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Mechanisms involved in the long-term modulation of tyrosine hydroxylase by endothelins in the olfactory bulb of normotensive rats

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ABSTRACT

The olfactory bulbs play a relevant role in the interaction between the animal and its environment. The existence of endothelin-1 and -3 in the rat olfactory bulbs suggests their role in the control of diverse functions regulated at this level. Tyrosine hydroxylase, a crucial enzyme in catecholamine biosynthesis, is tightly regulated by short- and long-term mechanisms. We have previously reported that in the olfactory bulbs endothelins participate in the short-term tyrosine hydroxylase regulation involving complex mechanisms. In the present work we studied the effect of long-term stimulation by endothelins on tyrosine hydroxylase in the rat olfactory bulbs. Our findings show that endothelin-1 and -3 modulated catecholaminergic transmission by increasing enzymatic activity. However, these peptides acted through different receptors and intracellular pathways. Endothelin-1 enhanced tyrosine hydroxylase activity through a super high affinity ET_A receptor and cAMP/PKA and CaMK-II pathways, whereas, endothelin-3 through a super high affinity atypical receptor coupled to cAMP/PKA, PLC/PKC and CaMK-II pathways. Endothelins also increased tyrosine hydroxylase mRNA and the enzyme total level as well as the phosphorylation of Ser 19, 31 and 40 sites. Furthermore, both peptides stimulated dopamine turnover and reduced its endogenous content.

These findings support that endothelins are involved in the long-term regulation of tyrosine hydroxylase, leading to an increase in the catecholaminergic activity which might be implicated in the development and/or maintenance of diverse pathologies involving the olfactory bulbs.

1. Introduction

Endothelins (ETs) are a family of vasoactive peptides comprised by three isoforms ET-1, ET-2 and ET-3 (Kuwaki et al., 1997; Schneider et al., 2007). ETs biological effects are mediated through the activation of two well characterized G-protein coupled receptors (GPCRs) named ET_A and ET_B (Kuwaki et al., 1997; Schneider et al., 2007). However, the observation of atypical responses in the presence of selective ET_A and ET_B antagonists and agonists support the existence of atypical receptors (ET_{AX} or ET_{BX}) (Nambi et al., 1997; di Nunzio et al., 2004; Perfume et al., 2007, 2008; Nabhen et al., 2009; Hope et al., 2010). In addition, a third receptor subtype named ET_C which displays high affinity for ET-3 was cloned in *Xenopus laevis*, and although functional studies support its existence in mammals it has not been cloned in this specie yet (Karne et al., 1993). The endothelinergic system and other vasoactive peptides like angiotensins and natriuretic peptides are present in different areas of the mammalian central nervous system (CNS) including the olfactory bulbs (OBs) (Kohzuki et al., 1991; Langub et al., 1995; Kurokawa et al., 1997; Wright and Harding, 1997).

The OB is an extension of the rostral telencephalon that plays a relevant role in the interaction between the animal and its environment (Cain, 1974). It is related to regions of the limbic system like the amygdala, septum, pyriform cortex and frontoorbito cortex as well as the preoptical area, and the ventromedial and posterior nuclei of the hypothalamus (Kawasaki et al., 1980; Song and Leonard, 2005). Most of these brain regions and areas are closely related to the control of the cardiovascular function and mood disorders. The loss of connection with the forebrain and midbrain produced by OB removal (olfactory bulbectomy) results in various neurochemical, behavioral and physiological changes

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(Moffitt et al., 2002; Song and Leonard, 2005). Impairment of the functional responses is related to changes in neurotransmitters like norepinephrine (NE), dopamine (DA), serotonine, GABA, glutamate and acetylcholine (Kelly et al., 1997; Moffitt et al., 2002; Masini et al., 2004). Furthermore, bilateral olfactory bulbectomy is an animal model of depression, a pathology frequently associated with cardiovascular diseases (Davis et al., 2008; Grippo and Johnson, 2009). The excessive activation of the sympathetic nervous system appears to be one of the mechanisms involved in the development of hypertension and heart failure in depressive disorders (Davis et al., 2008; Grippo and Johnson, 2009).

Several studies show that olfactory bulbectomy alters the concentration of brain NE and DA (Moffitt et al., 2002; Masini et al., 2004; Song and Leonard, 2005). Catecholamines (DA, NE, and epinephrine) are involved in the regulation of numerous biological functions including the cardiovascular activity. The biosynthetic pathway is regulated by tyrosine hydroxylase (TH), the enzyme which catalyzes the hydrolysis of L-tyrosine to L-Dopa. TH is a specific marker of catecholaminergic neurons and is expressed in the CNS including the OB (Cigola et al., 1998). The regulation of TH is a complex process which involves both short- and long-term mechanisms. Short-term modulatory mechanisms (seconds to minutes) include feedback inhibition, allosteric modulation and phosphorylation, whereas long-term regulatory mechanisms (hours to days) include transcriptional regulation, RNA stability, alternative RNA splicing and translational regulation (Kumer and Vrana, 1996; Dunkley et al., 2004).

We have recently reported that in the OB both, ET-1 and ET-3, increase TH activity presumably through the activation of an atypical receptor coupled to the adenylyl cyclase/protein kinase A (PKA), phospholipase C (PLC)/DAG/protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase II (CamK-II) pathways (Nabhen et al., 2009). In addition, increased intracellular calcium resulting from its release by ryanodine sensitive stores and its influx from the extracellular compartment is also involved. Furthermore, ETs increase TH phosphorylation at Ser-19 and Ser-40 sites without affecting Ser-31 or total protein levels (Nabhen et al., 2009). However, the role of the interaction between ETs and the catecholaminergic transmission in the OB remains to be fully elucidated.

In the present study we sought to establish the role of ET-1 and ET-3 in the long-term regulation of TH activity and to characterize the receptors and intracellular mechanisms involved. Present findings show that both ETs modulated catecholaminergic transmission through an increase in TH activity by enhancing its mRNA and protein synthesis and the phosphorylation of 19, 31 and 40 serine residues. Both ETs stimulated different receptors and intracellular pathways. ET-1 increased TH activity through a super high affinity ET_A receptor activation involving cAMP/PKA and CaMK-II pathways, whereas ET-3 through a super high affinity atypical receptor coupled to the cAMP/PKA, CaMK-II and PLC/PKC pathways.

2. Experimental procedures

2.1. Animals and chemicals

Male Sprague–Dawley rats weighing between 250 and 300 g (from the Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires) were used in the experiments. Rats were housed in steel cages, and maintained in a controlled room (temperature and humidity between 20–23 °C and 50–60%, respectively) with a 12 h light/dark cycle. All animals had free access to water and commercial chow.

The following drugs and reagents were used: ET-1, ET-3, (N,N-hexamethylene) carbamoyl-Leu-D-Trp(CHO)-D-Trp (BQ-610), and N-cis-2,6-dimethylpiperidinocarbonyl-L-γ-methyl-Leu-D-l-methoxycarbonil-Trp-D-Nle (BQ-788), 8–21 (IRL-1620) and sarafotoxin S6b (SRTx-b) (American Peptide Company Inc., CA, USA). Catalase, desipramine hydrochloride, L-DOPA, bisindolylmaleimide I (GF-109203x), N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide 2HCl (H-89), hydrocortisone, pargyline hydrochloride, suramin (SMN), 1-[N,O-bis-(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), 6-methyl-tetrahydrobiopterin and L-tyrosine (MP Biomedicals LLC, CA, USA). L-[3,5-^{3}H]tyrosine and DL-[7-^{3}H]NE (PerkinElmer Life and Analytical Sciences, MA, USA); PVDF membrane (GE Healthcare, Amersham Biosciences, UK); anti-actin polyclonal antibody (Ab) (Actin-Ab), anti-TH monoclonal Ab (TH-Ab), 8-bromoadenosine-3′,5′-cyclic monophosphate (8Br-cAMP), 8-bromoguanosine-3′,5′-cyclic monophosphate (8Br-cGMP), forskolin (FSK), Nω-nitro-L-arginine methyl ester (L-NAME), phorbol 12-myristate 13-acetate (PMA), protease inhibitor cocktail and 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexy]-1H-pyrrole-2,5-dione (U-73122) (Sigma, MO, USA). Rabbit anti-TH phospho-Ser-19, -31 and -40 (19 Ser-P, 31 Ser-P and 40 Ser-P, respectively) (Invitrogene, CA, USA). Peroxidase conjugated anti-mouse Ab, and anti-rabbit Ab (Pierce, USA) and minimum essential medium MEM/EBSS (HyClone Lab. Inv., UT, USA). Other reagents were of analytical or molecular biology quality and obtained from standard sources.

