Expression of c-Fos protein in medial septum/diagonal band of Broca and CA3 region, associated with the temporary inactivation of the supramammillary area.

Abstract

The supramammillary (SuM) area is part of the diencephalic nuclei comprising the mammillary bodies, and is a key structure in the memory and spatial learning processes. It is a critical region in the modulation/generation of hippocampal theta rhythm. In addition, many papers have recently shown a clear involvement of this structure in the processes of spatial learning and memory in animal models, although it is still not known how it modulates spatial navigation and response emotional.

The aim of the present research was to study the effect of the temporary inactivation of the SuM area on synaptic plasticity of crucial structures in the formation of spatial memory and emotional response.

Sprague-Dawley rats were asigned in three groups: a control group where the animals were not subjected to any treatment, and two groups where the rats received microinjections of tetrodotoxin (TTX) in the SuM area (5 ng diluted in 0.5 µl of saline) or saline (0.5 µl). The microinjections were administered 90 minutes before the perfusion. Later, cellular activity in medial septum/diagonal band of Broca (MS/DBB) and CA3 region of the dorsal hippocampus was assessed, by measuring the immediate early gene *c-fos*.

The results show a clear hiperactivity cellular in medial septum/diagonal band of Broca and a clear hypoactivity cellular in the CA3 region of the hippocampus when there was a functional inactivation of the SuM area. It suggests that the SuM area seems to be part of the connection and information input pathways to CA3 region of the hippocampal formation, key for proper functioning in spatial memory and emotional response.

Key words: supramammillary area, CA3 region, medial septum-diagonal band of Broca, spatial learning, tetrodotoxin, c-Fos.

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1. INTRODUCTION

Previous studies have shown the implication of the supramammillary (SuM) area in spatial learning and emotional response tasks (Aggleton et al. 1995, Gray and McNaughton 2000). More recently, it has been demonstrated that permanent and temporary lesion intra-SuM caused a clear affectation in spatial behavior and emotion in rats (Shahidi et al. 2004, Aranda et al. 2006, 2008), however, little is known about the molecular mechanism which makes the SuM a relevant structure in the execution of these tasks.

Given the connectivity that this nucleus has with other regions of the brain (Aggleton et al. 2010), it could be suggested that the SuM modulates spatial memory and anxiety response by means of its dense network of connections with regions such as the hippocampus.

The pattern of projections of the SuM area suggests that it can modulate hippocampal function in two ways: directly through the hippocampal projections (supramammillo-hippocampal pathway) and indirectly through the medial septum/diagonal band of Broca (MS/DBB) region (supramammillo-septo-hippocampal pathways). SuM projections to the hippocampus originate mainly in the lateral two-thirds of the SuM area (Vertes and Kocsis 1997), innervating the dentate gyrus and the CA2/CA3 region of Ammon's horn in the dorsal hippocampus (Nitsch and Leranth 1994, Kiss et al. 2000). The projections of the SuM area to hippocampus contains mainly glutamatergic neurons (Kiss et al. 2000). In addition, the SuM area receives input from the hippocampus, but this input is of less importance (Pan and McNaugton 2004). On the other hand, in the indirect via, the supramammillo-septum pathway contains glutamatergic, cholinergic and GABAergic neurons (Borgheyi and Freund 1998, Kiss et al. 2000). These excitatory synapses in turn project to hippocampus (Kiss et al. 2000). Finally, the septo-hippocampal pathway contains two major classes of neurons: cholinergic and GABAergic cells (Vertes and Kocsis 1997).

