Article

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Repeated Amphetamine Exposure Blunted Angiotensin II-Induced Responses Mediated by AT_1 Receptors

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Background: Angiotensin II, is critical in regulating the sympathetic and neuroendocrine systems through angiotensin II type 1 receptors (AT_1-R). Angiotensin II intracerebral administration increases water and sodium intake, as well as renal sodium excretion. Previously, our group has shown that AT_1-R is involved in behavioral and neurochemical sensitization induced by amphetamine. We aimed to assess the physiological output, behavioral, and neurochemical responses to intracerebral angiotensin II administration, via the AT_1-R, twenty-one days after amphetamine administration.

Material and Methods: Male Wistar rats received daily vehicle or AT_1-R antagonist (oral) for 10 days, and amphetamine or saline intraperitoneal (i.p.) from day 6 to 10. On day 25 they were implanted with an intracerebral cannula. On day 32, the animals received intracerebral angiotensin II. First group: the animals were tested in a free choice paradigm for 2% NaCl and water intake, and sacrificed for neuronal activity assessment via c-Fos immunohistochemistry. Second group: urine samples were collected for electrolyte determination. Third group: the animals were tested in the plus maze or the hole board for anxiety and working memory evaluation, respectively, and sacrificed for c-Fos immunohistochemistry.

Results: Amphetamine exposure blunted the increase in sodium intake ($p = 0.0022$), and potentiated the natriuretic ($p = 0.0043$) and kaliuretic effect ($p = 0.0002$) induced by angiotensin II. Moreover, amphetamine exposure prevented the expression of the anxiogenic effect (drug effect $p < 0.0001$) and the memory deficit ($p = 0.1314$) induced by cerebral angiotensin II administration. Amph decreased c-Fos immunoreactivity in nucleus tractus solitarii (NTS) $p = 0.0037$; paraventricular nucleus (PVN) $p = 0.0047$; Central amygdala (CeA) $p = 0.0008$; Basolateral amygdala (BLA) $p = 0.0018$; increased in hippocampus region CA1 $p = 0.0043$; CA3 $p = 0.026$; and dentate gyrus (DG) $p = 0.0057$. The blockade of AT_1-R prevented these alterations (sodium intake $p = 0.0421$ natriuresis $p = 0.019$; kaliuresis $p = 0.196$; working memory ($p < 0.0001$); but no the anxiogenic response to angiotensin II (drug effect $p < 0.0001$); as well as the c-Fos changes (NTS $p = 0.0052$; PVN $p = 0.029$; CeA $p = 0.0002$; BLA $p = 0.0021$; CA1 $p = 0.0026$; CA3 $p = 0.022$; and DG $p = 0.0016$).

Conclusion: Most of the long-lasting AT_1-R altered responses to brain angiotensin II administration induced by repeated amphetamine exposure could be prevented by AT_1-R blockade.

Keywords: angiotensin II; AT_1-receptor; amphetamine; working memory; anxiety

Introduction

The brain renin-angiotensin system (BRAS), modulates several processes, such as hydro-electrolytic homeostasis [1], anxiety [2–4], and cognition [5,6]. Most of the known physiological functions of angiotensin II (ANG II) are mediated by angiotensin II type 1 receptors (AT_1-R) [7]. Broad evidence supports the intricate crosstalk between ANG II and the dopamine system, through the AT_1-R [8–10]. This interaction has a main role in the already described ANG II central effects on cardiovascular control, water intake, and more complex behaviors [11,12]. A high density of AT_1-R has been described in dopamine-rich regions such as the dorsal striatum, hypothalamus, hippocampus, nucleus accumbens, and ventral pallidum [9,13]. Our lab has shown that changes in dopamine neurotransmission induced by amphetamine (Amph) exposure altered the AT_1-R functioning, even seven days after the last administration of this psychostimulant [14]. Several long-term neuroadaptations mediated by AT_1-R and induced by Amph administration were observed as behavioral and neurochemical changes unmasked by the psychostimulant re-exposure. ANG II intracerebroventricular (icv) administration has been found to increase renal sodium excretion,

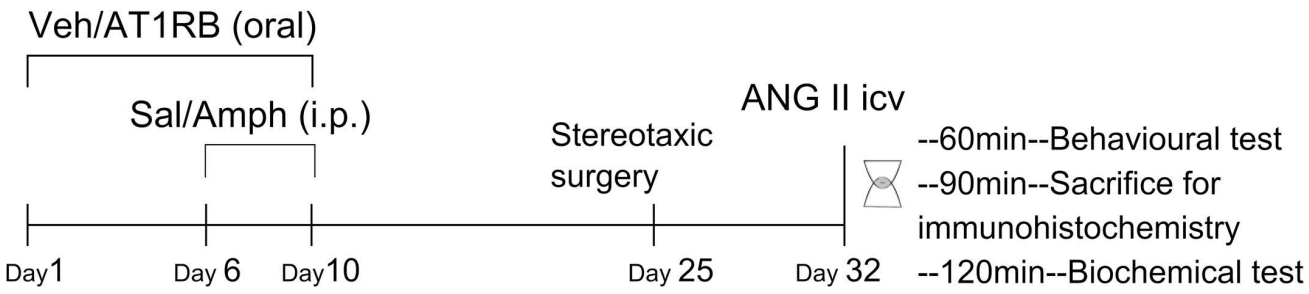

Fig. 1. Experimental design. Schematic representation of experimental protocol used in this work. Veh, vehicle; AT_1RB, angiotensin II type 1 receptors blockade; Sal, saline; Amph, amphetamine; ANG II, angiotensin II; icv, intracerebroventricular; ip, intraperitoneal. The artwork was conducted with Inkscape software 1.4.X (Free and Open-source Software licensed under the General Public License. Free software Foundation, Inc., Boston, MA, USA).

and water and sodium intake [13,15,16], as well as decrease plasma renin activity and increase vasopressin and oxytocin secretion [12,17]. Furthermore, we have previously reported that repeated Amph administered seven days before, markedly reduced the sodium intake induced by ANG II icv meanwhile water intake remains unaffected [18], supporting the idea that differential AT_1-R roles in water and saline intake stimulated by ANG II. Moreover, these altered responses were accompanied by altered activation (increased c-Fos expression) of the brain areas related to the physiological roles described for ANG II icv. In this work, we aimed to analyze whether the neuroadaptations induced by Amph administration, which modifies the AT_1-R response to ANG II icv, are long-lasting and could be observed 21 days after the last Amph administration; and whether AT_1-R blockade prevents or attenuates these alterations. We also analyzed behavioral responses associated with anxiety, memory working, and neuronal activation in the related brain areas.

Materials and Methods

Animals

Adult male Wistar rats (8 weeks; 250–320 g) from our colony (Faculty of Chemical Sciences, National University of Córdoba, Argentina) were randomly housed in groups of five per cage and maintained under controlled temperature (21 ± 1 °C), humidity (40–60% Relative Humidity, and 12-h light-dark cycle conditions (lights from 8:00 AM to 8:00 PM) with free access to water and standard laboratory rodent chow (Rat chow: Na^+ content: 0.35%; K^+ content: 0.96%).