2.2. Experimental protocol

Experiments were performed following the recommendations of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication 1985, Revised 1996) and approved by the Institutional Animal Care Committee of the Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires. Animals were killed by decapitation between 9:00 and 12:00 h and the OB quickly removed according to Palkovits and Brownstein (1988). Tissues were pre-incubated in a Dubnoff incubator for 30 min at 37 °C in Krebs bicarbonate solution supplemented with minimum essential medium MEM/EBSS (KBSS), pH 7.4, and bubbled with a gas mixture (95% O_2–5% CO_2) under continuous shaking. To determine the effect of ET-1 or ET-3 long term exposure on TH activity and expression, tissues were incubated for 240 min in the absence or in the presence of ETs (control and experimental groups, respectively). The following antagonists or inhibitors were added 15 min before and during the incubation period: 100 nM and 100 pM BQ-610 (ET_A receptor antagonist), 100 nM and 100 pM BQ-788 (ET_B receptor antagonist), 500 nM SMN (G-protein inhibitor), 10 μM L-NAME (NO synthase (NOS) inhibitor), 500 nM H-89, 10 μM U-73122, 100 nM GF-109203x, 1 μM KN-62 (PKA, PLC, PKC and CaMK-II inhibitors, respectively). In other experiments, the OBs were incubated for 240 min in the presence of 300 nM and 100 pM SRT-x ($ET_{A/B}$ agonist), 1 μM and 100 pM IRL-1620 (ET_B agonist), 100 μM 8Br-cGMP or 100 μM 8Br-cAMP (cGMP and cAMP analogue, respectively); and 1 μM PMA or 20 μM forskolin (PKC and adenylyl cyclase activator, respectively).

2.2.1. Determination of TH activity

TH activity was assessed as reported by Reinhard et al. (1986). Briefly, following the incubation period OBs were homogenized in 500 μl buffer (5 mM KH_2PO_4 and 0.2% Triton X-100, pH 7.0). After saving an aliquot for protein determination, samples were centrifuged for 10 min at 10,000 × g at 4 °C, and an aliquot of the supernatant was incubated for 20 min at 37 °C with 50 mM HEPES (pH 7.0), containing 15 nmol L-tyrosine with 0.5 μCi ^{3}H-tyrosine, 420 mM β-mercaptoethanol, 1000 U catalase, and 0.75 mM 6-methyl-tetrahydrobiopterin. The reaction was stopped by the addition of 1 ml 7.5% activated charcoal suspension in 1 N HCl. The final mixture was vortexed and centrifuged at 500 × g for 10 min followed by the supernatant separation where ^3H_2O was determined by conventional scintillation methods. Blank values were obtained by omitting 6-methyl-tetrahydrobiopterin from the mixture. Recovered ^3H_2O was determined as described by Reinhard et al. (1986). Results were expressed as percentage of control group ± SEM.

2.2.2. TH Western blot assay

OBs were homogenized in lysis buffer (20 mM Tris–Cl^- pH: 7.4, 1 mM PMSF, 5 mM EDTA, 25 mM NaF, 1% Triton X-100, 1% protease inhibitor cocktail) and then centrifuged for 20 min at 4 °C. An aliquot of the supernatant was saved for protein determination and the remaining sample mixed with LAEMMLI buffer (62.5 mM Tris–Cl^- pH: 6.8, 2% SDS, 5% B-ME, 10% glycerol, 4% bromophenol blue), boiled for 5 min, and then subjected to SDS-PAGE gel at 100 V for 2.30 h. Gels were then transferred to PVDF membranes at 100 V for 75 min. The membranes were blocked overnight at 4 °C in blocked solution (5% non-fat powder milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T)) and the gels were stained overnight at 4 °C with Coomasie Blue. The membranes were washed with TBS-T, and incubated with TH-Ab, 19 Ser-P, 31 Ser-P, 40 Ser-P (1/2000 overnight at 4 °C); β-actin Ab (1/2000, 1 h at room temperature), peroxidase conjugated anti-mouse Ab or anti-rabbit Ab (1/5000, 1 h at room temperature). After the final wash with TBS-T the bands were detected with Super Signal West Femto kit (Pierce, IL, USA). Bands were analyzed by densitometry and normalized to β-actin. Results were expressed as percentage of control group ± SEM.

2.2.3. Quantitative real-time RT-PCR for TH mRNA

After the incubation period total RNA was extracted from individual OB samples using MasterPure™ Purification Kit (Epicentre Biotechnologies, WI, USA) according to the manufacturer's protocol. Contaminating DNA was removed from the RNA by incubating each sample with RQ-1 RNase-Free DNase (Promega, WI, USA) at 37 °C for 40 min. RNA concentration was determined by spectrophotometry.

The cDNA was reversed transcripted from 2 μg RNA using 200 U M-MuLV Reverse Transcriptase and 20 U RNasin RNase inhibitor (Bionner, Korea), 10 μM

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dNTPs and 5 μg oligo (dT) primer. Specific mRNA level in each sample was measured in the ROTOR GENE-Q termocicler (Qiagen, Germany) in a 20 μl final volume. Each reaction was performed using 10 μl of 2× Real Mix containing Eva-green fluorocrome (Biodynamics, Argentina), 0.5 μl specific primers (10 μM) and 1 μl RT product. Amplification consisted of 35 cycles of denaturation at 94 °C for 15 s, annealing at 54 °C for 45 s, and extension at 72 °C for 30 s. Fluorescence signals were monitored sequentially for each sample once per cycle at the end of extension. All samples were assessed in duplicate.

An external standard RNA concentration curve for each primer pair was generated using pooled RNA samples and verified by agarose gel electrophoresis. For each experiment, the specificity of PCR products was confirmed by melting curves analysis showing the presence of a single cDNA product per primer pair, and by agarose gel electrophoresis revealing a single band of the predicted molecular weight for each product. To correct for minor variability among samples, TH expression was normalized to its expression level of the housekeeping gene β-actin. Relative expression levels quantification was carried out using the standard curve method. The following primers were used:

TH	Sense	5′-AGGGCTGCTGTCTTCCTAC-3′
	Antisense	5′-GCTGTGTCTGGGTCAAAGG-3′
β-Actin	Sense	5′-TTCTGTGTGGATTGGT GGCTCTA-3′
	Antisense	5′-CTGCTTGCTGATCCACATCTG-3′.

TH primers amplificate between the nucleotides 1539 and 1620 (80 pb) of the mRNA, whereas β-actin primers include the fragment 1096 and 1177 (81 pb) of the mRNA.

