Neuroscience 340 (2017) 521–529

ANGIOTENSIN II AT_1 RECEPTORS MEDIATE NEURONAL SENSITIZATION AND SUSTAINED BLOOD PRESSURE RESPONSE INDUCED BY A SINGLE INJECTION OF AMPHETAMINE

N. A. MARCHESE,[a1] M. C. PAZ,[a1] X. CAEIRO,[b] F. M. DADAM,[b] G. BAIARDI,[c] M. F. PEREZ[a] AND C. BREGONZIO[a*]

[a] *Instituto de Farmacología Experimental Córdoba (IFEC-CONICET), Departamento de Farmacología, Facultad de Ciencias Químicas Universidad Nacional de Córdoba, Córdoba, Argentina*

[b] *Instituto de Investigación Médica Mercedes y Martín Ferreyra (INIMEC-CONICET), Universidad Nacional de Córdoba, Córdoba, Argentina*

[c] *Laboratorio de Neurofarmacología, (IIBYT-CONICET) Universidad Nacional de Córdoba, Córdoba, Argentina*

Abstract—A single exposure to amphetamine induces neurochemical sensitization in striatal areas. The neuropeptide angiotensin II, through AT_1 receptors (AT_1-R) activation, is involved in these responses. However, amphetamine-induced alterations can be extended to extra-striatal areas involved in blood pressure control and their physiological outcomes. Our aim for the present study was to analyze the possible role for AT_1-R in these events using a two-injection protocol and to further characterize the proposed AT_1-R antagonism protocol. Central effect of orally administered AT_1-R blocker (Candesartan, 3 mg/kg p.o. $\times$ 5 days) in male Wistar rats was analyzed by spontaneous activity of neurons within locus coeruleus. In another group of animals pretreated with the AT_1-R blocker or vehicle, sensitization was achieved by a single administration of amphetamine (5 mg/kg i.p. – day 6) followed by a 3-week period off drug. On day 27, after receiving an amphetamine challenge (0.5 mg/kg i.p.), we evaluated: (1) the sensitized c-Fos expression in locus coeruleus (LC), nucleus of the solitary tract (NTS), caudal ventrolateral medulla (A1) and central amygdala (CeAmy); and (2) the blood pressure response. AT_1-R blockade decreased LC neurons' spontaneous firing rate. Moreover, sensitized c-Fos immunoreactivity in TH + neurons was found in LC and NTS; and both responses were blunted by the AT_1-R blocker pretreatment. Meanwhile, no differences were found neither in CeAmy nor A1. Sensitized blood pressure response was observed as sustained changes in mean arterial pressure and was effectively prevented by AT_1-R blockade. Our results extend AT_1-R role in amphetamine-induced sensitization over noradrenergic nuclei and their cardiovascular output. **© 2016 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: angiotensin II AT_1 receptors, amphetamine sensitization, c-Fos/TH immunoreactivity, locus coeruleus, nucleus of the solitary tract, blood pressure response.

INTRODUCTION

Since it was first described at the early beginnings of the 20th century, Angiotensin II (Ang II) has been identified as a multifunctional peptide with hormonal and paracrine actions. Among tissue-specific and locally acting renin–angiotensin systems (RAS), brain Ang II, acting through AT_1 receptors (AT_1-R), has been related to regulation of physiological responses such as fluid homeostasis, thirst, vasopressin release and autonomic control of blood pressure (Saavedra, 1992; Llorens-Cortes and Mendelsohn, 2002). In the past decades brain RAS has become an important research target in neurogenic hypertension, stress responses, neurodegenerative diseases, alcohol consumption and psychostimulant-induced adaptations (Maul et al., 2005; Paz et al., 2011, 2013, 2014; Saavedra et al., 2011; Labandeira-Garcia et al., 2012).

Blood pressure maintenance by Ang II implies peripheral stimulant actions over the heart and vascular tone, as well as central activity in forebrain structures (lacking blood–brain barrier, circulating Ang II-responders) and brainstem structures (locally produced Ang II-responders) (Llorens-Cortes and Mendelsohn, 2002; Watanabe et al., 2010; Wright and Harding, 2013). Furthermore, locally produced Ang II regulates noradrenergic activity in brain areas involved in blood pressure control, such as the nucleus of the solitary tract (NTS), rostral and caudal ventrolateral medulla (C1 and A1), central amygdala (CeAmy) and locus coeruleus (LC) (Murphy et al., 1994; Watanabe et al., 2010; Johnson et al., 2015). Besides, LC provides noradrenergic innervation to the cortex and participates in the central sympathetic stimulation induced by stress (Berridge and Waterhouse, 2003; Carrasco and Van de Kar, 2003). Early studies showed that exogenously applied Ang II

*Corresponding author. Address: Instituto de Farmacología Experimental Córdoba (IFEC-CONICET), Departamento de Farmacología, Facultad de Ciencias Químicas Universidad Nacional de Córdoba, Haya de la Torre S/N, esquina Medina Allende, Edificio Nuevo de Ciencias I Ciudad Universitaria Córdoba, Argentina.
E-mail address: bregonzio@fcq.unc.edu.ar (C. Bregonzio).

[1] The authors contributed equally to this work.

Abbreviations: A1, caudal ventrolateral medulla; Amph, Amphetamine; AT_1-R, AT_1 receptors; CeAmy, central amygdala; LC, locus coeruleus; NA, noradrenaline; NTS, nucleus of the solitary tract; RAS, renin–angiotensin systems; TH, tyrosine hydroxylase.

http://dx.doi.org/10.1016/j.neuroscience.2016.11.006
0306-4522/© 2016 IBRO. Published by Elsevier Ltd. All rights reserved.

depressed the depolarizing effect of glutamate and excitatory postsynaptic potentials in LC slices through AT_2 receptors (AT_2-R) stimulation (Xiong and Marshall, 1994). Later on, stress exposure was shown to decrease AT_2-R binding in the LC, in contrast to the increase observed in AT_1-R at forebrain and brainstem structures (Peng and Phillips, 2001; Saavedra et al., 2006). However, AT_1-R antagonism prevents the increase in tyrosine hydroxylase (TH) mRNA and decrease in AT_2-R binding in LC observed after stress exposure, as well as the activation of the sympatho-adrenal response. These findings suggest that brain AT_1-R antagonism prevents the stress-induced increase in central sympathetic drive by indirect effects, possibly requiring AT_2-R participation (Armando et al., 2001). Furthermore, pretreatment with AT_1-R antagonists prevented the increase in TH mRNA in the LC observed after central administration of Ang II (Seltzer et al., 2004). Thus, it seems that AT_1-R antagonists have indirect effects over LC activity; that may include inhibition of brainstem AT_1-R located in the NTS and the area postrema (Bregonzio et al., 2008), along with a decrease in blood pressure and changes in the baroreflex response that follow AT_1-R antagonism (Hasser et al., 2000). Regarding the CeAmy activity over blood pressure control, locally acting Ang II in this area was found to be involved in the pressor response elicited by cocaine through AT_1-R activation (Watanabe et al., 2010).