Experimental Design

To evaluate the AT_1-R involvement in the neuroadaptive responses induced by Amph, the animals received an AT_1-R antagonist (candesartan) or vehicle, once a day for 10 days (by gavage using a feeding needle). From day 6 until day 10, they also received an intraperitoneal injection of saline or Amph daily (Fig. 1). As a consequence, four experimental groups were assessed: vehicle-saline (Veh-Sal), AT_1-R blockade-saline (AT_1RB-Sal), vehicle-amphetamine (Veh-Amph), and AT_1-R blockade-amphetamine (AT_1RB-Amph). On day 25, all animals were implanted with brain cannulae by stereotaxic surgery and left undisturbed in their home cages for 7 days for recovery. On day 32, the animals received ANG II (dose: 400 pmol) icv and were randomly divided into groups for different analyses detailed next:

Hydro-electrolytic analysis:

○ Sodium and water Intake and c-Fos expression: one day before the test day, the animals were individually housed for one hour and the basal water and sodium intake was determined in all animals after a saline solution icv administration. On test day, the animals were once again housed individually for one hour and received a microinjection of ANG II icv. Each test cage had access to two burets, one filled with water and the other with a 2% NaCl solution. The volume of both solutions consumed was measured one hour after saline and ANG II icv injections. The animals were sacrificed by transcardial perfusion, ninety minutes after ANG II icv administration for c-Fos immunoreactivity.

○ Urine profile assessment: for the first two hours after the ANG II icv injection, the animals were individually housed in metabolic cages with free access to water. The urine samples were collected in clean plastic tubes, centrifuged at 10,000 G and the supernatant was stored at –20 °C. Urinary sodium and potassium were determined using the ion-selective method and a Roche-Hitachi Autoanalyzer, Cobas c311, in collaboration with the Central Laboratory of the Provincial San Roque Hospital in Córdoba, Argentina. After the urine sample collection, the animals were euthanized by using carbon dioxide inhalation.

○ Working memory and anxiety assessment and c-Fos expression: the animals were evaluated in the hole board or the plus maze test one hour after the ANG II icv injection. The immunoreactivity for c-Fos was evaluated ninety minutes after ANG II icv administration.

Drugs

D-amphetamine sulfate (Amph, Sigma-Aldrich, Saint Louis, MO, USA) was dissolved in 0.9% NaCl immediately before use. The dose was chosen considering previous work [19].

Candesartan cilexetil (Phoenix Laboratories, Buenos Aires, Argentina) was dissolved in a 0.1 N solution of sodium bicarbonate, the solution was protected from light and stored at 4 °C. The dose was chosen based on previous work [10].

Angiotensin II (ANG II, Sigma-Aldrich) was dissolved in 0.9% saline (NaCl) immediately before use. The dose of 400 pm was chosen considering previous work [20]. The microinjection volume used was 2 µL/side.

Stereotaxic Surgery

The stereotaxic surgery protocol was adapted from our previous work by Casarsa *et al.* [18]. The animals were anesthetized with ketamine/xylazine (75/5 mg/kg i.p.) and placed in a stereotaxic apparatus (Model 963 of 100 µm precision, David Kopf Instruments, Tujunga, CA, USA) without ear damage. Guide cannulas (stainless steel, made from BD Precision Glide hypodermic needles, 23 G gauge, and 11 mm length) were stereotaxically implanted into both lateral ventricles of the brain (bregma: –1.0 mm; lateral ± 1.7 mm; depth 3.5 mm) [21]. The guide cannulas were then fixed to the skull with acrylic cement and stainless steel screws. Stainless steel plugs (made from Deltajet dental hypodermic needles, 30 G gauge, and 11 mm length) were inserted into the guide cannulas to prevent obstruction. Two cannulas were implanted in each animal's brain to minimize the number of individuals required. If one cannula is obstructed, the administration of the complete volume is performed on the non-obstructed side. After the surgery, the animals were left undisturbed in their home cage for 7 days for recovery. Only animals with proper cannula placement were considered for different analyses.

Intracerebroventricular Injection

The animals were habituated in the behavior room for one hour. Then, they were removed from their cages, covered gently with a cloth, and manually restrained while 400 pmol of ANG II (in 4 µL pH 7.4 physiological solution) or vehicle (physiological solution) were administered into both lateral ventricles. A stainless steel injection needle with a 30 G gauge and 11 mm length connected to a 10 mL microsyringe (LOT#437742, Hamilton Company, Bellefonte, PA, USA) through a P20 polyethylene tube was inserted into the guide cannulas, delivering a total volume of 4 µL/rat into the lateral ventricles. The injection needle remained in the ventricle for twenty seconds to allow diffusion. The dosage was selected based on preliminary work [20,22].

Metabolic Cages

To collect the urine the animals were placed in metabolic cages, manufactured by stainless steel with validated dimensions with a surface of 320 cm^2 and a height of 14 cm. There is no urine washover and no potential for urine to enter the feces tube. Separation is immediate and complete. The animals have access to a bottle of water. The feeder chamber is located outside the cage.

Hole Board Test

To evaluate the AT_1-R involvement in working memory changes induced by repeated Amph exposure, the hole board test was performed one hour after ANG II (400 pmol) icv challenge administration. This behavioral test was adapted from our previous works [23]. Briefly, the animals were habituated to the behavioral room for one hour. After that, they received the ANG II icv challenge and, one hour later, they were located in a perforated open field (60 cm × 60 cm × 30 cm) that contained a central hole and four lateral holes situated 10 cm from the edge (each hole had a diameter of 3 cm). The behavior was recorded through a video camera for five minutes and the number of head dipping (inserting the head into each hole) was manually and blindly measured. The exploring rate (N° exploration per hole/ total of explorations to all holes) was calculated for each hole. The probability of exploring each hole is 1/5. Considering this, for the analysis we used the exploring index: exploring rate/probability of exploring each hole. It is assumed that a more homogeneous distribution reflects better working memory performance, as the animal remembers previously explored holes in the short term and does not continue with the exploration. On the contrary, a working memory deficit is observed as a significant difference in the exploration between the two most explored holes.

Elevated Plus Maze Test

To evaluate the AT_1-R involvement in anxiety-like behavior induced by repeated Amph exposure, the elevated plus maze test was performed one hour after an ANG II icv challenge administration. The elevated plus maze apparatus consisted of two open arms (50 cm × 10 cm) and two closed arms (50 cm × 10 cm × 40 cm), positioned 50 cm above the floor and illuminated with dim light [24,25]. The animals were placed in the maze's center, facing an open arm [26]. Behavior was recorded for five minutes by using video cameras. The time spent in the open arms and the number of entries into open and closed arms (Total entries) were manually and blindly measured. The time spent in the open arms is considered an anxiety index while the number of total entries indicates locomotor or exploratory activity [25,26]. An entry into an arm is defined as all four paws of the animal being placed within it [27].