It is not only the existence of these connections which indicates that the SuM area may influence spatial memory and emotional behavior, modulating the functional activity of these regions. Moreover, there is evidence that disruption of the SuM area affects the neurophysiological properties of the hippocampus. It is widely known that one of the neurophysiological properties of hippocampal neurons is the presence of theta rhythm, a frequency band oscillating between 4 and 8 Hz (Pan and McNaughton 2004). The theta rhythm is not an intrinsic property of hippocampal neurons, but under the control of a pacemaker located in the medial septal region (Pan and McNaughton 2004). In recent years, together with the importance of the medial septum, it has been observed that the SuM area is a critical link in the modulation /generation of this rhythm in the hippocampus, possibly modulating the activity of the medial septum (Vertes and Kocsis 1997, Kirk 1998). The present study was carried out with the aim of examining how inactivation by TTX of the supramammillary area of the hypothalamus, affects the neuronal plasticity of the MS/DBB and CA3 region of the hippocampus. Therefore, this investigation was designed to study the immunoreactivity of the c-Fos protein in the MS/DBB and hippocampal CA3 region, which receives both direct of the SuM area and indirect SuM projections through the MS/DBB (Fig 1). This protein is the product of the immediate expression gene called *c-fos.* This gene is stimulated by numerous events such as an increase in intracellular calcium, cell activation through numerous neurotransmitters such as excitatory amino acids (glutamate/aspartate), electrical stimulation, behavioral training, etc. (Rose 1991, Nicolaev et al. 1992) and is a well validated measure of cell activity (Albasser et al. 2010, Nowak et al. 2012). In this study, we selected immunohistochemistry determination of the protein product of the *c-fos* gene, or c-Fos protein, whose peak accumulation in the cell nucleus takes place between 60 and 120 minutes after stimulation (Wirtshafter et al. 1998). Furthermore, quantification of the immunoreactivity of the c-Fos protein in the MS/DBB and CA3 region of the hippocampus was performed using a stereological method. The stereological quantification allowed us to obtain unbiased estimates of quantitative parameters such as numerical density (Nv) to use in this latest study.

2.METHODS

2.1. Animals

For this study, 20 male Sprague-Dawley rats were used, with initial weights of 300-450 grams. All animals were supplied by the experimental animals breeding facility, Criffa S.A. (Barcelona, Spain).The animals were housed under standard conditions: a temperature of 22 +/- 3° C and constant 12 hours light/darkness cycles (8:00 a.m./20:00 p.m.) throughout the entire experiment. All animals were given access to food and water *ad libitum*. The animals were assigned to the following groups:
-Control group (n = 5): Animals belonging to this group were not subjected to any treatment, remaining in their cages until vascular perfusion.

-Saline group (SAL) (n = 7): Animals in this group were subjected to a surgical procedure whereby a guide cannula was implanted at the SuM area level, thereafter, they received saline.

-Tetrodotoxin group (TTX) (n = 8): Animals underwent stereotaxic implantation of guide cannulas in the SuM area, thereafter they received tetrodotoxin.

The experiments were done in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) for the care and use of laboratory animals.

2.2. Surgery

The rats underwent surgery after a minimum of 10 days in the laboratory. Before initiation of the surgical procedure, animals were anesthetized with equithesin (3.5 ml / Kg i.p.) and after 5 minutes were administered atropine sulfate (Braun Medical, Inc.) (0.05 ml / Kg i.p.) to alleviate respiratory congestion of the general anesthesia. The equithesin was obtained by mixing differents drug compounds: first, 0,5 gr of pentobarbital sodium (Sigma, Steinheim, Germany) and 5,87 mL of absolute ethanol were mixed (A); secondly, 2,7 gr chloral hydrate (Panreac Química, SA, Barcelona, Spain) and 21,83 mL propilene glicol (Panreac Química, SA, Barcelona, Spain) were mixed (B), finally, 1,08 gr of magnesium sulfate (Panreac Química, SA, Barcelona, Spain) and 24,33 mL distilled water were mixed (C). The compounds were mixed (A + B + C), and stored at room

temperature protected of the light. Having established the analgesic level reached by the animal, taking the dura mater as reference, the guide cannula (AP: -4.5 mm from bregma, at an angle of 6 ° to the vertical medial 0.9 mm and 8 mm deep with respect to the skull at the midline) was inserted according to the stereotaxic atlas of Paxinos and Watson (Paxinos and Watson 1998). Two screws were inserted into the skull and the cannula was fixed to them with dental cement. The cannula was then closed with a stylet. All animals were placed in individual cages and received paracetamol 300 mg/Kg (Upsamedica SL) diluted in 500 ml of water for 3 days after the operation.