Sustained changes in central nervous system (CNS) pathways, a phenomenon known as neuroplasticity, can be induced by prior experiences in order to modify the future outcome of behavioral and physiological responses. Time-dependent neuroplastic changes can be induced by psychostimulants or stress exposure, and visualized later on as sensitized responses (Stewart and Badiani, 1993; Kalivas, 2007). In the sensitization process two temporal phases can be distinguished: induction and expression. Using a two-injection sensitization protocol the changes in responsiveness are induced by a single psychostimulant administration, and revealed afterward by a second one. This protocol is particularly useful to study the long-lasting effects of drugs of abuse and to isolate the two events that take place in the sensitization phenomenon (Vanderschuren et al., 1999; Valjent et al., 2010; Paz et al., 2011, 2013). Even though this process has been long studied in striatal areas, sensitized responses to psychostimulants are not circumscribed to those areas. In this sense, the same phenomenon has been observed in noradrenergic brain nuclei such as LC, NTS and C1, underlying altered autonomic responses (Beveridge et al., 2004; McPherson and Lawrence, 2006).

Our previous findings showed the involvement of AT_1-R in the development and expression of behavioral and neurochemical sensitization induced by Amphetamine (Amph) in striatal areas (Paz et al., 2011, 2013). Moreover, we observed that Amph exposure modified brain RAS components and AT_1-R functionality in the same brain structures (Paz et al., 2014; Casarsa et al., 2015). Given that, there is no available evidence of Ang II involvement in the sensitized response to psychostimulants in extra-striatal noradrenergic areas, the aim of the present study was to analyze the possible role of AT_1-R in this phenomenon. Furthermore, we aimed to identify the existence of sensitization in the blood-pressure response to Amph involving AT_1-R activation.

EXPERIMENTAL PROCEDURES

Animals

A total of 85 adult male Wistar rats (250–330 g) from our colony at *Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Argentina* were used. The animals were maintained at 20–24 °C under a 12-h light–dark cycle (lights on at 07 a.m.) with free access to food and water through all the experiment. One week before the beginning of treatment rats were randomly housed in groups of four per cage.

All procedures were handled in accordance with the NIH Guide for the Care and Use of Laboratory Animals as approved by the Animal Care and Use Committee of the *Facultad de Ciencias Químicas Universidad Nacional de Córdoba, Argentina.*

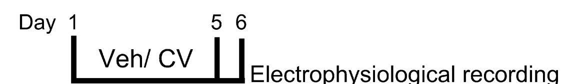
Drugs

D-amphetamine sulfate (Sigma Chemical Co.) was dissolved in 0.9% saline and administered intraperitoneally (i.p.) or intravenously (i.v.). AT_1-R antagonist Candesartan (CV, Laboratorios Phoenix, Buenos Aires, Argentina) was dissolved in $NaHCO_3$ 0.1 N and orally administered using a feeding needle (intragastric). The doses for each drug were chosen considering previous work (Vanderschuren et al., 1999; Paz et al., 2011, 2013).

Experimental protocol 1

A total of nine animals received CV (3 mg/kg, $n = 4$) or vehicle (Veh, $n = 5$) once a day for 5 days. Twenty-four hours after the last administration they were sacrificed for electrophysiological recording (Fig. 1A).

A Experimental protocol 1



B Experimental protocol 2

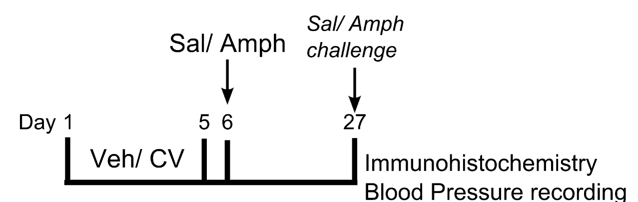


Fig. 1. Experimental protocols in the present work. (A) Experimental protocol of AT_1-R blockade. (B) Experimental protocol of AT_1-R blockade followed by Amph-sensitization two-injection protocol.

Experimental protocol 2

A total of 76 animals were randomly assigned to the treatments. Animals received CV (3 mg/kg) or Veh once a day for 5 days. Twenty-four hours after the last administration they were injected with Amph 5 mg/kg i.p. or saline. Four groups were defined as follows: vehicle-saline (Veh-Sal, $n = 21$), candesartan-saline (CV-Sal, $n = 18$), vehicle-amphetamine (Veh-Amph, $n = 18$) and candesartan-amphetamine (CV-Amph, $n = 19$). Animals were left undisturbed in their home cages until the day of the experiment. Twenty-one days after rats received the 5 mg/kg dose of Amph or saline, they were injected with either saline or Amph (0.5 mg/kg i.p. or i.v.) and then the experiments were performed as indicated in the immunohistochemistry and blood pressure recording section (Fig. 1B).

Extracellular single-cell recordings on LC neurons

Animals from Veh and CV groups, described in the Experimental Protocol 1 section, were used for extracellular single-unit recordings. The animals were anesthetized with chloral hydrate (400 mg/kg i.p.) and, if needed, anesthesia was maintained throughout the experiment with supplementary doses administered through a dorsal tail vein. The techniques used for extracellular single-cell recording have already been described in detail elsewhere (Perez et al., 2002). Briefly, rats' skulls were exposed, and a hole was drilled above the LC coordinates using a stereotaxic frame. According to Paxinos and Watsons' atlas coordinates (2009) an electrode was lowered to the top of LC by using a hydraulic microdrive in the following coordinates with respect to bregma: AP = −9.6/9.8 mm; L = 1.1/1.3 mm; DV (below the dura) = −5.5/6.5 mm. Noradrenergic neurons in LC display the following characteristics: (a) positive–negative action potentials lasting approximately 2 ms, often with a notch between the initial segment and the somatodendritic spike component; (b) a firing rate of 0.5–3.0 spike/s; (c) burst of firing followed by a quiescent period in response to pinching of the contralateral paw. These are the properties to fulfill electrophysiological criteria for the identification of LC noradrenergic cells (Aghajanian et al., 1977; Cedarbaum and Aghajanian, 1977; Ramirez and Wang, 1986a,b; Pavcovich et al., 1990). The number of spontaneously active cells per track (five-track average per animal) and their firing rate were assessed. The firing rate was obtained from the counted cells that displayed a signal-to-noise ratio of 2:1 or more. Electrode potentials displayed on an oscilloscope were previously passed through a high-impedance amplifier. The electrical signals were passed through a window discriminator and screened on an audio amplifier. Once the experiment was ended, the electrode location was marked by passing a 25-mA cathodal current through the recording electrode (15 min) and a spot of Fast green dye was deposited. Rats were then perfused with phosphate-buffered 10% formalin solution to later obtain serial frozen sections (50-mm thick) and trace the dye spot.