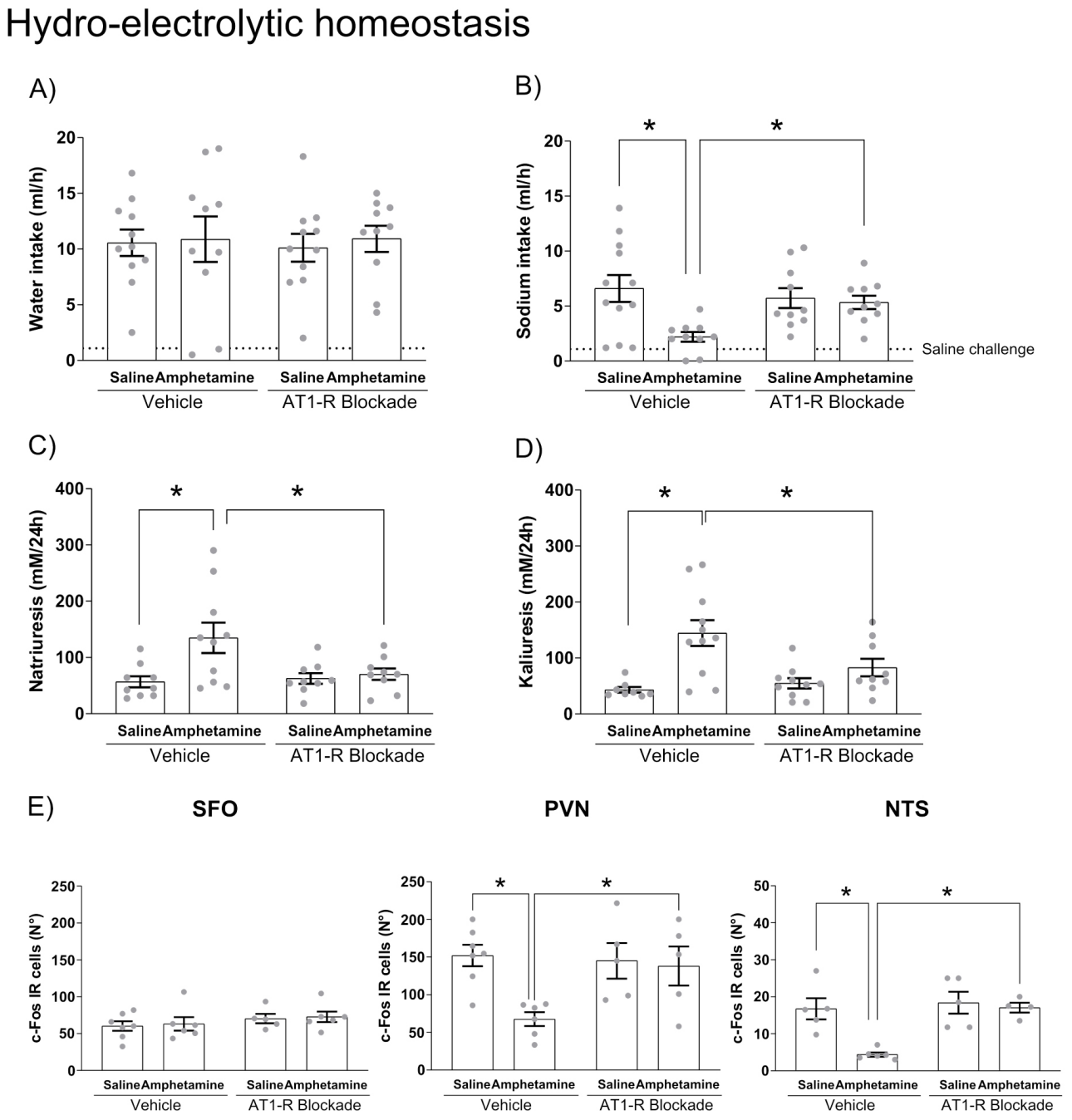

Fig. 2. Amph-administration altered the future physiological response to ANG II intracerebroventricular (icv), via AT_1-R activation. The graph shows the behavioral and neuronal activation responses related to hydro-electrolytic homeostasis observed in response to a challenge of ANG II icv in the four experimental groups. (A) Water intake. (B) Sodium intake. (C) Natriuresis. (D) Kaliuresis. (E) Neuronal activation is observed as c-Fos immunoreactivity (IR) in subfornical organ (SFO), paraventricular nucleus (PVN), and nucleus tractus solitarii (NTS). The values are presented as mean ± standard error of the mean (SEM), *$p < 0.05$. N = 10–12 for behavioral/biochemical tests. N = 5–7 for immunohistochemistry.

Immunohistochemistry

The immunohistochemistry procedure was adapted from our previous work by Casarsa *et al.* [18]. Briefly, ninety minutes following the administration of the challenge dose of ANG II icv, the animals were anesthetized with Urethane 50% (100 mg/kg) and perfused transcardially using 250 mL of saline/heparin (200 µm/L) solution using the pump (10 mL per minute; Model 777200-12 200RM, Barnant company, Barrington, IL, USA), followed by 400 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brains were fixed in the same solution overnight and then stored at 4 °C in a 30% sucrose/PB solution. Coronal sections (40 µm) were ob-

tained using a freezing microtome (Leica CM1510S-1, Leica microsystems, Wetzlar, Germany) and collected in 0.01 M phosphate buffer saline (PBS, pH 7.4); from the following brain regions: Central amygdala (CeA) and Basolateral amygdala (BLA; Bregma –2.80 mm), subfornical organ (SFO; Bregma –1.40 mm), nucleus tractus solitarii (NTS; Bregma –13.80 mm), paraventricular nucleus (PVN; distance from bregma –0.92 mm to –2.12 mm), and hippocampus region CA1, CA2, CA3, and dentate gyrus (DG; Bregma –1.30 mm). Then, the brain sections were placed free-floating in a 10% H_2O_2/10% methanol solution, washed three times with PBS 0.01 M, and then in 0.1 M PBS/10% normal horse serum (NHS-GIBCO, Auckland, New Zealand) solution solution, for two hours each. They were incubated free-floating overnight, at room temperature in the rabbit anti-c-Fos antibody (1:10,000; Ab-5; Oncogene Science, Manhasset, NY, USA)/2% NHS/ 0.3% Triton X-100 (Sigma-Aldrich; LOT: #MKBW8386V; St. Louis, MO, USA)/0.1 M PBS solution. The following day, the sections were incubated with biotin-labeled universal secondary antibody (diluted 1:500; BA-1000; Z1203, Vector Laboratories, Burlingame, CA, USA) and avidin-biotin-peroxidase complex (ABC, 1:200; Vector Laboratories) for two hours each, at room temperature. The peroxidase label was detected with diaminobenzidine hydrochloride (0.5 mg/mL, Sigma-Aldrich) and hydrogen peroxide. The solution was intensified with 1% cobalt chloride and 1% nickel ammonium sulfate giving the c-Fos positive nucleus a dark blue tone. Finally, they were washed three times with PBS 0.01 M, ten minutes each. They were mounted on gelatinized slides, air-dried overnight, dehydrated, cleared in xylene, and placed under a coverslip with DPX mountant medium (LOT: 441319/1; filing code: 34702126; Sigma-Aldrich; St. Louis, MO, USA). Images were obtained using a computerized system that included a microscope (Leica DM4000B, Leica microsystems, Wetzlar, Germany) with a DFC digital camera (Leica microsystems, Wetzlar, Germany). C-Fos immunoreactive neurons were identified by dense black staining of the nucleus and counted by setting a size range for cellular nuclei (8 to 12 µm diameter) using IMAGE J software (1.52i, National Institute of Health, Bethesda, MD, USA) from the National Institutes of Health as previously reported [18]. The counting was done manually and blindly blinded to the experimental groups. Images (40×) from two sections and both hemispheres were taken for each brain area and the final value obtained was the average of the four counted sections used for the statistical analysis.