2.3. Determination of dopamine endogenous content and turnover

Quantification of DA and 3,4-dihydroxyphenylacetic acid (DOPAC) content in OB samples was carried out by means of high performance liquid chromatography using a Phenomenex Luna 5 μm, C18, 100 mm × 1 mm column (Phenomenex, Torrance, CA), and LC-4C electrochemical detector with glassy carbon electrode (BAS, West Lafayette, IN). The working electrode was set at +0.70 V respect to an Ag/AgCl reference electrode. The mobile phase contained 0.76 M $NaH_2PO_4 \cdot H_2O$, 0.5 mM EDTA, 1.2 mM 1-octane sulfonic acid, and 5% acetonitrile, and pH was adjusted to 2.8. The limit of quantification of DA and DOPAC was 0.2 and 0.5 ng/ml, respectively.

2.4. Statistical analysis

Results were expressed as means ± SEM. The statistical analysis was performed by ANOVA followed by the Student–Newman–Keuls test. p-Values of 0.05 or less were considered statistically significant.

3. Results

In order to assess the long-term effect of ET-1 and ET-3 on TH activity in the OB of normotensive rats, a concentration-response study was performed. Results showed that 10 pM and 10 fM ET-1 and 10 pM and 10 fM ET-3 increased TH activity by 44%, 20%, 52% and 45%, respectively (Fig. 1). However, 10 nM ET-1 or ET-3 failed to affect the enzyme activity (Fig. 1).

To identify the ET receptor that mediated the effect of ET-1 and ET-3 on TH activity, tissues were pre-treated with 100 nM BQ-610 and 100 nM BQ-788 (ET_A and ET_B antagonists, respectively). None of the antagonists modified basal TH activity (Fig. 2A and B). The selective ET_A antagonist prevented the increase in TH activity induced by ET-1 without affecting ET-3 response (Fig. 2A and B). On the other hand, ET_B receptor blockade by BQ-788 did not modify the increase in TH activity evoked by both ETs (Fig. 2A and B). These results support that the ET_A receptor and a non-conventional or atypical receptor were involved in ET-1 and ET-3 response, respectively. In addition, OBs were incubated with 300 nM SRT-x and 1 μM IRL-1620 ($ET_{A/B}$ and ET_B agonist). IRL-1620 failed to affect TH activity but SRT-x enhanced, supporting ET_A activation.

Since ET_B receptor blockade did not affect ETs response and the major effect on TH activity was with ETs at pM concentrations OBs were pre-treated with both ET receptor antagonists (100 pM BQ-610 and 100 pM BQ-788). Fig. 3A and B shows that the ET_A receptor mediated ET-1 response. However, the effect of ET-3 was

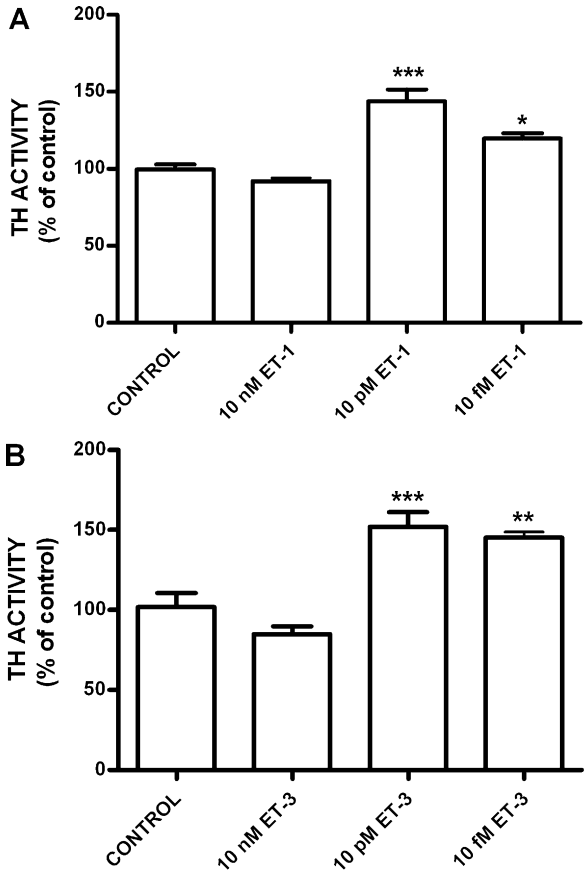

Fig. 1. Effect of endothelin-1 and endothelin-3 (ET-1 and ET-3) on tyrosine hydroxylase (TH) activity in the olfactory bulb (OB). The OBs were incubated with increasing concentrations of ET-1 (A) and ET-3 (B). TH activity was assessed as described in experimental procedures and it was expressed as percentage of control. $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control. Number of experiments: 6–7.

prevented by both antagonists supporting the participation of a non-conventional or atypical receptor with a different pharmacological profile. To further confirm the participation of ET receptors, tissues were incubated with 100 pM SRT-x and 100 pM IRL-1620. SRT-x increased TH activity whereas IRL-1620 did not modify it, thus confirming ET_A activation in ET-1 response (Fig. 3C).

Given that the conventional ET receptors are GPCRs and the pharmacological studies using ETs selective antagonists did not allow us to identify the receptor involved in ET-3 response, the OBs were pre-treated with 500 nM SMN (a G-protein inhibitor at this concentration) (Höller et al., 1999). Results showed that SMN did not modify TH activity, but it abolished ET-1 and ET-3 response (Fig. 4).

ETs receptors trigger diverse intracellular signalling pathways that in turn activate different kinases (PKC, PKG and PKA) involved in TH regulation (Kumer and Vrana, 1996; Dunkley, 2004). In order to investigate the participation of the PLC/PKC pathway, we studied the effect of ET-1 and ET-3 on TH activity in the presence of selective inhibitors (U-73122 and GF-109203x, respectively). Results showed that U-73122 and GF-109203x did not affect basal TH activity but they prevented the increase induced by ET-3 without affecting ET-1 response (Fig. 5A and B). To confirm PKC involvement in ET-3 effect on TH activity, the OBs were incubated with the PKC activator PMA. Activation of PKC by PMA led to an increase in TH activity (Fig. 5C).

To determine whether the cAMP/PKA pathway participated in ET-1 and ET-3 response experiments were carried out in the presence of H-89 (PKA inhibitor). Results showed that PKA

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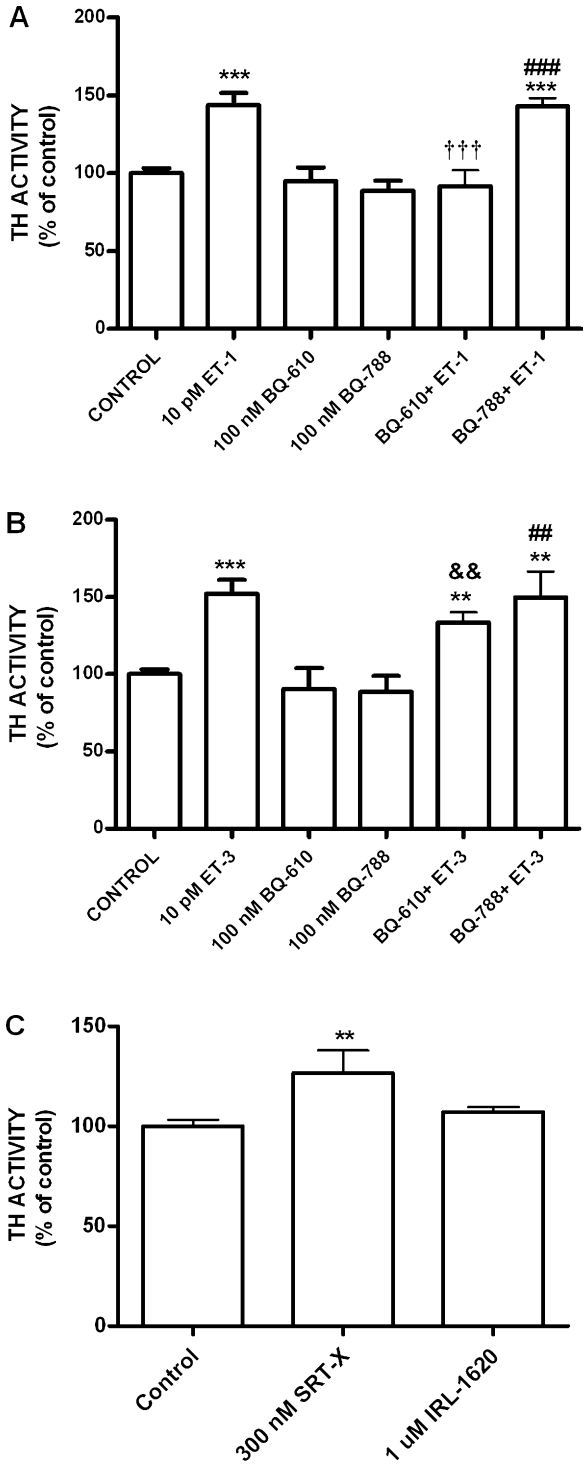