Staining procedure for c-Fos and c-Fos/Tyrosine hydroxylase immunohistochemistry

Animals from the four groups described in the Experimental Protocol 2 section were prepared for brain fixation ninety minutes after receiving the saline/Amph (0.5 mg/kg) challenge. The animals were anesthetized with chloral hydrate 16% (400 mg/kg i.p.) and perfused transcardially with 0.9% saline and heparin (200 μl/L), followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brains were removed and stored at 4 °C in PB containing 30% sucrose. Coronal sections of 40 μm were cut using a freezing microtome (Leica CM15105).

Using a double-labeling avidin–biotin–peroxidase procedure free-floating sections were first processed for c-Fos immunoreactivity and for TH staining afterward (Paz et al., 2013; Marchese et al., 2016). Briefly, sections were incubated overnight at room temperature with a rabbit anti-c-Fos antibody (Ab-5; Oncogene Science, Manhasset, NY, USA), diluted 1:20000 in PB 0.1 M, with 2% NHS (Natocor, Villa Carlos Paz, Córdoba, Argentina) and 0.3% Triton X-100 (Flucka Analytical). On the next day, sections were incubated at room temperature with biotin-labeled universal secondary antibody (1:2000 in 2% NHS-PB), and avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA; 1:200 in 2% NHS-PB), for 2 h each. The peroxidase label was detected with diaminobenzidine hydrochloride (Sigma Chemical Co., St Louis, MO, USA) using an intensified solution with 1% cobalt chloride and 1% nickel ammonium sulfate. The final result is a blue-black nuclear reaction product.

Later, the c-Fos-labeled sections were incubated for 48 h at 4 °C with mouse monoclonal anti-TH antibody (Millipore, Tecnolab S.A., 1:5000 in PB with 2% NHS and 0.3% Triton X-100) and later with goat biotin-labeled anti-mouse as secondary antibody (Jackson Laboratories (P) Ltd., diluted 1:5000 in 2% NHS-PB for 2 h). TH immunoreactivity was detected by an unintensified solution of diaminobenzidine hydrochloride, resulting in a cytoplasmic brown reaction product.

Cytoarchitectural and quantitative analyses

Images were obtained by a DFC Leica digital (with a contrast enhancement device) attached to a Leica DM 4000B microscope. Studied brain areas were identified and delimited according to Paxinos and Watson's atlas (2009). Analyses were performed at the LC (bregma: −9.60 mm to −9.96 mm), NTS and A1 (bregma: −13.68 mm to −14.04 mm), and CeAmy (bregma: −2.52 mm to −2.92 mm). The IMAGE J software from the National Institutes of Health (NIH) was used to image analyses. c-Fos IR nuclei (blue-black) were identified and counted only in positive TH cells labeled by cytoplasmic brown staining. For every studied area, counting was performed bilaterally in two sections and the total number was standardized for the counted area (on each photograph). The final value obtained was the average of the four counting. Taking into account that the size and

section thickness of nuclei did not change between experimental and control groups, any systematic error should be identical for all groups. Hence, the results are meant to provide relative data on expression of c-Fos/TH immunoreactivity but are not meant to be accurate estimates of absolute cell counts. Counting of c-Fos/TH IR cells was performed blinded to the experimental groups.

Surgical procedures-cannulation of vessels

On day 27 of the Experimental Protocol 2 animals from the four groups were anesthetized with urethane (1.5 g/kg ip). The rats' left femoral artery and vein were implanted with heparin–saline (50 U/ml)-filled polyethylene catheters (PE-50: 0.039 in. OD, 0.023 in. ID) for blood pressure recording and drug administration, respectively. The administered drugs were saline, Amph 0.5 mg/kg or an additional dose of anesthetics when needed. The arterial catheter was connected to a blood pressure transducer and PowerLab data-acquisition system (ADInstruments, Sydney, Australia). Blood pressure was continuously recorded, and heart rate (HR) was calculated by an internal rate-meter in the data acquisition system (Caeiro and Vivas, 2008).

Blood pressure record- data collection and analysis

Basal values for blood pressure were assessed for at least 20 min, afterward the animals received a saline injection and data were recorded for another 20 min, finally the animals received an Amph dose of 0.5 mg/kg and blood pressure values were recorded across 20 min. Values for mean arterial pressure (MAP – mm Hg) and HR (beats/min) were taken as the average values measured over a 2-min period for each selected time. The injections were not started in any animal that did not have stable blood pressure and heart rate. The differences in MAP and HR during the experimental protocol were obtained by subtracting the mean values from 2 min before the drug i.v. administration.

Statistical analyses

Data are reported as means ± SEM or median with interquartile range, according to the fulfillment (or not) of the Gaussian distribution and homoscedasticity assumptions. For experiments following Experimental Protocol 1 the study design used Mann–Whitney test comparing Veh and CV groups. The study design in Experimental Protocol 2 used a two-way ANOVA for c-Fos and c-Fos/TH immunoreactivity evaluation, and the Ray–Scheires–Hare test for MAP and HR values. In both cases the analyses considered Veh and CV as pretreatment factors and Sal and Amph as treatment factors. Analyses were performed separately for Sal and Amph challenge received 21 days after the treatment. If an interaction and/or main effect was observed, pairwise comparisons were made using the Bonferroni post-test (following ANOVA); and Mann–Whitney test with Bonferroni's correction (following Ray-Scheires-Hare test). A value of $p < 0.05$ was considered significant. The analyses were performed using Graph Pad Prism 6 software and IBM SPSS Statistic 22 software (SPSS Software for Business Analytics, IBM Software).

RESULTS

CV pretreatment modifies central catecholaminergic activity

LC-noradrenergic neurons spontaneous activity. To study the effects of CV pretreatment over central catecholaminergic neurotransmission, the LC-noradrenergic neurons activity was evaluated on Veh and CV treated rats, using the extracellular single-unit recording method (Fig. 1A/Fig. 2). Animals receiving CV for 5 days exhibited a diminished neuronal activity on LC-noradrenergic neurons, which was assessed by the decreased firing rate 24 h after the last administration when compared with control group (Veh) ($U_{(5,4,0.05)} = 0$, $p < 0.05$ – Fig. 2B). The number of active spontaneous cells per track was not modified by CV administration ($U_{(5,4,\ 0.05)} = 7.5$, $p > 0.05$ – Fig. 2A).

CV pretreatment prevents Amph-induced sensitized response

Activation pattern of c-Fos and c-Fos/tyrosine hydroxylase. c-Fos/TH immunoreactivity assay was performed to identify differential activation patterns in noradrenergic nuclei (LC, NTS and A1). Meanwhile, CeAmy was analyzed solely for c-Fos immunoreactivity. This approach was selected because the increased synthesis of c-Fos protein, 1–2 h post-stimulation, correlates with increased neural activity in a wide range of neural systems (Morgan and Curran, 1989; Nordquist et al., 2008). Furthermore, c-Fos pattern has been widely used to assess neuronal sensitization in dopamine-innervated areas after psychostimulants exposure (Nordquist et al., 2008; Paz et al., 2013).