Statistical Analysis

Data were analyzed by using Two-way Analysis of Variance (ANOVA). Two-way ANOVA repeated measure was used to analyze the Hole board test. To analyze the elevated plus maze test after acute ANG II icv, a *T*-test was performed. One-way ANOVA was used for the biochemical analysis. Multiple comparisons were made by using Bonferroni's post hoc test. A value of $p < 0.05$ was considered significant and the results are reported as means ± standard error of the mean (SEM). The analyses were performed by using GraphPad Prism® 8.02 software (GraphPad Software, Inc., San Diego, CA, USA). The artwork was conducted with Inkscape software (Free and Open-source Software licensed under the General Public License. Free Software Foundation, Inc., Boston, MA, USA).

Results

Effect of Previous Amph Exposure over Hydro-Electrolytic Homeostasis in Response to ANG II and the Role of the AT_1-R

Water and Sodium Appetite

After the ANG II challenge, the ANOVA showed no significant differences in water intake in any of the groups ($p > 0.05$). This suggests that neither Amph nor AT_1-R antagonist administration induces long-term alterations over the known effect of ANG II on water intake (Table 1; Fig. 2A). Previous Amph exposure blunted the ANG II-increased sodium intake, and the AT_1-R blockade prevented it ($p < 0.05$) (Table 1; Fig. 2B).

Biochemical Profile in Urine Natriuresis and Kaliuresis

Amph administration exacerbated the ANG II icv increased natriuresis and kaliuresis ($p < 0.01$). These alterations were prevented by the AT_1-R blockade (Table 1; Fig. 2C,D respectively) ($p < 0.05$). No differences among experimental groups were found in the urine volume, creatinine, and body weight ($p > 0.05$) (Table 1).

Neuronal Activation in Brain Areas Related to Hydro-Electrolytic Homeostasis

Previous Amph exposure reduced the number of c-Fos immunoreactivity (IR) cells on PVN and NTS in response to an ANG II icv administration ($p < 0.01$). The AT_1-R blockade prevented this alteration ($p < 0.05$). The ANOVA showed no significant differences between the groups in the SFO area (Table 1; Fig. 2E) ($p > 0.05$). The representative microphotographs and the brain areas analyzed are shown in Fig. 3.

Previous Amph Exposure Blunted the Effect of ANG II icv over Working Memory

Hole Board Test

The data are presented for all treatments in decreasing order, enabling discrimination to see if the treatments altered the exploration pattern between the holes. Considering that the probability of exploring each hole is 1/5 (figured as zero in the graph, Fig. 4A), a positive value indicates that the hole was explored more times than expected. In contrast, a negative value indicates it was explored fewer times than expected. In Bonferroni's post hoc test, the ex-

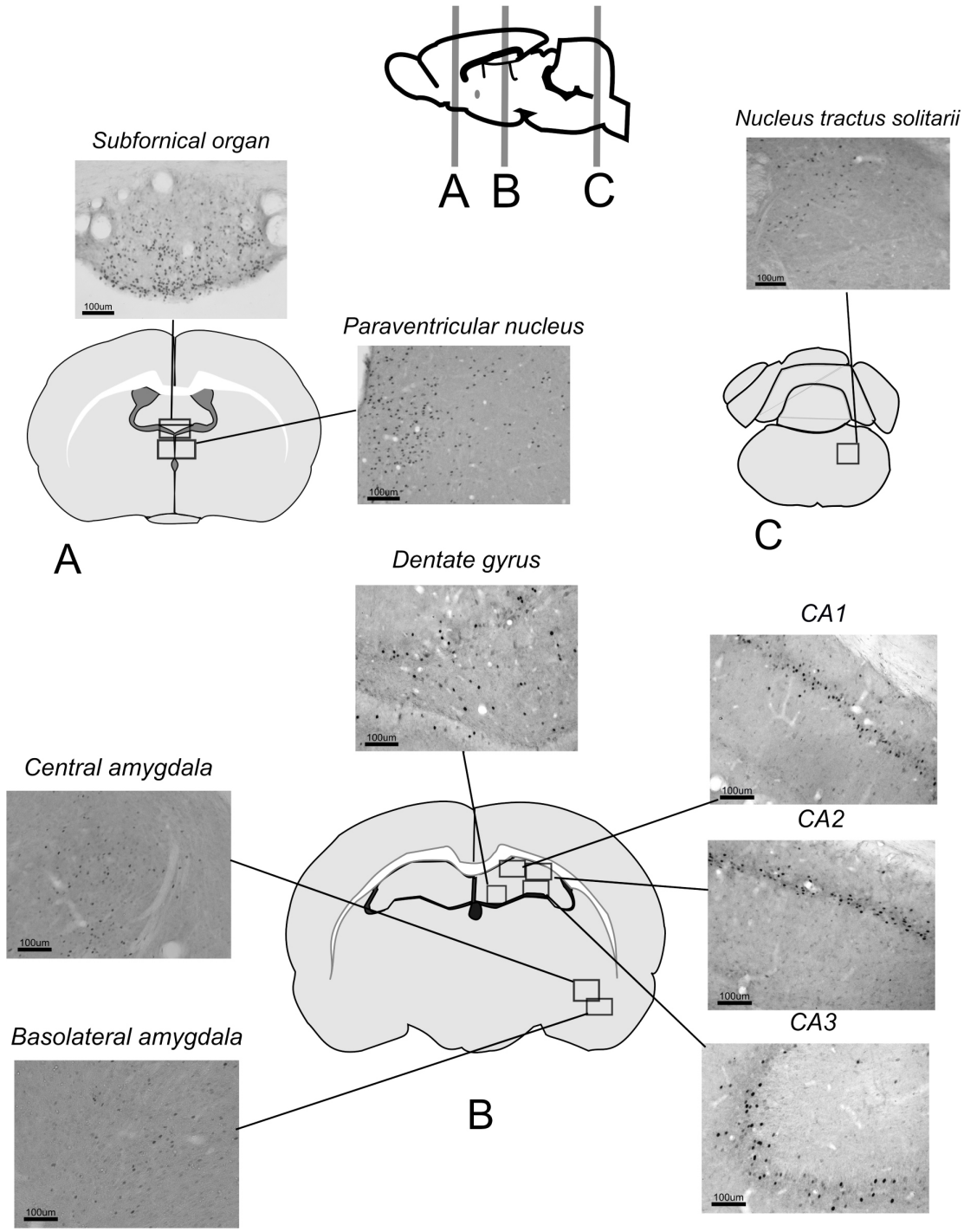

Fig. 3. Representative microphotographs showing c-Fos immunostaining and the brain areas analyzed. The c-Fos positive cells are evidenced as dense black dots. Top: sagittal representation of rat brain and the location of coronal sections used in the present work. Bottom: the coronal sections of the brain. (A) Paraventricular nucleus (PVN; distance from bregma –0.92 mm to –2.12 mm) and subfornical organ (SFO; Bregma –1.40 mm). (B) Central amygdala (CeA) and Basolateral amygdala (BLA; Bregma –2.80 mm), and hippocampus region CA1, CA2, CA3, and dentate gyrus (DG; Bregma –1.30 mm). (C) Nucleus tractus solitarii (NTS; Bregma –13.80 mm).