Fig. 2. Effect of endothelin receptor blockade on tyrosine hydroxylase (TH) activity in the olfactory bulb (OB). The OBs were pre-incubated with 10 nM BQ-610 (ET_A antagonist) (A), 100 nM BQ-788 (ET_B antagonist) (A) and further incubated with ET-1 and ET-3 or incubated with 300 nM SRT-x and 1 μM IRL-1620 ($ET_{A/B}$ and ET_B agonist) (C). TH activity was assessed as described in experimental procedures and it was expressed as percentage of control. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control; $^{†††}p < 0.001$ vs. ET-1; $^{\&\&}p < 0.01$ vs. BQ-610; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. BQ-788. Number of experiments: 6–8.

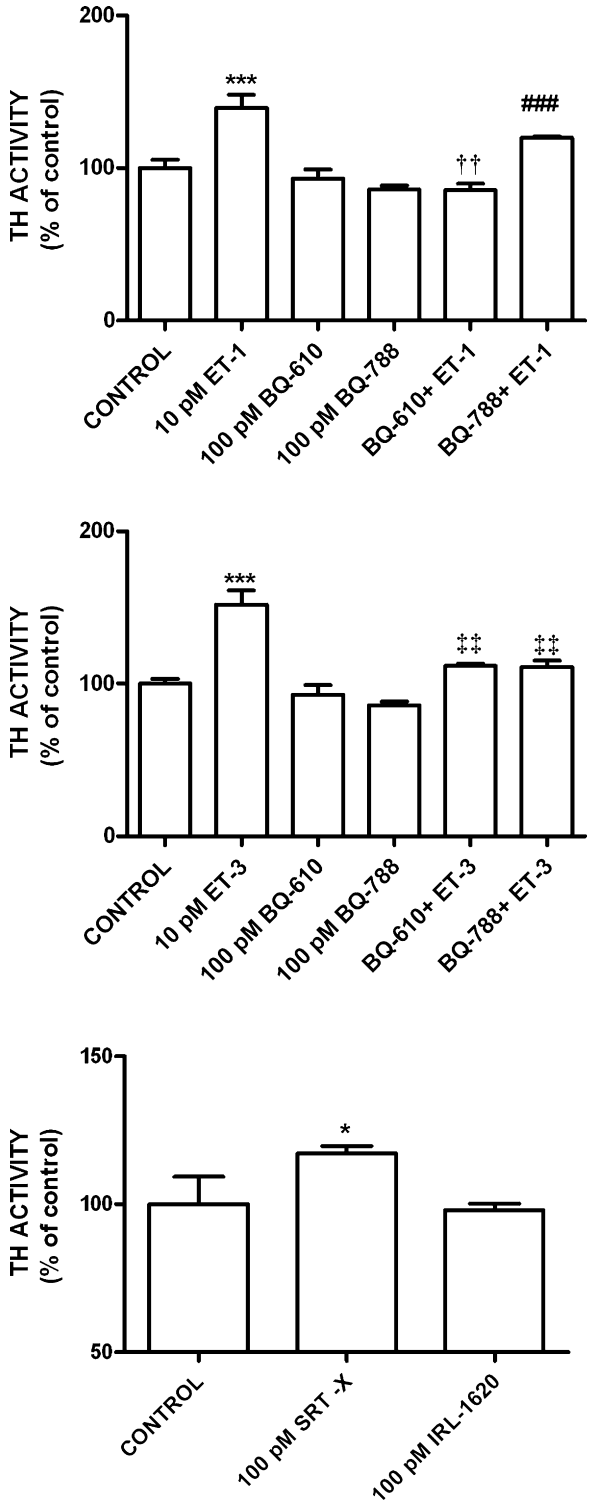

Fig. 3. Effect of endothelin receptor blockade on tyrosine hydroxylase (TH) activity in the olfactory bulb (OB). The OBs were pre-incubated with 100 pM BQ-610 (ET_A antagonist) (A) and 100 pM BQ-788 (ET_B antagonist) (A) and further incubated with ET-1 and ET-3 or incubated with 100 pM SRT-x and 100 pM IRL-1620 ($ET_{A/B}$ and ET_B agonist) (C). TH activity was assessed as described in experimental procedures and it was expressed as percentage of control. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control; $^{††}p < 0.01$ vs. ET-1; $^{‡‡}p < 0.01$ vs. ET-3; $^{\#\#\#}p < 0.01$ vs. BQ-788. Number of experiments: 6–8.

inhibition did not modify basal TH activity, but it abolished the increase in the enzyme activity induced by both ETs (Fig. 6A). To further confirm the contribution of this pathway, experiments were carried out in the presence of a cAMP analogue (100 μM 8Br-cAMP) and an adenylyl cyclase activator (20 μM FSK). Results showed that both 8Br-cAMP and FSK increased TH activity (Fig. 6B).

The participation of CaMK-II was assessed by pre-treating the tissues with KN-62 (CaMK-II inhibitor). Blockade of CaMK-II did

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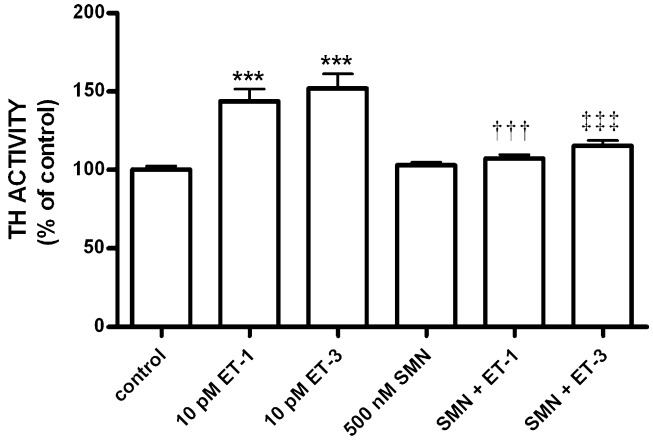

Fig. 4. Effect of a G-protein inhibitor on endothelin-1 and -3 (ET-1 and ET-3) response on tyrosine hydroxylase (TH) activity in the olfactory bulb (OB). The OBs were pre-treated with the G-protein inhibitor suramin (SMN) and further incubated with ET-1 and ET-3. TH activity was assessed as described in experimental procedures and it was expressed as percentage of control. $^{***}p < 0.001$ vs. control; $^{†††}p < 0.001$ vs. ET-1; $^{‡‡‡}p < 0.001$ vs. ET-3. Number of experiments: 6–7.

not affect the enzyme basal activity, but it prevented ETs response (Fig. 7).