As shown in Fig. 2 sensitized neuronal activation to an Amph challenge (0.5 mg/kg i.p.) is observed in previously Amph-treated animals within LC (Fig. 3A) and NTS (Fig. 3B). Pretreatment with CV significantly prevented the development of the sensitized response. The results obtained from the two-way ANOVA for the number of c-Fos/TH IR cells in LC indicated a significant effect for **pretreatment** $F_{(1,33)} = 4.78$, $p < 0.05$, **treatment** $F_{(1,33)} = 4.21$ $p < 0.05$ and **interaction** $F_{(1,33)} = 4.57$ $p < 0.05$, Bonferroni's post hoc comparisons indicated that the Veh-Amph group was significantly different from all groups ($p < 0.05$). Meanwhile, in NTS significant effects were found for **pretreatment** $F_{(1,21)} = 5.39$ $p < 0.05$, **treatment** $F_{(1,21)} = 7.14$ $p < 0.05$ and **interaction** $F_{(1,21)} = 4.84$ $p < 0.05$. Bonferroni's post hoc comparisons indicated that Veh-Amph group was significantly different from all groups ($p < 0.05$).

Amph challenge did not induce a sensitized response in A1 and Ce Amy, because no significant differences were found between groups in c-Fos/TH and c-Fos IR neurons, respectively (Table 2).

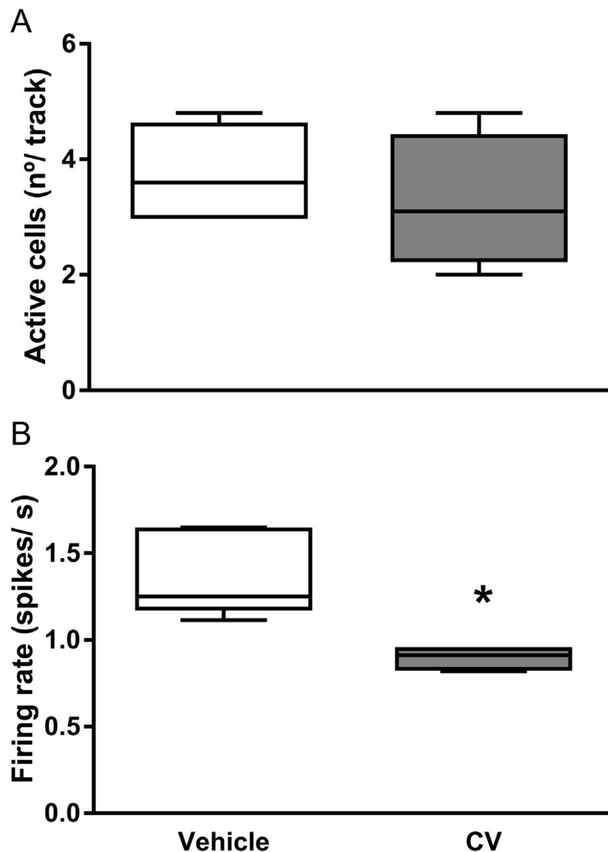


Fig. 2. Spontaneous neuronal activity within locus coeruleus, effect of AT_1-R blockade. Box and whiskers graphs show (A) number of spontaneously active cells (cells per track) within the LC and (B) their average firing rate (spikes/s). $^{*}p < 0.05$ different from Veh-treated animals. Values are expressed as median with interquartile range and maximum and minimum value (Veh $n = 5$; CV $n = 4$).

There were no significant differences in neuronal activation pattern after a Saline challenge in any of the studied areas, pointing out the specificity of the observed response after an Amph challenge (Table 1).

Interestingly, a separate t-test analysis indicated that c-Fos/TH IR neurons in LC of Veh-Sal groups are significantly different when receiving a Sal or Amph challenge $t_{(13;\ 0.05)} = 2.7$ $p < 0.05$, this difference is not observed in NTS $t_{(10;\ 0.05)} = 0.38$ $p > 0.05$. These results can be observed in Fig. 3A and B, where the dotted line represents the average number of c-Fos/TH IR from Veh-Sal group when receiving a saline challenge.

Blood pressure response. Taking into account the sensitized neuronal activation observed in LC and NTS, we aimed to determine whether a single Amph injection would affect the blood pressure response evoked by an Amph challenge 21 days after treatment. Likewise, we attempted to evaluate AT_1-R role in the Amph-induced alterations over blood pressure response (according to experimental protocol 2 – Fig. 1B). There were no differences in MAP increase elicited by Amph challenge 2 min after its administration (**pretreatment** $H_{(1)} = 0.11$, **treatment** $H_{(1)} = 2.56$ and **interaction** $H_{(1)} = 1.21$; $p > 0.05$ in all cases – Fig. 4B). However, when changes in MAP were analyzed 20 min after Amph challenge a significant effect was found for **treatment** $H_{(1)} = 6.22$ $p < 0.05$ and **interaction** $H_{(1)} = 4.0.7$ $p < 0.05$ (Fig. 4B). Mann–Whitney with Bonferroni's correction post hoc comparison indicated that only Veh-Amph is statistically different from control group (Veh-Sal; $p < 0.017$). Thus, the altered response induced by a single injection of Amph was observed as a sustained increase in MAP and prevented by CV pretreatment.

In basal conditions nor after a saline challenge MAP values were not statistically different between groups (basal: **pretreatment** $H_{(1)} = 0.65$, **treatment** $H_{(1)} = 0.45$ and **interaction** $H_{(1)} = 0.81$; $p > 0.05$ in all cases – Fig. 4A – saline: **pretreatment** $H_{(1)} = 0.99$, **treatment** $H_{(1)} = 0.0003$ and **interaction** $H_{(1)} = 0.51$; $p > 0.05$ in all cases). No statistical differences were observed in HR values between treatment groups (data not shown).

DISCUSSION

The results presented in this work show that an orally-administered AT_1-R antagonist prevents the sensitized noradrenergic response induced by Amph over brain areas related with blood pressure control and its physiological output (MAP). Amph-induced sensitization was evidenced by an Amph challenge as increased c-Fos expression in noradrenergic neurons (within LC and NTS) and sustained increase in blood pressure response. The AT_1-R antagonist, CV, administered for 5 days had central effects over noradrenergic transmission, evidenced by diminished electrical activity within LC. Remarkably, it was found that AT_1-R blockade prevented the development of the sensitized noradrenergic response in LC and NTS and the sustained increase in blood pressure elicited by an Amph challenge.