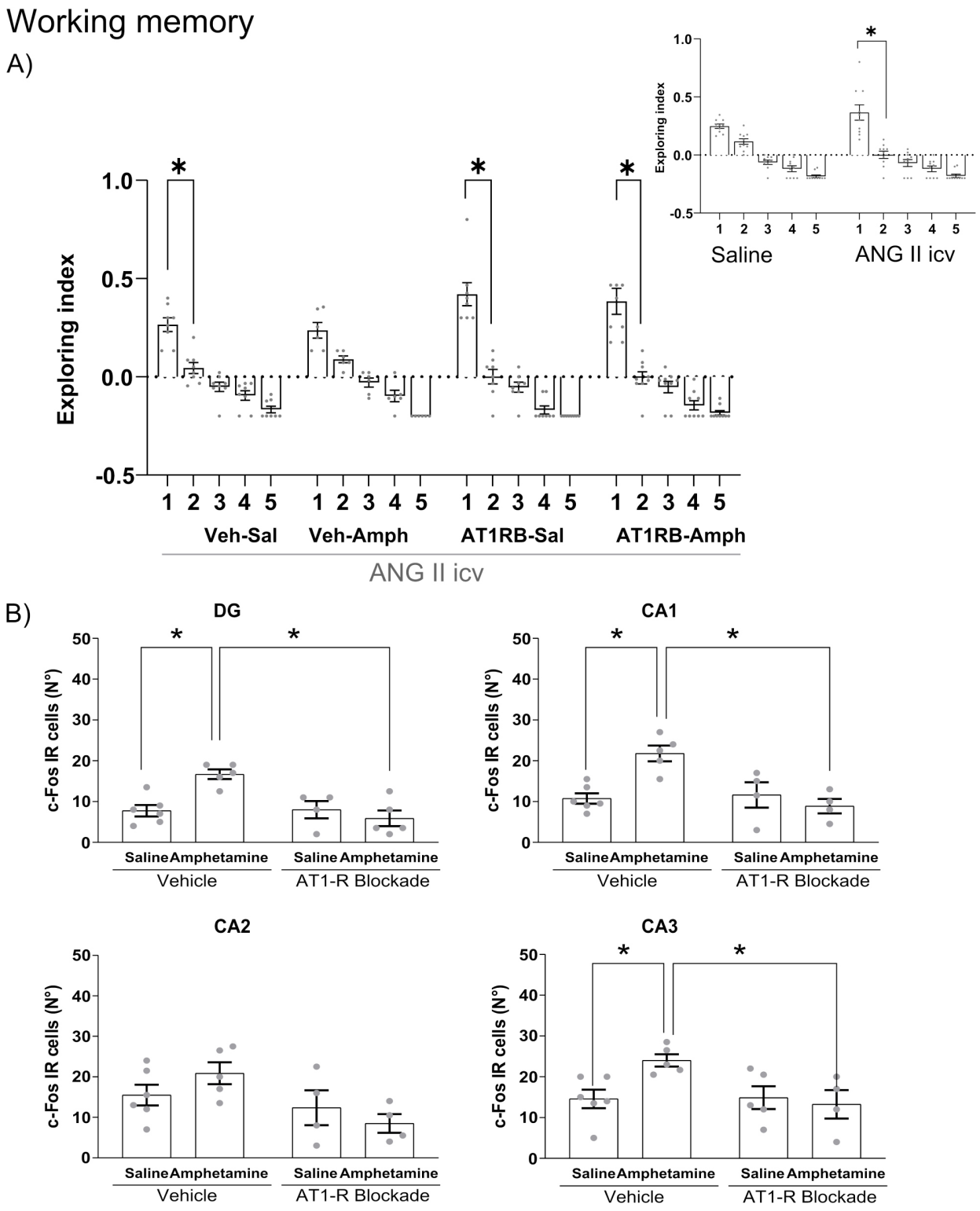

Fig. 4. Amph-administration blunted the memory deficit induced by ANG II icv, via AT_1-R activation. (A) The graph shows the difference between the exploring rate (head dipping) and the expected probability of exploring each hole. Cero in the graph indicates the probability of exploring each hole (1/5). Positive values indicate that the hole was explored more times than expected; while the negative, the hole was explored less times than expected. The significant difference between the first and second explored holes suggests a working memory deficit. (A-inset) The graph shows the working memory deficit observed after an acute ANG II icv administration. (B) Neuronal activation in CA1, CA2, CA3, and dentate gyrus was evaluated by c-Fos immunoreactivity. AT_1RB, AT_1-R blockade. The values are presented as mean ± SEM, $*p < 0.05$. N = 10–12 for the Hole board test. N = 5–7 for immunohistochemistry.

Table 1. Hydro-electrolytic homeostasis.