2.3. SuM area microinjections

Before microinjection, the animal was restrained by hand and the cannula stylet was removed and replaced with the injection needle (30-gauge) connected to polyethylene tubing (P_{50}) and a 10 µl Hamilton srynge (Hamilton, Reno, NV). The needle was inserted 1 mm beyond the tip of the cannula. Syringe flow was regulated by a microinjection pump (Harvard Apparatus Boston, USA), which administered 0.5 µl of saline (0.9% NaCl) or 5 ng of tetrodotoxin (TTX Sigma, St Louis, Mo) diluted in 0.5 µl of saline (0.9% NaCl) for 2 minutes. The needle was kept in place for another 1 minute before it was slowly withdrawn. TTX is a powerful sodium channel blocker (Narahashi 1972) that lasts for at least 90 minutes and is fully reversible 24 hours after administration (Zhuravin and Bures 1991, Aranda et al. 2008) (Fig 2).

2.4. c-Fos immunohistochemistry

All animals were deeply anesthetized with sodium pentobarbital (60 mg/Kg, i.p.) (Sigma-Aldrich, Steinheim, Germany), 90 minutes after finishing the TTX microinjections or solution saline. Later, the perfusion was performed in all animals by means of the artery aorta, the first, with phospate buffered saline (PBS) (0.1 M, Ph 7.4) for 10 minutes, followed by 4% paraformaldehyde in PBS (0.1 M, Ph 7.4) for 15 minutes. The brains were postfixed in 4% paraformaldehyde for 24 hours until they sank in PBS containing 30% sucrose (Panreac Química, S.A., Madrid, Spain). These tissues were stored at -70ºC until the day of the seriation. Histological sections were obtained with a thickness of 30 µm and were stored at -20ºC until of the inmunohistochemical study. Four series of tissues were

obtained, with 5-6 sections of them containing the region in MS/DBB and 8-10 sections in CA3 region aproximately. A serie was used for the immunohistochemical study, and other serie, for verify that the guide cannulas were correctly implanted.

The immunohistochemical process was performed on flotation. After some washes for 10 minutes at room temperature, the endogenous peroxidase was deactivated with a PBS (0.01 M, pH 7.4) containing hydrogen peroxide (0.6%) for 30 minutes in the dark. The sections were subsequently washed three times in PBS, and later, a wash with a solution PBS containing normal ram serum was performed (1%) and Tritón X-100 (3%), then incubated with a primary antibody (anti-c-Fos (Santa Cruz biotechnology Inc., CL, USA; 1/5000) for 12-18 hours at 4ºC. This is a polyclonal antibody obtained in rabbit, wich recognizes 3-16 amino acids of the N-terminal region of human c-Fos protein p_{62}. Following multiple washes in PBS, the sections were incubated with a secondary antibody, biotin conjugated (anti-rabbit IgG, dilution 1/200). They were washed three times in PBS and were incubated with avidin-biotin-peroxidase complex (system ABC, Lab Vector) for 1 hour at room temperature. Finally, DAB (Sigma-Aldrich, Madrid, Spain) for 5 minutes in the dark was used for visualization of c-Fos immunoreactivity. After some washes with PBS, the sections were then dehydrated through a series of increasing degree alcohols, cleared with xylene, and mounted in DPX (Sigma-Aldrich, Madrid, Spain) for microscopic observation.

The number of c-Fos positive nuclei in MS/DBB and the CA3 region of the hippocampus was quantified by the stereological methods. Stereology as a discipline that consists of a set of statistical-geometric procedures in order to obtain quantitative information of three-dimensional structures from their two-dimensional images (Cruz-Orive and Weibel 1990). Once the objects under study had been obtained, quantification was done using a microscope (Olimpus BX-51, Germany) with monitored plate and connected to computerized interface to guide a MT12 microcator that control the movements in z-axis. The software analysis (CAST GRID. Olympus, Denmark) made a random sampling out within each selected area, using dissectors of 1885 μm^2 and 20 µm thick. Thus, by multiplying the area of the dissector by its height, we obtained the volume of each dissector. Multiplying this volume by the number of dissectors employed, the reference volume was obtained.