To assess the contribution of the NO pathway the OBs were pre-treated with a NOs inhibitor (L-NAME). Blockade of NOs by L-NAME failed to affect either, basal or ETs-stimulated TH activity (Fig. 8A). To further confirm these results, OBs were incubated in the presence of a cGMP analogue (8Br-cGMP). Fig. 8B shows that 8Br-cGMP failed to affect basal TH activity.

To determine whether ETs modulated TH activity by changes in the translational rate, total TH protein was analyzed by Western blotting. Results show that ET-1 and ET-3 increased total TH expression (Fig. 9A). Furthermore, both ETs also augmented phosphorylated 19, 31 and 40 serine sites (Fig. 9B–D).

In order to evaluate whether increased levels of TH protein and activity in the OB in response to ETs were associated with changes in TH-mRNA, its expression was measured by quantitative real-time RT-PCR. Fig. 10 shows that TH-mRNA content was increased by ET-1 and ET-3 by approximately six-fold.

As catecholamine biosynthesis is tightly related to the neuronal release, we studied the effect of 10 pM ET-1 and ET-3 on DA turnover and endogenous content in OB by HPLC. Both ETs decreased DA endogenous content (Fig. 11A) without modifying DOPAC concentration (Fig. 11 B). In addition, both peptides increased DA turnover (DOPAC/DA) in the OBs of normotensive rats (Fig. 11C).

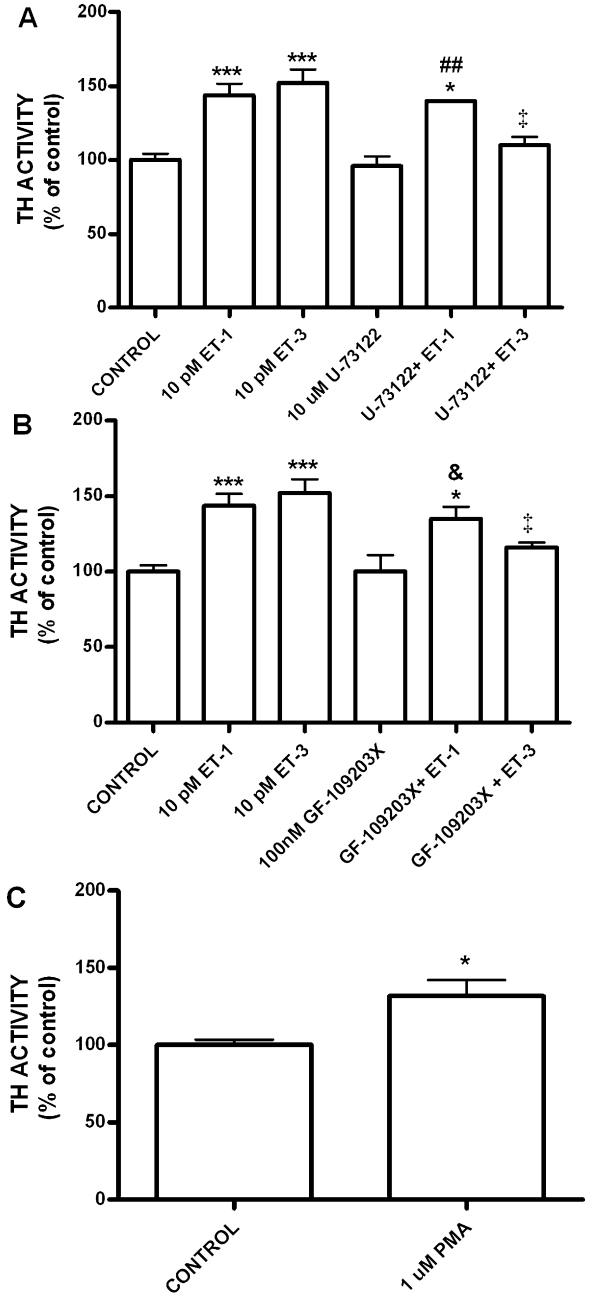

Fig. 5. Participation of PLC and PKC in endothelin-1 and -3 (ET-1 and ET-3) effect on tyrosine hydroxylase (TH) activity in the olfactory bulb (OB). The OBs were pre-treated with U-73122 (PLC inhibitor) (A), GF-109203x (PKC inhibitor) (B) and further incubated with ET-1 and ET-3 or incubated with PMA (PKC activator) (C). TH activity was assessed as described in experimental procedures and it was expressed as percentage of control. $^{*}p < 0.05$ and $^{***}p < 0.001$ vs. control; $^{‡}p < 0.05$ vs. ET-3; $^{\#\#}p < 0.01$ vs. U-73122; $^{\&}p < 0.05$ vs. GF-109203x. Number of experiments: 6–8.

4. Discussion

The major finding of the present study was that in the OB the long-term exposure to ET-1 and ET-3 increased TH activity through the activation of different ETs receptors and multiple signalling pathways. In addition, both ETs increased TH-mRNA and total protein levels but also the phosphorylation of serine residues at 19, 31 and 40 sites.

The OBs are part of the limbic system and project to the forebrain, and through a connection in the amygdala, to the midbrain (Cain, 1974; Song and Leonard, 2005). The limbic forebrain plays an important role in the regulation of emotions, stress and activities associated with changes in the cardiovascular function (Ku, 2006). In this regard, stressor exposure elicits a physiological response characterized by increased temperature, heart rate, and blood pressure (Harkin et al., 2002). In addition, stress decreases neurogenesis in the OB and increases c-fos expression in this and other regions related to cardiovascular and mood regulation such as the cortical areas, the amygdala and the hypothalamus (Mineur et al., 2007; Roche et al., 2007).

The major depressive disorder is a psychological condition that affects the mental and physical health of the patient. Interestingly, central areas involved in the olfaction process are often altered in major depression, suggesting that the dysfunction of olfactory processing may occur (Atanasova et al., 2008). Furthermore, a lower olfactory sensitivity and diverging impairments in olfactory perception have been observed in patients with major depression (Pause et al., 2003; Lombion-Pouthier et al., 2006). In addition, the removal of OB in rats generates behavioral, endocrine, immune and

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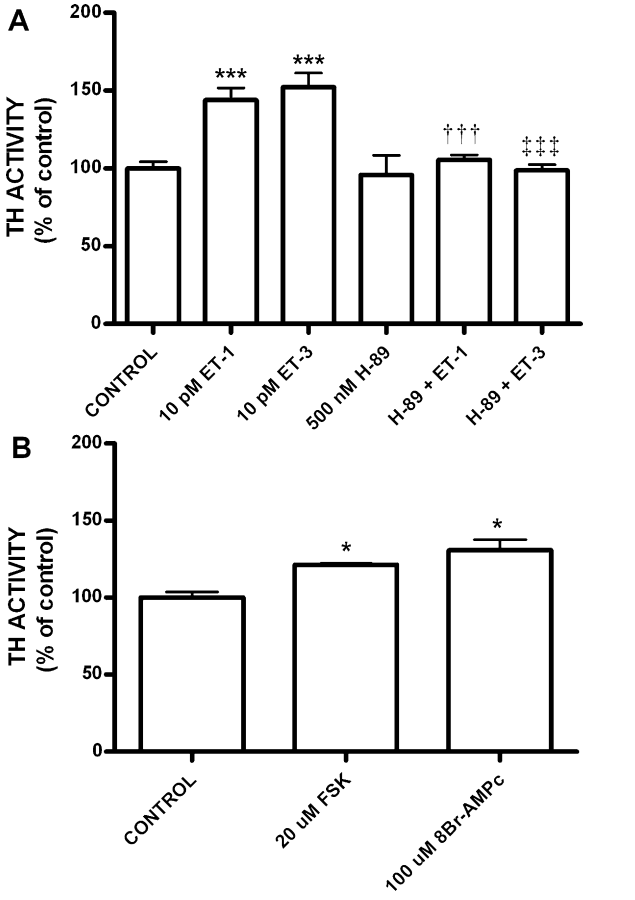

Fig. 6. Participation of the adenylyl cyclase pathway in endothelin-1 and -3 (ET-1 and ET-3) response on tyrosine hydroxylase (TH) activity in the olfactory bulb (OB). The OBs were pre-treated with H-89 (PKA inhibitor) and further incubated with ET-1 and ET-3 (A) or incubated with FSK (adenylyl cyclase activator) and 8Br-cAMP (cAMP analogue) (B). TH activity was assessed as described in experimental procedures and it was expressed as percentage of control. $^{*}p < 0.05$ and $^{***}p < 0.001$ vs. control; $^{\dagger\dagger\dagger}p < 0.001$ vs. ET-1; $^{\ddagger\ddagger\ddagger}p < 0.001$ vs. ET-3. Number of experiments: 6–8.