Behavioral and biochemical test		Water intake			Sodium intake			Natriuresis			Kaliuresis	
	interaction	F (1, 38) = 0.028	$p = 0.87$	*interaction*	F (1, 38) = 4.960	$p = 0.032$*	*interaction*	F (1, 33) = 4.413	$p = 0.043$*	*interaction*	F (1, 34) = 5.003	$p = 0.032$*
	drug	F (1, 38) = 0.156	$p = 0.695$	*drug*	F (1, 38) = 7.053	$p = 0.012$*	*drug*	F (1, 33) = 6.501	$p = 0.016$*	*drug*	F (1, 34) = 15.681	$p = 0.0004$*
	treatment	F (1, 38) = 0.019	$p = 0.89$	*treatment*	F (1, 38) = 1.564	$p = 0.219$	*treatment*	F (1, 33) = 3.041	$p = 0.091$	*treatment*	F (1, 34) = 2.338	$p = 0.135$
				bonferroni's posthoc		*p*	bonferroni's posthoc		*p*	bonferroni's posthoc		*p*
				Veh-Sal vs Veh-Amph		0.0022*	Veh-Sal vs Veh-Amph		0.0043*	Veh-Sal vs Veh-Amph		0.0002*
				Veh-Amph vs AT_1-R blockade-Amph		0.0421*	Veh-Amph vs AT_1-R blockade-Amph		0.019*	Veh-Amph vs AT_1-R blockade-Amph		0.0196*
Neuronal activation		c-Fos NTS			c-Fos PVN			c-Fos SFO				
	interaction	F (1, 16) = 6.451	$p = 0.022$*	*interaction*	F (1, 19) = 4.513	$p = 0.047$*	*interaction*	F (1, 20) = 0.0013	$p = 0.972$			
	drug	F (1, 16) = 9.957	$p = 0.006$*	*drug*	F (1, 19) = 6.277	$p = 0.021$*	*drug*	F (1, 20) = 0.1265	$p = 0.726$			
	treatment	F (1, 16) = 10.89	$p = 0.0045$*	*treatment*	F (1, 19) = 3.027	$p = 0.098$	*treatment*	F (1, 20) = 1.738	$p = 0.2023$			
	bonferroni's posthoc		*p*	bonferroni's posthoc		*p*						
	Veh-Sal vs Veh-Amph		0.0037*	Veh-Sal vs Veh-Amph		0.0047*						
	Veh-Amph vs AT_1-R blockade-Amph		0.0052*	Veh-Amph vs AT_1-R blockade-Amph		0.029*						
Biochemical parameters	group	volume (mL)/2 hs	creatinine (mg %)	body weight (g)								
	Veh-Sal	4.0 ± 1.3	33.4 ± 11.5	325.2 ± 5.6								
	AT_1-R blockade-Sal	4.4 ± 0.9	33.8 ± 9.9	326.9 ± 6.5								
	Veh-Amph	3.7 ± 1.1	32.0 ± 7.8	317.7 ± 5.2								
	AT_1-R blockade-Amph	5.0 ± 1.0	28.1 ± 9.4	329.2 ± 5.5								
	One-Way ANOVA	F = 0.2682; $p = 0.848$	F = 0.0713; $p = 0.975$	F = 0.7556; $p = 0.5246$								

Statistical results for hydro-electrolytic analysis and neuronal activation in the related brain areas: SFO, subfornical organ; PVN, paraventricular nucleus; NTS, nucleus tractus solitarii. Two-way Analysis of Variance (ANOVA) and Bonferroni's multiple comparisons. Interaction: drug*treatment interaction. Drug: saline/amphetamine effect. Treatment: vehicle/AT_1-R blockade effect. *$p < 0.05$. One-way ANOVA for biochemical parameters.

Table 2. Working memory.

Behavioural test	Visiting index*			Hole board test (acute ANG II icv)			*Visiting index each hole/probability of visiting each hole
	interaction	F (12, 108) = 2.055	$p = 0.026$*	*interaction*	F (4, 72) = 2.959	$p = 0.0254$*	
	hole	F (4, 108) = 118.2	$p < 0.0001$*	*hole*	F (4, 72) = 65.43	$p < 0.0001$*	
	treatment	F (3, 27) = 1.420	$p = 0.259$	*treatment*	F (1, 18) = 0.2432	$p = 0.6278$	
	Subject	F (27, 108) = 6.632×10^{-16}	$p > 0.9999$	*Subject*	F (18, 72) = 3.614×10^{-16}	$p > 0.9999$	
	bonferroni's posthoc between holes 1 vs 2		*p*	bonferroni's posthoc between holes 1 vs 2		*p*	
	Veh-Sal		0.0003*	saline		0.0718	
	AT_1RB-Sal		<0.0001*	ANG II icv		<0.0001*	
	Veh-Amph		0.1314				
	AT_1RB-Amph		<0.0001*				
Neuronal activation	c-Fos DG			c-Fos CA1			c-Fos CA2			c-Fos CA3	
	interaction	F (1, 16) = 11.16	$p = 0.0041$*	*interaction*	F (1, 15) = 11.88	$p = 0.0036$*	*interaction*	F (1, 15) = 2.360	$p = 0.1453$	*interaction*	F (1, 16) = 4.825	$p = 0.0431$*
	drug	F (1, 16) = 4.288	$p = 0.0549$	*drug*	F (1, 15) = 4.296	$p = 0.0559$	*drug*	F (1, 15) = 0.064	$p = 0.8040$	*drug*	F (1, 16) = 2.383	$p = 0.1422$
	treatment	F (1, 16) = 10.17	$p = 0.0057$*	*treatment*	F (1, 15) = 9.055	$p = 0.009$	*treatment*	F (1, 15) = 6.612	$p = 0.0213$	*treatment*	F (1, 16) = 4.290	$p = 0.0549$
	bonferroni's posthoc		*p*	bonferroni's posthoc		*p*				bonferroni's posthoc		*p*
	Veh-Sal vs Veh-Amph		0.0057*	Veh-Sal vs Veh-Amph		0.0043*				Veh-Sal vs Veh-Amph		0.026*
	Veh-Amph vs AT_1-R blockade-Amph		0.0016*	Veh-Amph vs AT_1-R blockade-Amph		0.0026*				Veh-Amph vs AT_1-R blockade-Amph		0.022*

Statistical results for the Hole board test and neuronal activation in brain areas related to working memory: The exploring index indicates the ratio between the exploring rate/probability of exploring (1/5) for each hole. DG, dentate gyrus; AT_1RB, AT_1-R blockade. Two-way ANOVA and Bonferroni's multiple comparisons. Interaction: drug*treatment interaction. Drug: saline/amphetamine effect. Treatment: vehicle/AT_1-R blockade effect. Hole board: Two-way ANOVA repeated measures and Bonferroni's multiple comparisons. Interaction: Hole*treatment interaction Hole: hole's exploring index. Treatment: experimental groups. *$p < 0.05$.

Table 3. Anxiety.

Behavioural test	Time spent in open arms			Total entries			Time spent in open arms (Acute ANG II icv)	
	interaction	F (1, 42) = 0.345	$p = 0.5601$	*interaction*	F (1, 42) = 0.008	$p = 0.9296$	*unpaired t test*	
	drug	F (1, 42) = 25.16	$p = 0.0001$*	*drug*	F (1, 42) = 0.298	$p = 0.5881$	*p-value*	<0.0001
	treatment	F (1, 42) = 0.2192	$p = 0.6421$	*treatment*	F (1, 42) = 0.658	$p = 0.4219$	*p-value summary*	
Neuronal activation	c-Fos BLA			c-Fos CeA				
	Interaction	F (1, 18) = 4.518	$p = 0.048$*	*interaction*	F (1, 20) = 10.85	$p = 0.0036$*		
	drug	F (1, 18) = 15.42	$p = 0.001$*	*drug*	F (1, 20) = 9.391	$p = 0.0061$*		
	treatment	F (1, 18) = 18.81	$p = 0.0004$*	*treatment*	F (1, 20) = 16.43	$p = 0.0006$*		
	bonferroni's posthoc		*p*	bonferroni's posthoc		*p*		
	Veh-Sal vs Veh-Amph		0.0018*	Veh-Sal vs Veh-Amph		0.0008*		
	Veh-Amph vs AT_1-R blockade-Amph		0.0021*	Veh-Amph vs AT_1-R blockade-Amph		0.0002*		

Statistical results for Elevated plus maze test and neuronal activation in brain areas related to anxiety: BLA, Basolateral amygdala; CeA, Central amygdala. Two-way ANOVA and Bonferroni's multiple comparisons. Interaction: drug*treatment interaction. Drug: saline/amphetamine effect. Treatment: vehicle/AT_1-R blockade effect. Unpaired *t*-test. *$p < 0.05$.

ploration rates of the most explored hole vs the second most explored hole were compared in each group. In this regard, acute ANG II icv administration induced working memory deficit (Table 2; Fig. 4A inset). The control groups (Veh-Sal and AT_1-R blockade-Sal) evidenced the cognitive deficit induced by ANG II icv. Repeated Amph exposure blunted the expression of the working memory impairment induced by ANG II icv administration. The animals treated with the AT_1-R Blocker and exposed to Amph showed a cognitive deficit induced by ANG II icv similar to the control groups (Table 2; Fig. 4A).

