.02 | *0.24 ± 0.02 | ***0.20 ± 0.03 | *0.25 ± 0.04 | **0.26 ± 0.03 |
| 120 | 5 | 5-HIAA/ 5-HT | 0.26 ± 0.02 | *0.25 ± 0.01 | ***0.19 ± 0.01 | 0.29 ± 0.04 | ***0.25 ± 0.05 | *0.26 ± 0.03 | **0.24 ± 0.03 |
| 30 | 10 | 5-HIAA/ 5-HT | 0.32 ± 0.02 | *0.26 ± 0.02 | ***0.24 ± 0.01 | 0.31 ± 0.03 | ***0.28 ± 0.02 | *0.29 ± 0.01 | **0.27 ± 0.03 |

P* ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001.

Striatum (S), Hypothalamus (H), Brain Stem (BS), Midbrain (MB), Frontal Cortex (FC), Right Hemispheres (RH), Left hemispheres (LH).

Some investigations with different neurotoxins reported similar results about our research. Chi *et al*. [17] detected a significant increase in the levels of 5-HT in male rats treated with a high dose of the mycotoxin T-2 in the different trial periods studied. Boyd *et al*. [18] also measured the levels of 5-HT and 5-HIAA in chickens and male rats fed during 30 days with the T-2 and they detected that 5-HT and 5-HIAA levels were increased in all the rat brain regions investigated. They reported that this increase appeared in the evaluated time intervals in all the rat brain regions studied, but not in chickens. They thought that T-2 toxin influences brain biogenic amine metabolism and concluded that there is an interspecies difference in the central effects of this mycotoxin.

The serotoninergic system is implicated in the control of numerous behavioral and physiological functions [15, 19]. The electrical stimulation of the serotoninergic neurons increases the synthesis and release of the neurotransmitter. After its synthesis, 5-HT can be stored in synaptic vesicles, liberated, re-uptaked or degraded. Some perturbation in any part of this mechanism can produce neurotoxicity. Therefore, considering this mechanism, we can propose some different hypothesis in order to explain 5-HT increases.

a) Different investigators have demonstrated that STX and TTX act in similar way [20-24] by blocking the voltage dependent sodium channels [25]. In the investigated regions, 5-HT increased levels might be due to a decrease in the releasing process caused by STX, provoking an accumulation in the brain tissue.

b) The degradation process could be altered. It may be produced by the inhibition of the monoamine oxidase (MAO), mitochondrial enzyme [26,27], which is responsible for the first stage of the degradation of biogenic amines, such as serotonin, adrenaline, noradrenaline, and dopamine.

With regard to 5-HIAA, lower dose does not present any significant change in brain stem, frontal cortex, midbrain, right hemisphere, and left hemisphere. Nevertheless, significant increases were detected in striatum and hypothalamus. With the higher dose, 5-HIAA levels were increased significantly in all the brain regions analyzed with exception of the frontal cortex.

A relationship between 5-HT and the 5-HIAA was observed. Boyd *et al*. [18] found a significant increase in hypothalamus, hippocampus, cerebellum, and brain cortex of male rats treated with T-2 mycotoxin. Nevertheless, when they analyzed the complete brain, no significant differences have been found. Byers *et al*. [28] found a strong relationship between the levels of 5-HT and 5-HIAA after the subchronic exposition to TCDD (tetrachlorodibenzo-p-dioxin) in some brain regions analyzed.

The increase of 5-HIAA levels, observed in some regions, can be due to the accumulation of 5-HT in different brain regions, which in some cases would entail to a greater production of the metabolite.

Stenfors *et al*. [29] suggested the use of 5-HIAA/5-HT ratio as an estimation of 5-HT release. Rollema [30] also mentioned that the ratio 5-HIAA/5-HT in brain tissue has often been used as a measure of 5-HT turnover. This study is in agreement with Rollema [30] and considers the ratio 5-HIAA/5-HT like a measure of 5-HT turnover.

About that ratio, in the striatum, there are no significant changes for both doses. This could be interpreted as in the

treated group the accumulation of 5-HT is balanced with its degradation. For this reason, although there is a greater amount of the metabolite in the treated group than in the control group, the index does not present any variation.

In the hypothalamus, in which there is an increase in 5-HIAA levels with both doses, it can be observed that the ratio is decreased, meaning that 5-HT degradation is lower than its accumulation. In midbrain, significant decreases were observed in the ratio (60 and 120 min) and it could be interpreted that its synthesis is greater than its degradation. With the higher dose, the ratio 5-HIAA/5-HT does not present significant changes and it could be interpreted that its degradation is in equilibrium with its synthesis. Gaggi *et al*. [31] reported signs of inhibition of the serotoninergic neurotransmission because they detected a decrease of the 5-HIAA/5-HT ratio, after the injection of 5-HT receptor agonist X-OHDPAT in rats.

For brain stem, frontal cortex, right and left hemispheres, with the lower dose, a decrease in the ratio 5.HIAA/5-HT has been observed. This result suggests that at least these regions present a decrease of the metabolism of 5-HT. About the higher dose in brain stem, frontal cortex, right and left hemispheres, it can be observed that the ratio 5-HIAA/5-HT presents significant decreases for all of them. It can be interpreted that 5-HT degradation is lower than its synthesis, with the consequent accumulation inside serotoninergic neurons. It has previously been mentioned that STX might disable the release process provoking an accumulation in the brain tissue.