Ang II has long been related with peripheral and central catecholaminergic activity. Initially, studies demonstrated that noradrenaline (NA) levels could be increased by Ang II administration in neuron-enriched primary brain cell cultures (Sumners et al., 1983). Later on, studies performed in animals showed that i.c.v. injection of a pressor dose of ANG II selectively increased levels of NA in noradrenergic nuclei, involved in blood pressure control such as LC and A1 (Sumners and Phillips, 1983). Recently, new studies showed that Ang II administration, acting through AT_1-R, reduced the delayed rectifier potassium channel current in CATH.a cells line, which possess phenotypic properties of noradrenergic neurons in catecholaminergic nuclei: TH, Dopamine-Beta-Hydroxylase, dopamine and NA production; and AT_1-R expression (Du et al., 2004). Moreover, Ang II positively modulates TH transcription in LC. This response is AT_1-R dependent, given that orally administered CV abolished the TH mRNA increase observed after i.c.v. administration of Ang II or cold stress exposure. (Seltzer et al., 2004; Bregonzio et al., 2008). In agreement with these results, in the present work it was found that peripheral CV administration was able to attenuate the spontaneous neuronal activity within LC. Our present

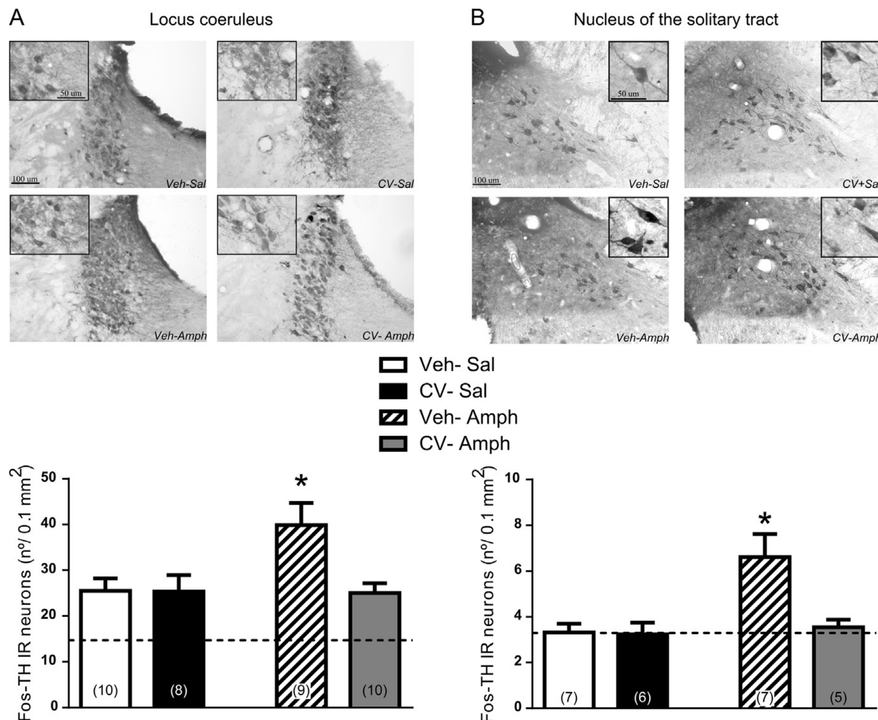


Fig. 3. Expression pattern of c-Fos/TH IR cells in (A) LC and (B) NTS. Microphotographs are representative of the studied areas for each experimental group. Graphs show average number of c-Fos/TH IR neurons from animals receiving an Amph challenge. $^{*}p < 0.05$ different from all groups. Values are means ± SEM; the number of animals for each group is indicated on graph bar. Dotted line indicates average number of c-Fos/TH IR from Veh-Sal group when receiving a saline challenge.

and previous results further validate the efficacy of orally administered CV to modify Ang II central actions over catecholaminergic activity.

It is known that Amph increases catecholaminergic neurotransmission and sensitizes multiple neuronal circuits modifying their future outcomes to pharmacological or non-pharmacological challenges (Pierce and Kalivas, 1997; Vanderschuren and Kalivas, 2000; Kalivas, 2007). We have previously shown that neurochemical and behavioral sensitization induced by Amph involves AT_1-R activation, because the increased c-Fos immunoreactivity in reward-processing areas (CPu and NAc) and the enhanced locomotor activity in response to an Amph challenge were prevented by AT_1-R blockade (Paz et al., 2011, 2013). Since dopaminergic neurotransmission hyperactivity has been described in Amph-induced sensitization, our previous results supported the AT_1-R modulatory role over this neurotransmitter pathway. In the present work, the Amph-induced sensitized c-Fos expression was observed in noradrenergic nuclei LC and NTS. However, the response elicited by Amph challenge between these brain areas was slightly different. While Amph challenge increased noradrenergic activation in control groups in LC, neuronal activation in NTS was observed only in Amph previously exposed animals. Interestingly, the neuronal sensitized activation in both brain areas was prevented by AT_1-R antagonist pretreatment. Moreover, no sensitized response was found in CeAmy or A1, and no differences were observed between groups in either case. In agreement with our findings, extra-striatal sensitization to Amph has been reported to occur in LC without changes in amygdala (McPherson and Lawrence, 2006). In the same way, other authors showed that cocaine self-administration in primates increases the neuronal activity in LC and NTS without changes in A1 (Beveridge et al., 2004). All together, these evidences indicate that Amph sensitization is not a generalized response, as it shows brain area specificity within catecholaminergic nuclei. Moreover, as CV prevented the sensitized neuronal acti-

Table 1. c-Fos expression pattern after saline challenge

	Veh-Sal	CV-Sal	Veh-Amph	CV-Amph	*p* value
c-Fos/TH IR NEURONS					
Locus coeruleus	14.8 ± 0.5 (5)	14.7 ± 1 (5)	14.8 ± 1.5 (5)	14.5 ± 1.1 (5)	Pre:0.85 Tra:0.93 Int:0.93
Nucleus of the solitary tract	3.5 ± 0.3 (5)	2.9 ± 0.3 (5)	3.2 ± 0.4 (5)	3.5 ± 0.5 (5)	Pre:0.71 Tra:0.62 Int:0.22
Caudal rostroventrolateral reticular nucleus (A1)	5.0 ± 0.5 (5)	5.7 ± 0.1 (5)	5.3 ± 0.3 (5)	5.5 ± 0.1 (5)	Pre:0.21 Tra:0.92 Int:0.42
c-Fos IR NEURONS					
Central amygdala	28.2 ± 6.9 (5)	20.0 ± 3.9 (5)	22.3 ± 3.7 (5)	22.7 ± 6.4 (5)	Pre:0.86 Tra:0.77 Int:0.79

Values indicate the number of IR neurons for c-Fos/TH (in LC; NTS and A1) and c-Fos (in CeAmy) when receiving a saline challenge for the four experimental groups. Values are means ± SEM; the number of animals for each group is indicated between brackets. p values are given for Pre: pretreatment, Tre: treatment and Int: interaction.