Neuronal Activation in Brain Areas Related to Working Memory

Previous Amph exposure increased the number of c-Fos IR cells immunostained in CA1, CA3, and Dentate gyrus in response to an ANG II challenge. These alterations were prevented by the AT_1-R blockade. No significant differences were observed in CA2 (Table 2; Fig. 4B). The representative microphotographs and the brain areas analyzed are shown in Fig. 3.

Previous Amph Exposure Blunted the Anxiogenic Effect of ANG II icv

Elevated Plus Maze Test

As previously reported, the acute administration of ANG II icv induced anxiogenic effects in naive rats (Table 3; Fig. 5A inset) ($p < 0.05$). The previous exposure to Amph avoided the decrease in the time spent in the open arms in response to the ANG II icv administration ($p < 0.05$). Interestingly, despite the AT_1-R blockade, the altered anxiogenic response to ANG II persisted. Specifically, the AT_1-R blockade-Amph group exhibited a significantly longer duration spent in the open arms than its control group (AT_1-R blockade-Sal), akin to the duration observed in the Veh-Amph group (Table 3; Fig. 5A) ($p < 0.05$). No statistically significant differences were found in total entries when comparing across experimental groups (see Table 3).

Neuronal Activation in Brain Areas Related to Anxiety

Previous Amph exposure decreased the number of c-Fos IR cells immunostained in BLA and CeA in response to an ANG II challenge, which was prevented by the AT_1-R blockade (Table 3; Fig. 5B) ($p < 0.01$). The representative microphotographs and the brain areas analyzed are shown in Fig. 3.

Discussion

Our results show that previous Amph exposure induces long-term neuroadaptations changing the future AT_1-R response to ANG II administration, which can be mostly prevented by AT_1-R blockade. This work goes along with previous reports from our lab showing that a history of psychostimulant exposure induces neuroadaptive changes in the brain RAS, highlighting the AT_1-R role [18,28–30].

Hydro-Electrolytic Homeostasis

The prior exposure to Amph significantly altered the hydro-electrolytic response to ANG II icv administration, observed in modified sodium intake, natriuresis, and kaliuresis, mediated by the AT_1-R. It is well-documented that ANG II, administered icv at the dosage used in this study, elicits a marked increase in sodium intake, natriuresis, and kaliuresis [12,15,16,31,32]. Consistent with prior findings, our study demonstrated a comparable response in control animals receiving either ANG II or saline. Interestingly, after the ANG II icv challenge, the animals exposed to Amph showed a reduced sodium intake alongside increased levels of natriuresis and kaliuresis compared with the control group. These altered responses are dependent on AT_1-R as were prevented by the AT_1-R antagonism. In a previous re-