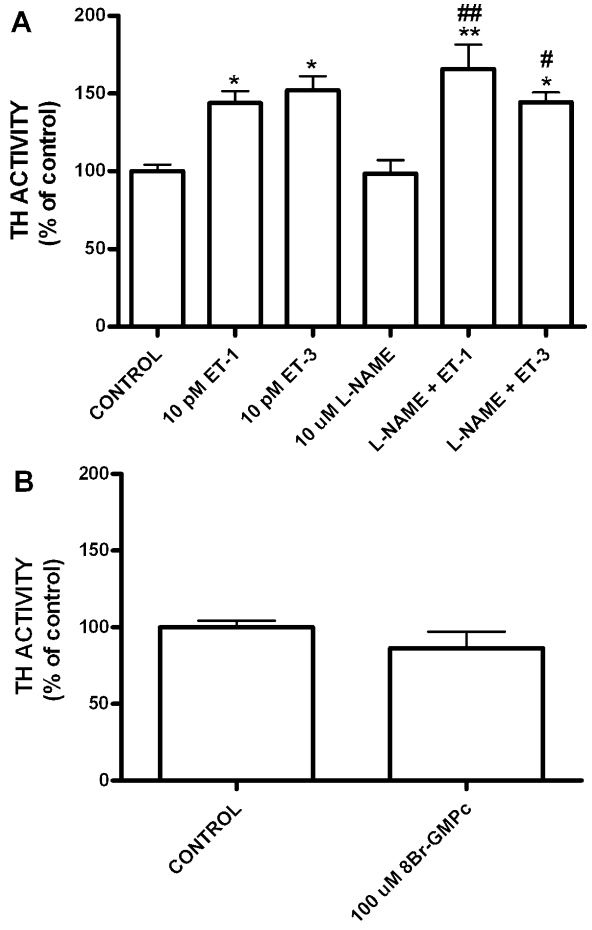

Fig. 8. Role of nitric oxide (NO) and cGMP in endothelin-1 and -3 (ET-1 and ET-3) effect on tyrosine hydroxylase (TH) activity in the olfactory bulb (OB). The OBs were pre-treated with L-NAME (NO synthase inhibitor) before the addition of ET-1 and ET-3 (A) or incubated with 8Br-cGMP (cGMP analogue) (B). TH activity was assessed as described in experimental procedures and it was expressed as percentage of control. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. L-NAME. Number of experiments: 6–8.

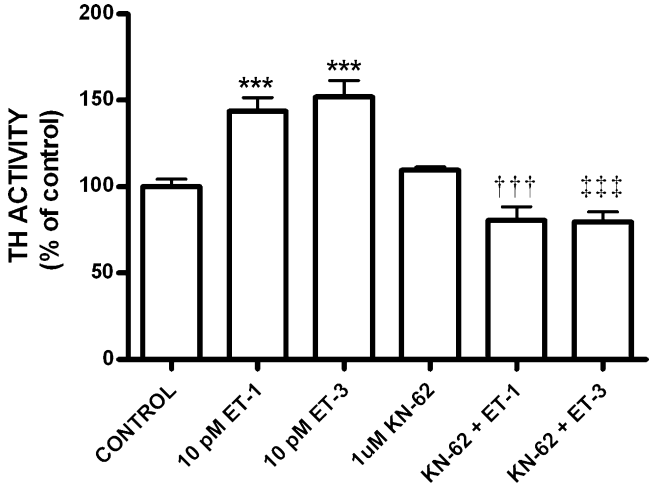

Fig. 7. Effect of CaMK-II on endothelin-1 and -3 (ET-1 and ET-3) response on tyrosine hydroxylase (TH) activity in the olfactory bulb (OB). The OBs were pre-treated with KN-62 (CaMK-II inhibitor) and further incubated with ET-1 and ET-3. TH activity was assessed as described in experimental procedures and it was expressed as percentage of control. $^{***}p < 0.001$ vs. control; $^{\dagger\dagger\dagger}p < 0.001$ vs. ET-1; $^{\ddagger\ddagger\ddagger}p < 0.001$ vs. ET-3. Number of experiments: 6–7.

neurotransmitter changes similar to those observed in patients with depression but chronic treatment with antidepressants blunts these symptoms (Kelly et al., 1997; Song and Leonard, 2005; Grippo and Johnson, 2009). Growing evidence supports the existence of a bidirectional association between depression and cardiovascular diseases. In this regard, depressive patients exhibit barorreflex impairment and increased heart rate (Lahmeyer and Bellur, 1987; Davis et al., 2008; Grippo and Johnson, 2009). Depression is also associated with excessive activation of the sympathetic nervous system that eventually increases cardiac risk (Barton et al., 2007; Grippo and Johnson, 2009). Catecholamines are important neurotransmitters involved in the development of mood disorders and its imbalance has also been observed in bulbectomyzed rats (Kelly et al., 1997; Song and Leonard, 2005). Taken together these findings suggest that the catecholaminergic impairment observed in major depression may result in an autonomic arousal responsible at least in part for the cardiovascular alterations showed by patients with depression.

ETs are localized in different regions of the CNS including the OB and are intimately involved in the control of sympathetic nerve activity and blood pressure (Koizumi et al., 1992; Kuwaki et al., 1997; Schneider et al., 2007). On the other hand, a very interesting observation made by Marslarova et al. (1995), is that the intracerebroventricular administration of ETs induces depressive symptoms in mice suggesting that these peptides are involved in

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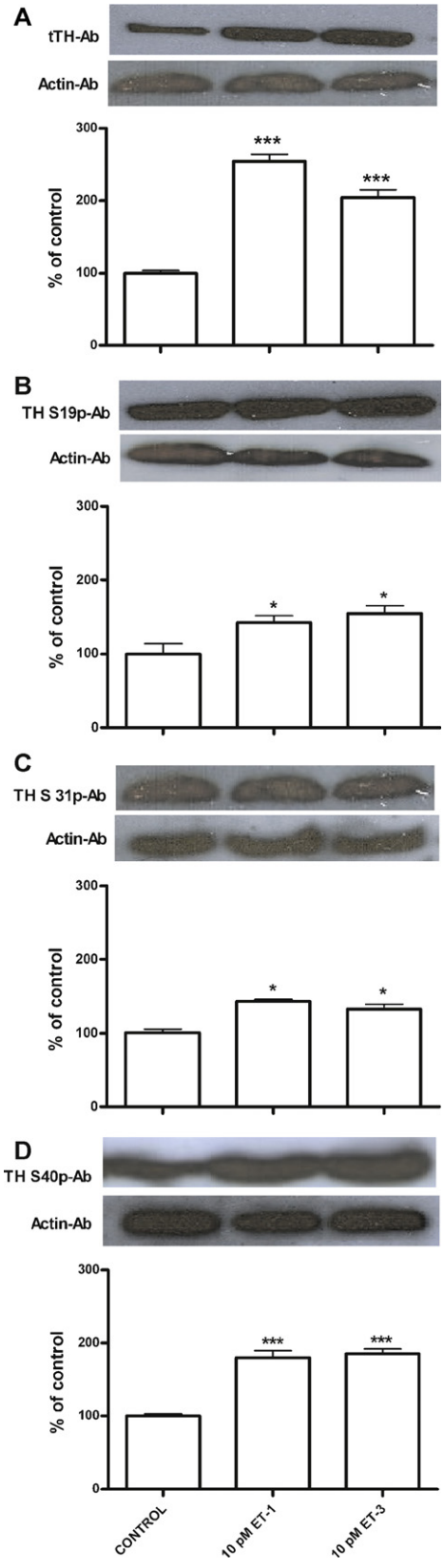