Table 2. c-Fos expression pattern after amphetamine challenge

	Veh-Sal	CV-Sal	Veh-Amph	CV-Amph	*p* value
c-Fos/TH IR NEURONS					
Caudal rostroventrolateral reticular nucleus (A1)	5.5 ± 0.4 (7)	5.6 ± 0.7 (7)	6.0 ± 0.7 (8)	5.4 ± 0.4 (8)	Pre:0.69 Tra:0.77 Int:0.57
c-Fos IR NEURONS					
Central amygdala	42.7 ± 5.3 (5)	38.9 ± 10.2 (6)	44.9 ± 9.6 (6)	33.4 ± 11.0 (5)	Pre:0.43 Tra:0.87 Int:0.69

Values indicate the number of IR neurons for c-Fos/TH (in A1) and c-Fos (in CeAmy) when receiving an Amph challenge for the four experimental groups. Values are means ± SEM; the number of animals for each group is indicated between brackets. p values are given for Pre: pretreatment, Tre: treatment and Int: interaction.

vation in LC and NTS our results extend AT_1-R involvement in Amph-induced sensitization regarding noradrenergic neurotransmission.

The physiological role of LC and NTS over cardiovascular responses has long been studied for acute blood pressure stimulus (Murphy et al., 1994; Lohmeier et al., 2002; Bundzikova-Osacka et al., 2015) and under different experimental conditions of essential and induced hypertension (Veerasingham et al., 2000; Chan et al., 2002; Lohmeier et al., 2002). In addition, animals with induced-hypertension showed increased levels of TH mRNA in LC and NTS; meanwhile increased angiotensinogen mRNA was found only in NTS (Maximino et al., 2006). Considering the sensitized noradrenergic response observed at LC and NTS in the present work we evaluated if an altered blood pressure response to an Amph challenge could be observed in Amph-sensitized animals. To this respect, our results show that previous Amph exposure induced a sustained increase in blood pressure response to a subsequent Amph challenge (up to 20 min). Similar, a sensitized blood pressure response was previously described for methamphetamine by Yoshida et al. (1993). Furthermore, the results presented here describe for the first time that Amph-induced blood pressure sensitized response involved AT_1-R activation, since previous CV administration abolished the persistent increase in blood pressure induced by Amph. Moreover, they point out that Amph-induced sensitized blood pressure response involves central noradrenergic sensitization and Ang II activity. Our results are in accordance with recently reviewed Ang II role in long-lasting changes over neural network involved in blood pressure control, associated with sensitized responses to hypertensinogenic stimuli (Johnson et al., 2015). The initially called “angiotensin auto-potentiation” (Godfraind, 1970) was described for several of its physiological actions; and, later on, it has been proposed as a sensitization process that may apply to the development of Ang II-hypertension models (Johnson et al., 2015). To this respect, non-pressor doses of Ang II (peripheral induction) sensitizes to future Ang II-induced hypertension (expression after delayed period) (Xue et al., 2012a). This phenomenon involves brain AT_1-R activation, given that it can be induced by Ang II i.c.v. administration and blunted by a centrally administered AT_1-R antagonist (Xue et al., 2012a). Furthermore, cross-sensitized responses are observed for different hypertensinogenic stimuli. For instance, Ang II-induced hyper-

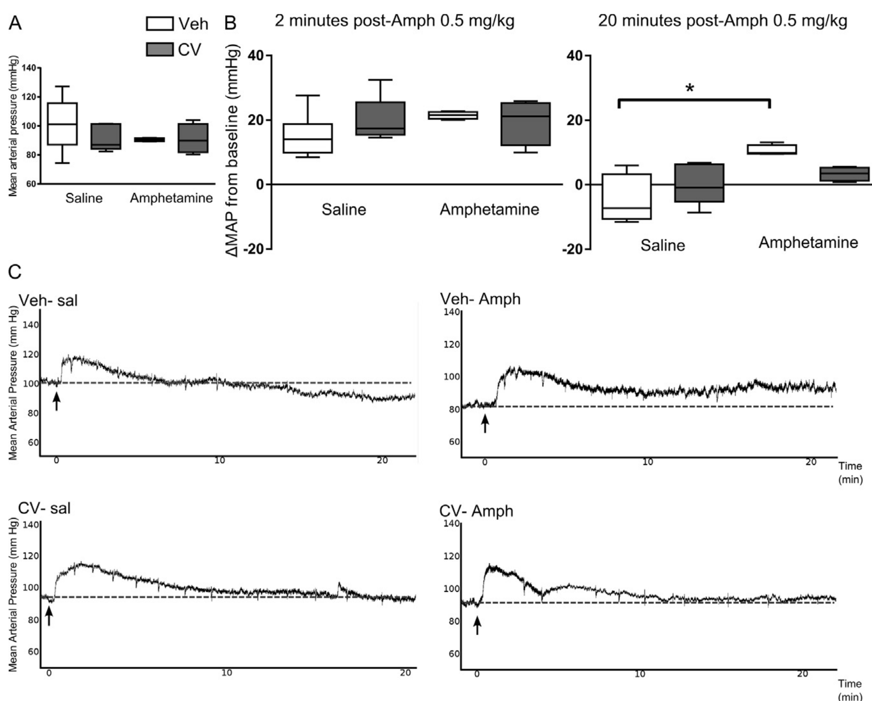


Fig. 4. Blood pressure response observed after an Amph challenge. Box and whiskers graphs show (A) Absolute basal values for mean arterial pressure in the four groups (Veh- Sal $n = 6$; CV-Sal $n = 5$; Veh-Anf $n = 4$; CV-Anf $n = 4$) and (B) Changes in mean arterial pressure 2 and 20 min after an Amph challenge. $^{*}p < 0.05$ different from Veh-Sal. Values are expressed as median with interquartile range and maximum and minimum value. Panel (C) shows representative plot for mean arterial pressure response of each experimental group.

tension has been observed by induction with aldosterone, high- and low-salt intake, high-fat diet and chronic stress exposure (Loria et al., 2010; Xue et al., 2012b, 2013, 2016); likewise, Ang II peripheral induction sensitizes to subsequent salt-sensitive hypertension (Clayton et al., 2014). Furthermore, it has been proposed that some common aspect might be triggering neuroplasticity and promoting blood pressure-sensitization (Johnson et al., 2015). This idea coincides with the proposed cross-sensitized response between drugs of abuse, stress and/or natural rewards (Goeders, 1998, 2002; Roitman et al., 2002; Acerbo and Johnson, 2011). In light of the present results, it is worth noting the involvement of brain AT_1-R in the induction of the central-controlled blood pressure sensitized response promoted by psychostimulant exposure.

CONCLUSION

Considering the physiological role of LC and NTS over cardiovascular functions the remaining question would be: can sensitized response be extended to other hypertensinogenic stimuli? Our findings bring into focus Amph as a prominent candidate to promote a sensitized blood pressure response and give rise to the possibility to make pharmacological interventions over noradrenergic system with orally administered AT_1-R blockers.