Later, the total number of c-Fos positive neuronal nuclei counted in all dissectors was divided by the total reference volume, thereby obtaining the number of c-Fos positive cells per unit volume (Nv).

For quantification, the MS/DBB and CA3 region of the dorsal hippocampus was delineated using the 4x/10x objetive (following the coordinates of Paxinos and Watson 1998 atlas) (from 1.20 mm to 0.20 mm for MS/DBB region and, from -1.6 mm to -3.3 mm to CA3 region).Within the selected region using 100x immersion objetive, the software created sampling dissectors randomly, on which the number of immunorective neuronal nuclei for c-Fos protein were counted. Quantification was done counting only the c-Fos positive nuclei falling within the dissector.

2.5. Statistics

Standar deviations were comparable in all groups, and the data had a normal distribution. So, the number of c-Fos immunoreactive neuronal nuclei estimated in MS/DBB and the CA3 region of the hippocampus were analyzed by means of an one-way analysis of variance (ANOVA), followed by an a posteriori test of statistical significance (Honestly Significant Difference test or HSD for unequal groups). All statistical analysis was performed using the Statistica version 7.0 program.

3. RESULTS

3.1. Verification of cannula placement

Two animals microinjected with TTX and one animal microinjected with solution saline were excluded from statistical analysis, due to the cannula tips being located more than 500 µm of the SuM area (Fig 3).

3.2. The effects of functional inactivation in SuM area on c-Fos expression in MS/DBB.

For the analysis of the effects of inactivating the SuM area on c-Fos immunoreactivity in the medial septum/diagonal band of Broca, a total of 5 control rats, 6 rats microinjected with a saline solution and 6 rats microinjected with TTX, were used. The results obtained in the medial septum/diagonal band of Broca show that microinjection of a saline in the SuM area causes an increase in c-Fos immunoreactivity, however, the effect of the microinjection experimented a significant increase in the number of immunopositive c-Fos in MS/DBB when the SuM area were inactivated with microinjections of TTX ($F_{2,14} = 16.76$; $p < 0.00019$; HSD: Control *vs* SAL; SAL *vs* TTX ($p < 0.05$)). (Fig 4A).

3.3. The effects of functional inactivation in SuM area on c-Fos expression in CA3 region of hippocampus.

For the analysis of the effects of inactivating the SuM area on c-Fos immunoreactivity in the CA3 region of the dorsal hippocampus, a total of 5 control rats, 6 rats microinjected with a saline solution and 6 rats microinjected with TTX, were used. The results obtained in the CA3 region of the dorsal hippocampus show that microinjection of a saline solution in the SuM area causes an increase in c-Fos immunoreactivity. However, the effect of the microinjection is eliminated by inactivation of the SuM area with the microinjections of TTX ($F_{2, 14} = 7.076$; $p<0.001$; HSD: SAL *vs* Control and TTX ($p<0.05$)). (Fig 5E).

4. DISCUSSION

The main finding of the present study was that the temporary inactivation of the supramammillary area increases c-Fos immunoreactivity in the medial septum/diagonal band of Broca while decreasing in the CA3 region of the hippocampus. This suggests that the functional inactivation of the SuM area induces changes of neuronal plasticity in essential structures for spatial memory and emotional response.

The results show that microinjections of saline in the SuM area induced an increase in the expression of c-Fos protein in the MS/DBB region and the CA3 field of the dorsal hippocampus. However, the

inactivation of the supramammillary area with TTX causes a significant increase in the number of c-Fos positive cells in MS/DBB (Fig 4C, D, E), in contrast with the control animals and the animals microinjected with saline. On the other hand, the TTX microinjections in the SuM area causes an important decrease in the number of c-Fos positive cells in the CA3 field of the dorsal hippocampus (Fig 5B, C, D), in contrast with the animals microinjected with saline and with the control animals. In this work, the c-Fos protein expression in the projection region of the SuM area was studied when it was functionally inactivated by microinjections of TTX. It is well known that injury or functional impairment of the hippocampus causes significant deficiency in the performance of spatial and emotional learning tasks (Morris et al. 1982, Gray and McNaughton 2000, Ramos 2009), leading to the onset of amnesia in humans (Meador et al. 1991, Kahana et al. 1999). Thus, the hypofunctionality observed in the CA3 region of hippocampus is a consequence of the temporary inactivation of the SuM area that may constitute a critical event which explains, at least in part, behavioral deficiency in memory tasks and anxiety response in some investigations (Shahidi et al. 2004, Aranda et al. 2006, 2008). As such, this hypoactivity allows to hypothesize that the SuM area projections to part of the CA3 region of the hippocampus transfer important information via MS/DBB, which enables hippocampus to normally carry out spatial memory as well as emotional response functions.