Fig. 9. Effect of endothelin-1 and -3 (ET-1 and ET-3) on the expression of total TH and the phosphorylation levels of the enzyme in the olfactory bulb (OB). The expression of total TH level (A) and the enzyme phosphorylated forms as Ser 19 (B), 31 (C) and 40 (D) were determined by Western blot analysis and normalized to β-actin as detailed in experimental procedures. $^{*}p < 0.05$ and $^{***}p < 0.001$ vs. control. The Western blot assays shown are representative of at least three or four independent experiments.

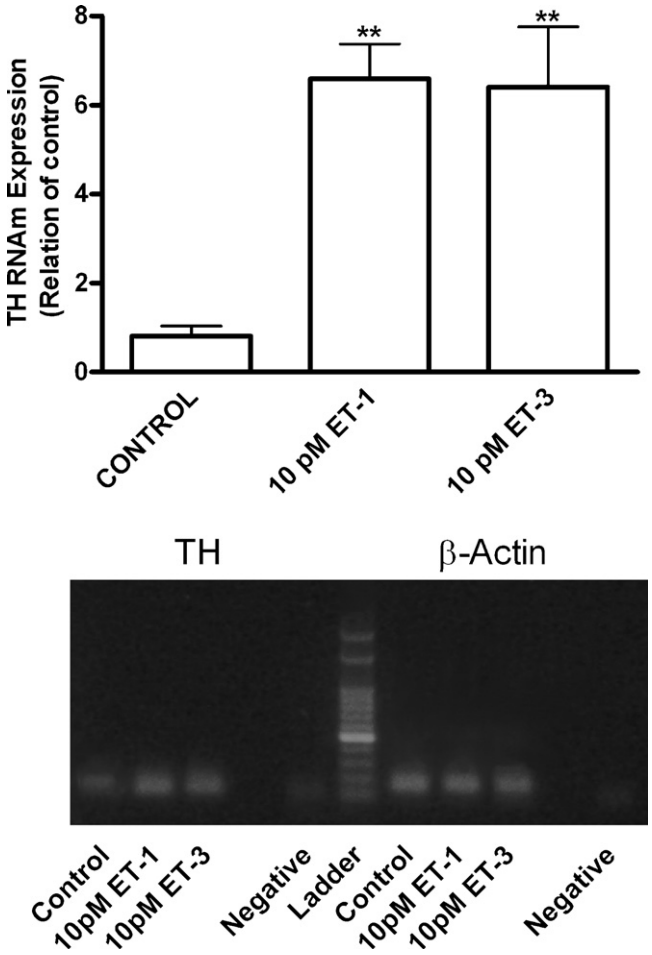

Fig. 10. Effect of endothelin-1 and -3 (ET-1 and ET-3) on the expression of TH mRNA in the olfactory bulb. The OBs were incubated with 10 pM ET-1 or ET-3. The expression of TH mRNA was determined as detailed in experimental procedures and expressed as relation of control. $^{**}p < 0.01$ vs. control. Number of experiments: 4. Then, a 1.5% agarose gel was performed to verify the presence of one PCR product in response to ETs with the specific primers for TH and β-actin, respectively.

the development of depression. Nevertheless, little is known regarding the relationship between ETs and depression.

Our previous findings show that 10 nM ET-1 and ET-3 are involved in the short-term regulation of TH activity in the OB of normotensive rats through the activation of an atypical or non conventional receptor coupled to the stimulation of the adenylyl cyclase/PKA, PLC/DAG/PKC and CaMK-II pathways (Nabhen et al., 2009). However, in the present work we observed that lower concentrations of ETs (10 pM and 10 fM) induced a significant increase in TH activity at 240 min, whereas 10 nM ETs failed to affect the enzyme activity. In this regard, Sokolovsky et al. (1992) reported the existence of a receptor subtype of "super high affinity" that binds ET in the pM concentration range, besides the typical receptors (high affinity) that respond to nM concentrations. These types of responses were identified in several regions of rat brain and atrium (Ambar and Sokolovsky, 1993; Sokolovsky, 1995). The disparity in the results of the short-term effect of ETs previously reported (Nabhen et al., 2009) and long-term response observed in the present study may result from the coexistence of both receptor subtypes (high and super high affinity) in the OBs. ET_A receptors mediated the increase in TH activity induced by ET-1 since the selective ET_A antagonist (BQ-610 100 nM and 100 pM) abolished the response and stimulation with SRT-x augmented it. This finding correlates with previous studies showing that the ET_A receptor displays higher affinity for ET-1 (Opgenorth, 1995;

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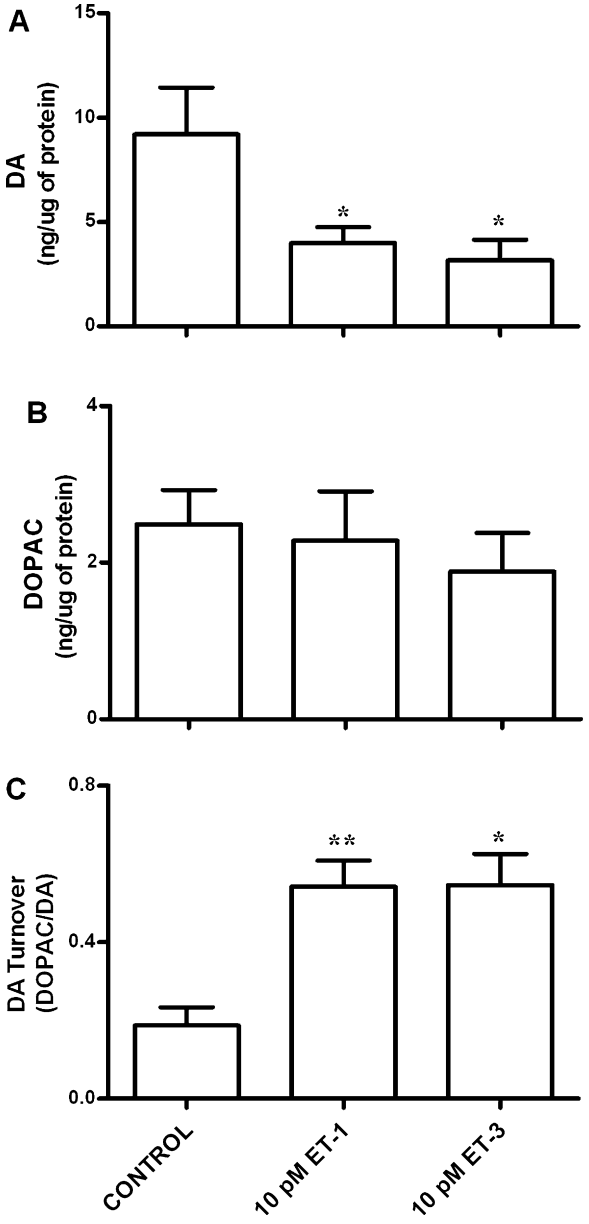

Fig. 11. Effect of endothelin-1 and -3 (ET-1 and ET-3) on dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC) endogenous content, and DA turnover in the olfactory bulb (OB). The OBs were incubated with 10 pM ET-1 or ET-3. DA (A) and DOPAC (B) endogenous content were determined by HPLC, and DA turnover (C) was expressed as DOPAC/DA ratio as detailed in experimental procedures. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control. Number of experiments: 4.