In summary, the systemic administration of STX induces an increase in the content of 5-HT in all the rat brain regions investigated. With respect to its metabolite 5-HIAA, just high STX dose increased its levels in all the brain regions analyzed. In our opinion, it will remain necessary to assess the effects on 5-HT release, by measuring changes in extracellular 5-HT using *in vivo* sampling technique in awake animals, because of which microdialysis would be the method of choice.

## 4. EXPERIMENTAL

### 4.1. Toxin and Reagents

Sodium hydroxide (NaOH), perchloric acid (HClO4), and hydrochloric acid (HCl) were purchased from Panreac (Madrid, Spain). Serotonin, 5-hidroxiindolacetic, octanesulfonic acid, 3-4 dihydroxy benzoic acid (DHBA-), ethylenediaminetetraacetic acid (EDTA) and potassium dihydrogen phosphate ($KH_2PO_4$) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Methanol LC grade was obtained from Panreac. Chromatographic-grade water was produced by a Milli-Q system (Millipore, MA, USA). Standard solution of STX was purchased from the Institute for Marine Bioscience, National Research Council, Certified Reference Material Program (NRC-CRM Halifax, Canada).

### 4.2. Animals

Male adult Sprague–Dawley rats (weighing between 150 and 200 g) were used in all the experiments. Animals were housed under monitored conditions of temperature (22 ± 2 º C) and photoperiod (light: dark 14:10 h) with free access to food and water. The experiments were performed according to the Guidelines of the European Union Council (2003/65/EU) for the use of laboratory animals [32].

### 4.3. Treatment

We studied two doses of STX, 5 and 10 μg $kg^{-1}$ body weigh (bw), in groups of 5 rats. Lower dose (approximately 0.5 mL suspension of STX) was injected i.p. using a syringe, amounting to the equivalent of 5 μg $kg^{-1}$ bw. Higher dose (approximately 1 mL STX suspension) was also administered i.p. amounting to the equivalent of 10 μg $kg^{-1}$ bw. After systemic administration, we just waited 30, 60 and 120 min (*experimental periods* of time, in which we studied STX effects on 5-HT and 5-HIAA levels) for lower dose and 30 min (experimental period) for the higher dose. After that, animals were sacrificed by cervical dislocation. As soon as the rats were killed, brains were removed and dissected in the following rat brain regions: striatum (S), hypothalamus (H), brain stem (BS), mid brain (MB), left hemisphere (LH), and right hemisphere (RH). Eight control rats were killed in parallel to both treated groups post-injection of 0.5-1.0 ml of saline solution.

### 4.4. Sample Preparation

Tissue samples were weighed, and then homogenized by sonication in a solution of 0.1 M $HClO_4$ (hemispheres in a proportion of 1:8; midbrain and brain stem in a proportion of 1:10; hypothalamus and striatum in a proportion of 1:20). Finally, samples were centrifuged at 16,000 g at 4º C, during 15 min. Supernatants were filtered through 0.22 μm nylon filters, and became cool until determination of 5-HT and 5-HIAA by High Performance Liquid Chromatographic with electrochemical detection (HPLC-EC) [33,34].

### 4.5. Recovery Procedure

The effectiveness of the extraction procedure of 5-HT and its metabolite 5-HIAA was also investigated. Three rat brains were dissected and processed in the same way as the sample. Each region was spiked with a well-known amount of 3-4-dihidroxibencilamina (DHBA), previous homogenization. DHBA is a catecholamine that does not exist in the cerebral tissue. The regions are spiked of such form that each one has a concentration of 50 pg/μL of DHBA. 20 μL of each spiked sample was injected in the HPLC-EC. The height of the spiked sample obtained was compared with the height of a DHBA standard previously processed. Recovery percentage of DHBA varies from 76% in the hemispheres to 91% in the hypothalamus.

### 4.6. Chromatographic Conditions for HPLC Analysis of 5-HT

The HPLC-ED analysis of 5-HT and 5-HIAA from the rat brain samples were carried out using a chromatographic system equipped with a Jasco PU-980 pump. The levels were quantified using the method described by Duran *et al*. [13]. The method was evaluated and optimized taking into account the effect of the particular biological matrix. The isocratic separation was achieved using Teknokroma Kromasil 100 C18 reversed-phase column (5 μm particle size and 15 x 0.46 cm). Column was eluted with a mobile phase consisting of 0.7 mM $KH_2PO4$, 0.1 mM octanesulfonic acid as an ion-

pairing reagent, 0.1 mM EDTA and 14 % methanol, pH = 3.5. The flow rate was maintained at 2 mL/min. The injected volume was 20 µL using a Rheodyne injection valve, the chromatograms obtained allowed the determination of 5-HT and its metabolite with a run time of 20 min. Detection of the substances was achieved using an ESA Coulochem III (ESA/Coulochem) with an analytical cell ESA Modelo 5011 and the data were analyzed by Cromanec XP 1.0 system. The amount of 5-HT and 5-HIAA in the samples of control and treated groups was calculated by comparing their peak heights with standard solution height. Results are reported as ng/g of tissue.

### 4.7. Statistical Analysis

Data are reported as mean ± SEM. For data regarding 5-HT determination, one-way ANOVA [11] and the Duncan test (as a *pos hoc* test) were used in order to compare results among the treatment and experimental times. The statistical package SPSS 14.0 was used. The level of significance was set at $*P \leq 0.05$, $*P \leq 0.01$, and $***P \leq 0.001$.

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