Acknowledgments—This study was supported by grants from CONICET 11220120100373CO-KB1 to Drs. Bregonzio y Perez, SECyT to Drs. Bregonzio y Perez, FonCyT PRESTAMO BID PICT 2011-2476 to Dr. Perez. The authors declare no conflicts of interest. The authors are grateful to Estela Salde and Lorena Mercado for their laboratory technical assistance.

REFERENCES

Acerbo MJ, Johnson AK (2011) Behavioral cross-sensitization between DOCA-induced sodium appetite and cocaine-induced locomotor behavior. Pharmacol Biochem Behav 98:440–448.

Aghajanian GK, Cedarbaum JM, Wang RY (1977) Evidence for norepinephrine-mediated collateral inhibition of locus coeruleus neurons. Brain Res 136:570–577.

Armando I, Carranza A, Nishimura Y, Hoe KL, Barontini M, Terron JA, Falcon-Neri A, Ito T, Juorio AV, Saavedra JM (2001) Peripheral administration of an angiotensin II AT(1) receptor antagonist decreases the hypothalamic-pituitary-adrenal response to isolation Stress. Endocrinology 142:3880–3889.

Berridge CW, Waterhouse BD (2003) The locus coeruleus-noradrenergic system: modulation of behavioral state and state-dependent cognitive processes. Brain Res Brain Res Rev 42:33–84.

Beveridge TJ, Smith HR, Nader MA, Porrino LJ (2004) Functional effects of cocaine self-administration in primate brain regions regulating cardiovascular function. Neurosci Lett 370:201–205.

Bregonzio C, Seltzer A, Armando I, Pavel J, Saavedra JM (2008) Angiotensin II AT(1) receptor blockade selectively enhances brain AT(2) receptor expression, and abolishes the cold-restraint stress-induced increase in tyrosine hydroxylase mRNA in the locus coeruleus of spontaneously hypertensive rats. Stress 11:457–466.

Bundzikova-Osacka J, Ghosal S, Packard BA, Ulrich-Lai YM, Herman JP (2015) Role of nucleus of the solitary tract noradrenergic neurons in post-stress cardiovascular and hormonal control in male rats. Stress 18:221–232.

Caeiro X, Vivas L (2008) Beta-endorphin in the median preoptic nucleus modulates the pressor response induced by subcutaneous hypertonic sodium chloride. Exp Neurol 210:59–66.

Carrasco GA, Van de Kar LD (2003) Neuroendocrine pharmacology of stress. Eur J Pharmacol 463:235–272.

Casarsa BS, Marinzalda MA, Marchese NA, Paz MC, Vivas L, Baiardi G, Bregonzio C (2015) A previous history of repeated amphetamine exposure modifies brain angiotensin II AT1 receptor functionality. Neuroscience 307:1–13.

Cedarbaum JM, Aghajanian GK (1977) Catecholamine receptors on locus coeruleus neurons: pharmacological characterization. Eur J Pharmacol 44:375–385.

Clayton SC, Zhang Z, Beltz T, Xue B, Johnson AK (2014) CNS neuroplasticity and salt-sensitive hypertension induced by prior treatment with subpressor doses of ANG II or aldosterone. Am J Physiol Regul Integr Comp Physiol 306:R908–R917.

Chan JY, Wang LL, Lee HY, Chan SH (2002) Augmented upregulation by c-fos of angiotensin subtype 1 receptor in nucleus tractus solitarii of spontaneously hypertensive rats. Hypertension 40:335–341.

Du JQ, Sun CW, Tang JS (2004) Effect of angiotensin II type 1 receptor on delayed rectifier potassium current in catecholaminergic CATH.a cells. Acta Pharmacol Sin 25:1145–1150.

Godfraind T (1970) Angiotensin auto-potentiation. Br J Pharmacol 40:542P–543P.

Goeders NE (1998) Stress, the hypothalamic-pituitary-adrenal axis, and vulnerability to drug abuse. NIDA Res Monogr 169:83–104.

Goeders NE (2002) Stress and cocaine addiction. J Pharmacol Exp Ther 301:785–789.

Hasser EM, Cunningham JT, Sullivan MJ, Curtis KS, Blaine EH, Hay M (2000) Area postrema and sympathetic nervous system effects of vasopressin and angiotensin II. Clin Exp Pharmacol Physiol 27:432–436.

Johnson AK, Zhang Z, Clayton SC, Beltz TG, Hurley SW, Thunhorst RL, Xue B (2015) The roles of sensitization and neuroplasticity in the long-term regulation of blood pressure and hypertension. Am J Physiol Regul Integr Comp Physiol 309:1309–R 1325.

Kalivas PW (2007) Cocaine and amphetamine-like psychostimulants: neurocircuitry and glutamate neuroplasticity. Dialogues Clin Neurosci 9:389–397.

Labandeira-Garcia JL, Rodriguez-Pallares J, Rodriguez-Perez AI, Garrido-Gil P, Villar-Cheda B, Valenzuela R, Guerra MJ (2012) Brain angiotensin and dopaminergic degeneration: relevance to Parkinson's disease. Am J Neurodegener Dis 1:226–244.

Lohmeier TE, Lohmeier JR, Warren S, May PJ, Cunningham JT (2002) Sustained activation of the central baroreceptor pathway in angiotensin hypertension. Hypertension 39:550–556.

Loria AS, Pollock DM, Pollock JS (2010) Early life stress sensitizes rats to angiotensin II-induced hypertension and vascular inflammation in adult life. Hypertension 55:494–499.

Llorens-Cortes C, Mendelsohn FA (2002) Organisation and functional role of the brain angiotensin system. J Renin Angiotensin Aldosterone Syst 3(Suppl 1):39–S 48.

Marchese NA, Artur de laVillarmois E, Basmadjian OM, Perez MF, Baiardi G, Bregonzio C (2016) Brain Angiotensin II AT1 receptors are involved in the acute and long-term amphetamine-induced neurocognitive alterations. Psychopharmacology 233:795–807.

Maul B, Krause W, Pankow K, Becker M, Gembardt F, Alenina N, Walther T, Bader M, Siems WE (2005) Central angiotensin II controls alcohol consumption via its AT1 receptor. FASEB J 19:1474–1481.

Maximino JR, Ferrari MF, Coelho EF, Fior-Chadi DR (2006) Time course analysis of tyrosine hydroxylase and angiotensinogen mRNA expression in central nervous system of rats submitted to experimental hypertension. Neurosci Res 55:292–299.

McPherson CS, Lawrence AJ (2006) Exposure to amphetamine in rats during periadolescence establishes behavioural and

extrastriatal neural sensitization in adulthood. Int J Neuropsychopharmacol 9:377–392.

Morgan JI, Curran T (1989) Stimulus-transcription coupling in neurons: role of cellular immediate-early genes. Trends Neurosci 12:459–462.

Murphy AZ, Ennis M, Shipley MT, Behbehani MM (1994) Directionally specific changes in arterial pressure induce differential patterns of fos expression in discrete areas of the rat brainstem: a double-labeling study for Fos and catecholamines. J Comp Neurol 349:36–50.