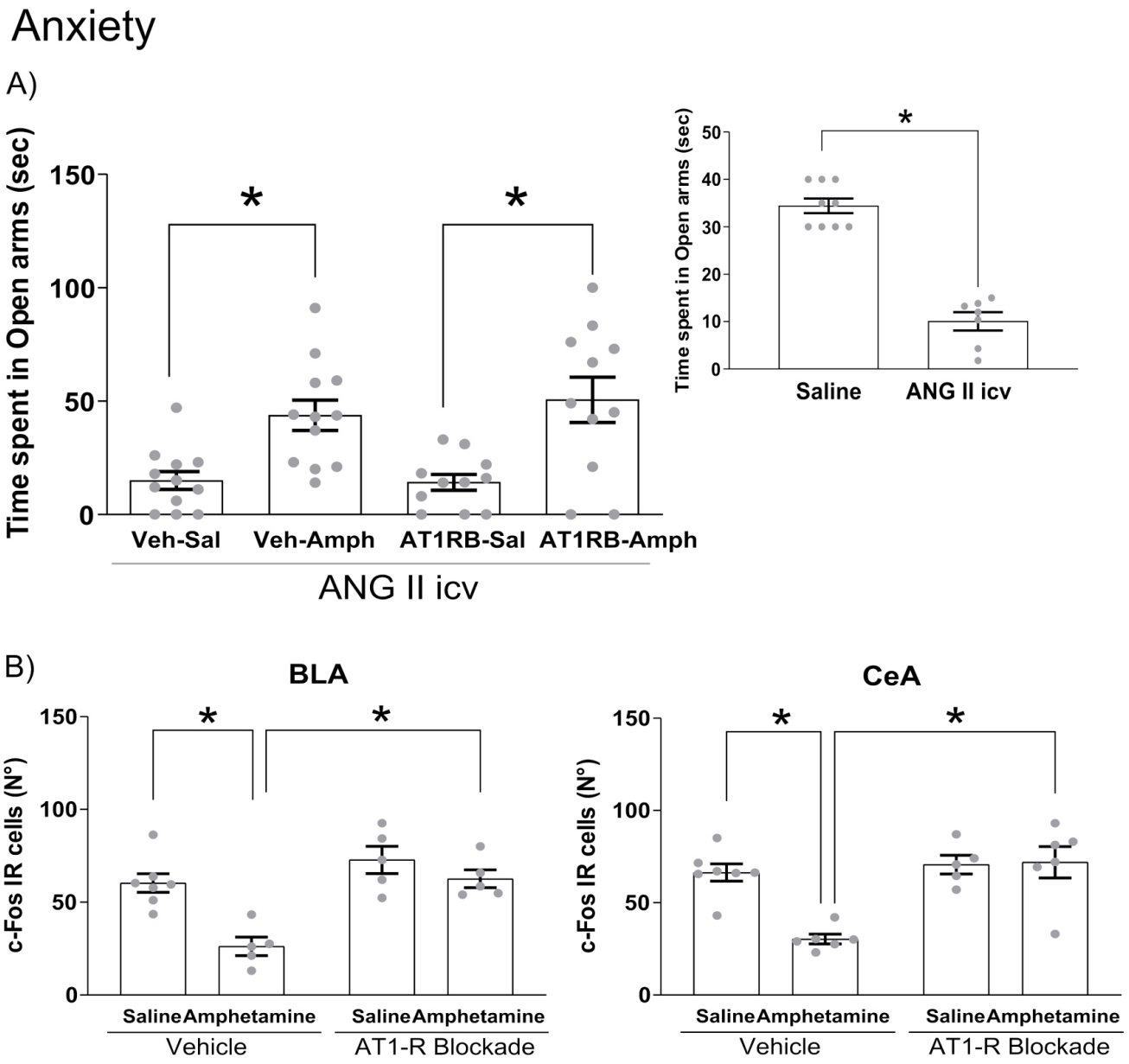

Fig. 5. Amph-administration blunted the ANG II icv anxiogenic effect independently of the AT_1-R activation. The graph shows the anxiety-related behavioral and neuronal activation responses to a challenge of ANG II icv administration, in the four experimental groups. (A) Time spent in the open arms in the elevated plus maze test. (A-inset) Time spent in the open arms in the elevated plus maze test after an acute ANG II icv administration. (B) Neuronal activation is observed as c-Fos IR in the central amygdala (CeA) and basolateral amygdala (BLA). AT_1RB, AT_1-R blockade. The values are presented as mean ± SEM, $*p < 0.05$. N = 11–13 for the behavioral test. N = 5–7 for immunohistochemistry.

port, we showed that the history of Amph exposure induced a similar desensitized response to ANG II icv, observed in a shorter withdrawal of this psychostimulant (7 days) [18]. One plausible explanation for the observed altered response in Amph-exposed animals involves the desensitization and internalization of AT_1-R. Numerous studies have demonstrated that persistent or repetitive stimulation by ANG II induces internalization of these receptors decreasing the response to ANG II [33–36].

Broad evidence has shown that the renin angiotensin-system (RAS) system is involved in osmoregulation and the AT_1-R is expressed in related-brain areas including SFO, NTS, and PVN [37,38]. In this regard, sodium depletion via stimulation of the BRAS was found to increase c-Fos expression in the SFO and PVN in rats, correlating with an increase in sodium intake [39,40]. Also, an injection of ANG II icv induces c-Fos IR in SFO and PVN and increases water intake in mice [41]. In line with the above results, we observed the classical increase of water and sodium intake

after ANG II icv administration in control animals. However, animals previously exposed to Amph showed a desensitized response to sodium intake as they did not show an exacerbated response to ANG II injection. This behavioral change was accompanied by a decreased c-Fos expression in NTS and PVN in the Amph-exposed animal. The alterations induced by Amph in sodium intake and c-Fos expression in NTS and PVN are dependent on AT_1-R as previous administration of candesartan prevented these alterations. Interestingly, the ANG II icv injection induced an increase in water intake independently of Amph or AT_1-R Blocker exposure as no differences in water intake were found among groups. This finding is supported by the absence of observed changes in the SFO, a brain region intricately associated with water intake rather than sodium consumption [32].

Working Memory

Extensive evidence suggests that hyperactivation of the BRAS has a significant impact on memory [42–44]. Indeed we observed that the dose of 400 pmol of ANG II icv induces impairment in working memory, evidenced by an altered exploration pattern in the Hole Board test. Furthermore, we found that animals previously exposed to Amph did not exhibit this memory impairment in response to ANG II icv. The lack of response in the Amph-exposed animals can be explained by desensitization/inactivation of AT_1-R as a result of overstimulation induced by repeated exposure to the psychostimulant. Moreover, this effect was prevented by the AT_1-R blocker administration. In line with these results, we previously reported that the repeated Amph exposure induced long-term attention deficit, which was reversed by the administration of an Amph challenge dose. Also, in that case, the AT_1-R blockade prevented all these changes [23]. Regarding the neuronal adaptations, it has been described that c-Fos expression in the cerebral cortex and the CA3 region of the dorsal hippocampus increases during spatial learning in the radial arm maze, and this specific increase in the hippocampal region is necessary for the formation of spatial memory [45]. This background supports the results obtained in the present study, where the impairment in working memory induced by ANG II icv is correlated with a decreased c-Fos expression in the CA1, CA3, and dentate gyrus regions of the hippocampus. In contrast, previous Amph administration prevented not only the expression of the ANG II-cognitive deficit but also the altered neuronal activation in the analyzed hippocampal areas. As the behavioral output, the neuronal activation alterations produced by exposure to the psychostimulant were prevented with AT_1-R blocker pretreatment. This phenomenon reinforces the theory of desensitization/inactivation of AT_1-R induced by repeated exposure to Amph.

Anxiety

The elevated plus maze test is a validated widely used paradigm for anxiety from a behavioral and pharmacological perspective [24,46]. It is well-known that ANG II has anxiogenic effects while the AT_1-R antagonists, such as candesartan and losartan, have anxiolytic effects [47]. In this sense, as was expected, ANG II icv administration induced anxiogenic effects. However, the groups previously exposed to Amph spent more time inside open arms despite the ANG II icv administration. Regarding neuronal activation, it has been observed that rats evaluated in the elevated plus maze test show increased c-Fos IR in the central amygdala, PVN, and LC, among other areas [48–50]. These brain areas also express c-Fos when animals are exposed to other anxiogenic stimuli (electric shock, predator odor, etc.) [51–54]. Our results showed that ANG II icv induces increased c-Fos expression in the central and basolateral amygdala, concurrent with a significant anxiogenic effect. Remarkably, the previous repeated exposure to Amph blunted the anxiogenic effect of icv ANG II and the increased c-Fos IR in the central and basolateral amygdala, and PVN. These results could be also explained by the AT_1-R desensitization/inactivation since the AT_1-R blocker administration blunted the decreased c-Fos expression after Amph exposure. However, the Amph administration prevented the anxiogenic response to ANG II icv independently of the AT_1-R blockade, suggesting a more complex neuroadaptation induced by Amph. Moreover, it has been suggested that the anxiolytic effect of the AT_1-R blockers is associated with an up-regulation of AT2 receptors in the brain [55]. It is possible to speculate that the lack of anxiogenic effect in response to ANG II icv could be relayed to the AT2 receptors stimulation instead of AT_1-R which seems to be unresponsive after Amph exposure.

Conclusion

All the evidence in this work shows that ANG II icv unmasks the neuroadaptations induced by Amph in brain AT_1-R, highlighting the crucial role of this receptor in the endurable sequels of Amph beyond the catecholaminergic system.

Availability of Data and Materials

The authors confirm that all data underlying the findings are fully available at https://rdu.unc.edu.ar/handle/11086/12863, National University of Córdoba.

Author Contributions

BSC: conceptualization, methodology, and performing the experiments. VBO: conceptualization, data curation, and formal analysis. OMB: formal and statistical analyses. MJP: investigation, validation, and writing. GB: methodology and validation. CB: methodology, vi-

sualization, supervision. VBO, MJP, and OMB wrote the manuscript, and CB revised it. All authors were involved in the drafting and critical revision of the manuscript. All authors have read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All procedures were performed by a qualified technician and approved by the Animal Care and Use Committee of the Faculty of Chemical Sciences, National University of Córdoba (Res. No. 270/18 and Res. No 1843/24), Argentina, in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, 1996.

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Conflict of Interest

The authors declare no conflict of interest.

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