There may be two pathways through which the inactivation of the SuM area can alter hippocampal function: directly, through the SuM area projections to the hippocampus (Vertes and McKenna 2000, Kiss et al. 2000) or indirectly without of the MS/DBB (Gonzalo-Ruiz et al. 1999, Kiss et al. 2000). Several results indicate that the modulation of the hipocamppal activity through the MS/DBB is crucial for the right functioning of this one. In this regard, electrophysiological studies by the McNaughton group indicate that a significant reduction occurs in the frequency of the theta rhythm of the hippocampus in animals with neurochemical lesions and/or temporal lesions of the SuM nucleus (Kirk and McNaughton 1991, 1993, Kosis and Vertes 1994). This reduction in the frequency of the theta rhythm is, in turn, related to alterations in emotional response and hippocampus-dependent learning (Pan and McNaughton 1997). Even when great importance is granted to mediation of the MS/DBB in the functional modulation of the SuM area, we can not rule out the

possibility that the direct projection of the SuM area to the dorsal hippocampus may contribute to the behavioral results observed in those studies (Shahidi et al. 2004, Aranda et al. 2008). Given this possibility, the study of c-Fos immunoreactivity in the MS/DBB region suggests that, at least, this projection is an important vehicle to transfer information of the SuM area to the CA3 region of the hippocampus. In fact, the results show that the temporary inactivation of SuM area causes a significant and substantial increase in c-Fos immunoreactivity in the MS/DBB. Considering the information obtained in this work, it is possible to suggest a mechanism of action.

In this way, it is well- established that the MS/DBB contains two populations of neurons, one of them being GABAergic and another one cholinergic (Wainer et al. 1985). The interaction between both neuronal populations in the MS/DBB regulates the hipocampal function, as well as the behavioral associated to itself.The electrofisiological studies show that cholinergic hippocampal afferent activation produces a desinhibition of pyramidal cells in this temporal region (Bernardo and Price 1982, Bland et al. 1996). Also, the intraseptal administration of a GABAergic (muscimol) agonist which reduces the functional activity of the cells medial septum, reduces significantly the levels of acetylcholine (Ach) in the hippocampus trougth of the GABAergic inhibition of the septo-hippocampal projection cells (Allen and Crawfort 1984). Recently, *in vitro* studies have shown that the stimulation of the septal region decreased the activity of the GABAergic interneurons of the hippocampus, increasing the general activity of the pyramidal neurons (Alreja et al. 2000).The activity of cholinergic neurons MS/DBB, containing $GABA_A$ receptors, is modulated by GABAergic interneurons. Pharmacological blockade of these receptors using GABA antagonists increases ACh release in the hippocampus, while stimulation with agonists exerts an opposite effect, reducing the release of ACh in the hippocampus (Moor et al. 1998a, 1988b).

Taking into account the information obtained in the present study regarding hypofunction in the CA3 region of hippocampus (reduced immunoreactivity c-Fos) when the SuM area was inactivated, this could be due to an increase in the functional activity of GABAergic interneurons MS/DBB. This increase of GABAergic activity in the MS/DBB inhibits cholinergic neurons that project to the hippocampus, causing the observed hypofunction. However, we can not rule out that increased

activation of the GABAergic projection neurons to the hippocampus, as well as inhibition of a set of recently described glutamatergic projection neurons (Manseau et al. 2005), may contribute to the hypofunction described in CA3 region. Furthermore, the temporary removal of the direct projection of the SuM area to the hippocampus, reducing glutamatergic stimulation of pyramidal neurons, could also contribute to the observed results in the works of Aranda group (2006, 2008), when inactivation supramammillary area impairs spatial memory and emotional response.