Schneider et al., 2007). Unfortunately, we were unable to identify the receptor involved in ET-3 response because blockade of ETs receptors either by BQ-610 or BQ-788 100 nM failed to modify ETs response. However, both antagonists (100 pM) prevented the increase in TH activity evoked by ET-3. The results obtained with the antagonists alone or combined may result from different pharmacological mechanisms. In this regard, studies carried out with the specific antagonist BQ-123 in the rat heart and brain capillary endothelial cells, demonstrated that the antagonist inhibitory action was competitive at the nanomolar sites (high affinity) and non-competitive at the picomolar sites (super high affinity) and was dependent on the experimental conditions (Sokolovsky, 1993; Vigne et al., 1993). Thus, the effect of ET antagonists should be analyzed with caution given that the inhibition pattern they exhibit may be more complex than a simple competitive inhibition so further studies are necessary to clarify this issue. Even though ET antagonists did not allow the identification of the ET receptor that mediated ET-3 response on TH activity, overall findings suggest that a super high affinity atypical receptor was involved. Atypical or non-conventional responses in the presence of ETs agonists and antagonists have been observed in diverse tissues leading to the concept of atypical receptors (ET_{AX} and ET_{BX} receptors) (Nambi et al., 1997; di Nunzio et al., 2004; Perfume et al., 2007, 2008; Nabhen et al., 2009; Hope et al., 2010). In addition, experimental evidence shows that ET_A and ET_B receptors can form homo and hetero dimmers, which may partially explain the atypical responses previously reported (Harada et al., 2002). Furthermore, functional and binding studies suggest the existence of an additional ET receptor named ET_C which displays high affinity for ET-3 but it has not been cloned in mammals yet (Karne et al., 1993; di Nunzio et al., 2004; Perfume et al., 2008). Although, we could not identify the receptor that mediated ET-3 response, we could confirm that it was a GPCR.

Both, ET_A and ET_B receptors are coupled to multiple signalling pathways depending on the specific G protein activated (Gs, Gq, Go, Gi), the tissue involved and the ligand concentration (Sokolovsky, 1995; Shraga-Levine and Sokolovsky, 2000). In this regard, it is well known that most of the kinases activated by ETs (PKA, PKC, PKG and CaMK-II) regulate TH activity (Kumer and Vrana, 1996; Dunkley et al., 2004). Present findings show that ET-1 increased TH activity through the stimulation of the cAMP/PKA and CaMK-II pathways but not the PLC/PKC pathway. In turn, ET-3 enhanced TH activity by activating the cAMP/PKA, CaMK-II and PLC/PKC pathways. However, NO did not contribute to ET-1 or ET-3 response on TH activity. In addition, it is possible that the stimulation of a particular cascade may activate other kinases by "cross-talk" mechanisms. Furthermore, we observed that both ETs enhanced TH activity by increasing the phosphorylation rate of serine residues (Ser 19, Ser 31 and Ser40) in the regulatory domain and total protein levels. These serine residues are substrate for direct phosphorylation by different protein kinases like ERK1/2, PKA, PKC, CaM-Kll and PKG. Thus, the kinases shown here to be activated by ETs may be responsible for the increase in TH phosphorylation. However, phosphorylation of each serine site does not affect TH activity equally (Kumer and Vrana, 1996; Dunkley et al., 2004). TH activity reflects the equilibrium between phosphorylation and dephosphorylation processes, and at longer times other factors like de novo synthesis also contribute to the resulting activity of the enzyme. Although phosphorylation and dephosphorylation are rapid processes, other authors reported that long term regulation of TH activity (hours to days) involves changes in total enzyme content as well as the phosphorylated forms (Guitart et al., 1990; Arbogast and Hyde, 2000; Gozal et al., 2005).

It is well known that phosphorylated TH is a more active form but simultaneously less stable (Kumer and Vrana, 1996). Phosphorylated TH has a short half life which may increase the enzyme turnover. Thus, it seems likely that some of the kinases activated by long term exposure to ETs may induce TH gene expression. It has been reported that PKA and PKC phosphorylate transcription factors like CREB, Fos and Jun which in turn activate TH transcription (Kumer and Vrana, 1996; Sun and Tank, 2003; Hebert et al., 2005). In these regard, present findings show that long term exposure to 10 pM ET-1 and ET-3 increased TH mRNA expression by approximately six-fold. Interestingly, no correlation between TH protein and mRNA was observed since the increase in total protein level was two-fold in response to both ETs. Thus, although both ETs augmented TH-mRNA expression, it may not be induced for a sufficient period of time to elicit an increase of functional TH protein (Kumer and Vrana, 2006; Xu et al., 2007; Tank et al., 2008). However, other possibility is that changes at the

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protein level may result from an increased transduction rate of previous existing TH-mRNA or its stability (Kumer and Vrana, 2006; Xu et al., 2007).

Catecholamines modulate TH activity by feedback inhibition (Goldstein, 1995; Kumer and Vrana, 1996). The OB receives centrifugal NE afferents from the locus coeruleus but only express DA cell bodies (Baker et al., 1983). As the long term effect on TH regulation in response to ETs could be specifically related to DA synthesis the metabolism of DA was assessed. ETs decreased DA endogenous content and increased turnover (DOPAC/DA ratio) which is an indicative of its utilization (Gómez et al., 2007; Scholl et al., 2010). These experimental observations suggest that the decrease in the amine endogenous content would facilitate BH_4 (cofactor) binding to TH thus triggering an increase in the enzyme activity (Goldstein, 1995; Kumer and Vrana, 1996).The numerous connections between OBs and other brain regions suggest that they mediate functions others than olfaction. Studies carried out in our laboratory show that in the OB of the DOCA-salt hypertensive rats the noradrenergic activity is increased, and that exposure to exogenous ETs modifies it (unpublished data). These results suggest that the catecholaminergic transmission in the OB is modified by vasoactive peptides in DOCA-salt hypertensive rats. However, more studies are necessary in order to elucidate the role of the OB in the regulation of the cardiovascular system.In summary, present findings show that ET-1 and ET-3 modulate catecholamine synthesis in the OB, a brain area directly exposed to environmental stimulations. Both ETs augmented TH mRNA and total protein level besides increasing the phosphorylation rate of Ser 19, 31 and 40 sites, thus favoring TH equilibrium towards a more active and less stable enzyme form (phosphorylated TH). On the other hand, increased DA turnover by ETs would displace the equilibrium TH-catecholamines to TH-biopterin (cofactor). Taken jointly, these findings support the increase in TH activity induced by both ETs.The physiological implication of the present findings lies in the fact that the relationship of organisms with their environment requires the maintenance of homeostasis. Stressors which constantly challenge this dynamic equilibrium comprise a long list of potentially adverse forces from emotional or physical sources. In this regard, chronic stress may cause a wide range of manifestations like depression and hypertension which in turn, have a bidirectional association (Davis et al., 2008; Grippo et al., 2002). Indeed, disrupted DA function is implicated in the pathophysiology of depression (Grippo and Johnson, 2009). Thus, this work represents an approach to the study of how exaggerated endothelinergic activation might be implicated in the behavioral and cardiovascular alterations caused at least in part by the impairment in the catecholaminergic transmission.

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