Nordquist RE, Vanderschuren LJ, Jonker AJ, Bergsma M, de Vries TJ, Pennartz CM, Voorn P (2008) Expression of amphetamine sensitization is associated with recruitment of a reactive neuronal population in the nucleus accumbens core. Psychopharmacology 198:113–126.

Pavcovich LA, Cancela LM, Volosin M, Molina VA, Ramirez OA (1990) Chronic stress-induced changes in locus coeruleus neuronal activity. Brain Res Bull 24:293–296.

Paxinos G, Watson C (2009) The rat brain in stereotaxic coordinates. Oxford: Elsevier.

Paz MC, Assis MA, Cabrera RJ, Cancela LM, Bregonzio C (2011) The AT(1) angiotensin II receptor blockade attenuates the development of amphetamine-induced behavioral sensitization in a two-injection protocol. Synapse 65:505–512.

Paz MC, Marchese NA, Cancela LM, Bregonzio C (2013) Angiotensin II AT(1) receptors are involved in neuronal activation induced by amphetamine in a two-injection protocol. Biomed Res Int 2013:534817.

Paz MC, Marchese NA, Stroppa MM, Gerez de Burgos NM, Imboden H, Baiardi G, Cancela LM, Bregonzio C (2014) Involvement of the brain renin-angiotensin system (RAS) in the neuroadaptive responses induced by amphetamine in a two-injection protocol. Behav Brain Res 272:314–323.

Peng JF, Phillips MI (2001) Opposite regulation of brain angiotensin type 1 and type 2 receptors in cold-induced hypertension. Regul Pept 97:91–102.

Perez MF, Maglio LE, Marchesini GR, Molina JC, Ramirez OA (2002) Environmental changes modify the expression of Diazepam withdrawal. Behav Brain Res 136:75–81.

Pierce RC, Kalivas PW (1997) A circuitry model of the expression of behavioral sensitization to amphetamine-like psychostimulants. Brain Res Brain Res Rev 25:192–216.

Ramirez OA, Wang RY (1986a) Electrophysiological evidence for locus coeruleus norepinephrine autoreceptor subsensitivity following subchronic administration of D-amphetamine. Brain Res 385:415–419.

Ramirez OA, Wang RY (1986b) Locus coeruleus norepinephrine-containing neurons: effects produced by acute and subchronic treatment with antipsychotic drugs and amphetamine. Brain Res 362:165–170.

Roitman MF, Na E, Anderson G, Jones TA, Bernstein IL (2002) Induction of a salt appetite alters dendritic morphology in nucleus accumbens and sensitizes rats to amphetamine. J Neurosci 22: RC225.

Saavedra JM (1992) Brain and pituitary angiotensin. Endocr Rev 13:329–380.

Saavedra JM, Armando I, Bregonzio C, Juorio A, Macova M, Pavel J, Sanchez-Lemus E (2006) A centrally acting, anxiolytic angiotensin II AT1 receptor antagonist prevents the isolation stress-induced decrease in cortical CRF1 receptor and benzodiazepine binding. Neuropsychopharmacology 31:1123–1134.

Saavedra JM, Sanchez-Lemus E, Benicky J (2011) Blockade of brain angiotensin II AT1 receptors ameliorates stress, anxiety, brain inflammation and ischemia: therapeutic implications. Psychoneuroendocrinology 36:1–18.

Seltzer A, Bregonzio C, Armando I, Baiardi G, Saavedra JM (2004) Oral administration of an AT1 receptor antagonist prevents the central effects of angiotensin II in spontaneously hypertensive rats. Brain Res 1028:9–18.

Stewart J, Badiani A (1993) Tolerance and sensitization to the behavioral effects of drugs. Behav Pharmacol 4:289–312.

Sumners C, Phillips MI (1983) Central injection of angiotensin II alters catecholamine activity in rat brain. Am J Physiol 244:R257–R263.

Sumners C, Phillips MI, Raizada MK (1983) Angiotensin II stimulates changes in the norepinephrine content of primary cultures of rat brain. Neurosci Lett 36:305–309.

Valjent E, Bertran-Gonzalez J, Aubier B, Greengard P, Herve D, Girault JA (2010) Mechanisms of locomotor sensitization to drugs of abuse in a two-injection protocol. Neuropsychopharmacology 35:401–415.

Vanderschuren LJ, Kalivas PW (2000) Alterations in dopaminergic and glutamatergic transmission in the induction and expression of behavioral sensitization: a critical review of preclinical studies. Psychopharmacology 151:99–120.

Vanderschuren LJ, Schmidt ED, De Vries TJ, Van Moorsel CA, Tilders FJ, Schoffelmeer AN (1999) A single exposure to amphetamine is sufficient to induce long-term behavioral, neuroendocrine, and neurochemical sensitization in rats. J Neurosci 19:9579–9586.

Veerasingham SJ, Vahid-Ansari F, Leenen FH (2000) Neuronal Fos-like immunoreactivity in ouabain-induced hypertension. Brain Res 876:17–21.

Watanabe MA, Kucenas S, Bowman TA, Ruhlman M, Knuepfer MM (2010) Angiotensin II and CRF receptors in the central nucleus of the amygdala mediate hemodynamic response variability to cocaine in conscious rats. Brain Res 1309:53–65.

Wright JW, Harding JW (2013) The brain renin-angiotensin system: a diversity of functions and implications for CNS diseases. Pflugers Arch 465:133–151.

Xiong H, Marshall KC (1994) Angiotensin II depresses glutamate depolarizations and excitatory postsynaptic potentials in locus coeruleus through angiotensin II subtype 2 receptors. Neuroscience 62:163–175.

Xue B, Thunhorst RL, Yu Y, Guo F, Beltz TG, Felder RB, Johnson AK (2016) Central renin-angiotensin system activation and inflammation induced by high-fat diet sensitize angiotensin II-elicited hypertension. Hypertension 67:163–170.

Xue B, Zhang Z, Beltz T, Guo F, Johnson AK (2013) Either sodium deprivation or sodium excess sensitizes angiotensin (ANG) II-induced hypertension. FASEB J 27:695.612.

Xue B, Zhang Z, Johnson RF, Johnson AK (2012a) Sensitization of slow pressor angiotensin II (Ang II)-initiated hypertension: induction of sensitization by prior Ang II treatment. Hypertension 59:459–466.

Xue B, Zhang Z, Roncari CF, Guo F, Johnson AK (2012b) Aldosterone acting through the central nervous system sensitizes angiotensin II-induced hypertension. Hypertension 60:1023–1030.

Yoshida K, Morimoto A, Makisumi T, Murakami N (1993) Cardiovascular, thermal and behavioral sensitization to methamphetamine in freely moving rats. J Pharmacol Exp Ther 267:1538–1543.

(Received 25 August 2016, Accepted 25 August 2016)
(Available online 14 November 2016)