Furthermore, this results are consistent with the results obtained in models of diencephalic amnesia (pyrithiamine thiamine induced deficiency), which described a severe disruption in the availability of Ach in the hippocampus and in the cortex (Roland and Savage 2007). Recently, Vann (2010) has suggested that the cholinergic impairment in this model could be the result of loss of efferent mammillary bodies. In this respect, the group of Beracochea (1995) showed the importance of the mammillary region in controlling cholinergic projections to the hippocampus and cortex. Damage to this region produces a significant reduction of choline uptake of sodium-dependent high affinity (SDHACU) in the hippocampus and cerebral cortex, indicating a loss of cholinergic inputs in these regions globally. Reinterpreting these experiments in which, at least partially, the SuM area is damaged, it is not surprising that this region contributes to the cholinergic impairment observed in patients with Wernicke-Korsakoff syndrome and in animal models of alcoholic encephalopathy. Therefore, cognitive and emotional deficits observed in humans and animals with functional alterations of SuM area, could be explained, in part, by the loss of cholinergic modulation of CA3 region function.

Further experiments will be necessary to determine the importance of the cholinergic and glutamatergic synapses of the SuM area with MS/DBB and the CA3 region of the hippocampus in the formation of spatial memory and emotional response.

5. CONCLUSIONS

In conclusion, the present study suggests that the SuM area maintains critical connections with the CA3 region of hippocampus through the MS/DBB. This could be an essential part in the input routes to the hippocampus, needed for the proper development of spatial memory and emotional response.

ACKNOWLEDGMENTS

This research was supported by University of Málaga. I thank Juan Gómez, Juan M. Enamorado, Francisca González and Dr Juan M. Manzaneque for their excellent technical assistance.

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Fig 1 Schematic representation of the experimental design used in the study of the c-Fos activity in MS/DBB and the CA3 region of hippocampus, when the supramammillary area is temporarily inactivated.

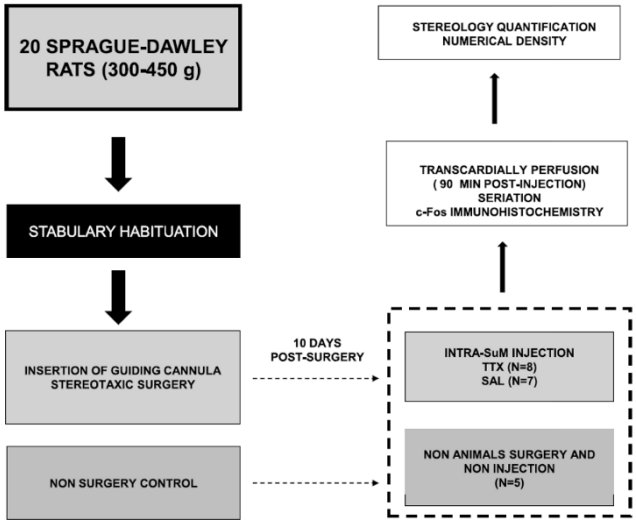

Fig 2 Representation of the process of tetrodotoxin (TTX) and saline microinjection, in the supramammillary area of hypothalamus. It shows the microinjection pump used, as well as, the Hamilton syringe (10 µl). The Hamilton syringe (10 µl) connected to polyethylene tubing (P50), through administered 0.5 µl of saline or TTX (5ng of tetrodotoxin diluted in 0.5 µl of saline) with a rate of administration 0.25 µl/minute. On the top image it is shown a coronal section in the SuM area where it is observed the guide cannula and injection cannula. The injection cannula was inserted 1 mm beyond the tip of the guide cannula .

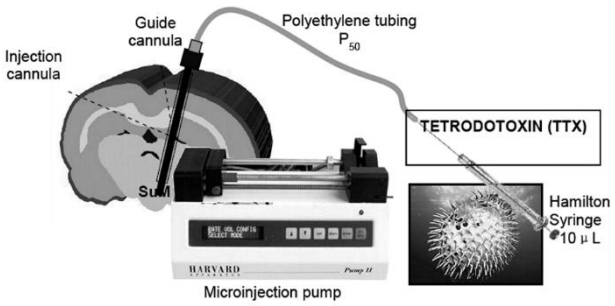

Fig 3 Schematic representation of placements of cannula tips targeted to the supramammillary area. (SuM: supramammillary nucleus; MM: medial mammaillary nucleus; LM: lateral mammillary

nucleus; mp: mammillary peduncle; mt: mammillothalamic tract; MRe: mammillary recess 3rd ventricle) (Paxinos and Watson, 1998).

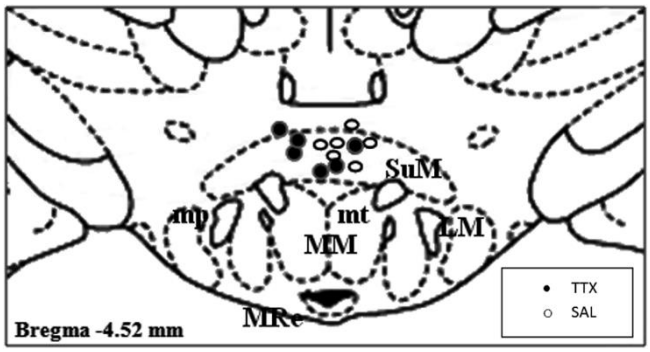

Fig 4 (A) Graphic representation showing the increase of c-Fos immunoreactivity in the MS/DBB region in rats subjected to microinfusion of TTX in the SuM area, when compared with the microinjection of saline in the same area, and control animals (* $p<0.05$ TTX vs Control and SAL; ** SAL vs Control and TTX). (B) Photograph showing the location of the MS/DBB region in coronal section of a rat brain immunostained for c-Fos protein. (C, D and E) Micrographs at the MS/DBB region showing c-Fos immunoreactivity in a control animal microinjected with saline (0.9%), and with tetrodotoxin (TTX; 5 ng) in supramammillary area. Scales: B: 1000 μm; C, D, E: 100 μm.

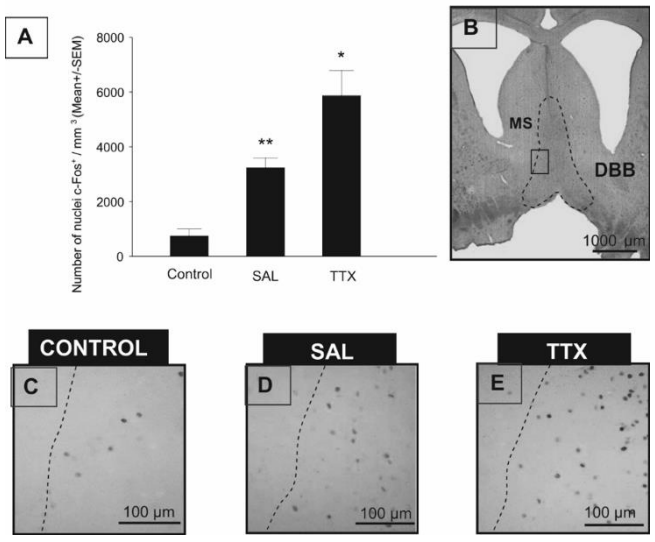

Fig 5 (A) Photograph showing the location of the CA3 region in the left dorsal hippocampus in a coronal section of a rat brain immunostained for c-Fos protein. (B, C and D) Micrographs at the CA3

region showing c-Fos immunoreactivity in a control animal microinjected with saline (0.9%), and with tetrodotoxin (TTX; 5 ng) in supramammillary area. Scales: A: 375 μm; B, C y D: 100 μm. (E) Graphic representation showing the reduction of c-Fos immunoreactivity in the CA3 region of the dorsal hippocampus in rats subjected to microinfusion of TTX in the SuM area, when compared with the microinjection of saline (0.9%) in the same area, and control animals (* $p<0.05$, SAL vs Control, SAL vs TTX).