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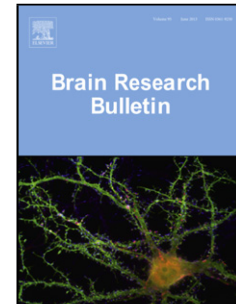

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Involvement of the brain Renin-Angiotensin System (RAS) in the neuroadaptive responses induced by amphetamine in a two-injection protocol

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*Equal contribution

Running title: Brain RAS and amphetamine induced-neuroadaptation

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ABSTRACT

A single or repeated exposure to psychostimulants induces long-lasting neuroadaptative changes. Different neurotransmitter systems are involved in these responses including the neuropeptide angiotensin II. Our study tested the hypothesis that the neuroadaptative changes induced by amphetamine produce alterations in brain RAS components that are involved in the expression of the locomotor sensitization to the psychostimulant drug. Wistar male rats, pretreated with amphetamine were used 7 or 21 days later to study AT_1 receptors by immunohistochemistry and western blot and also angiotensinogen mRNA and protein in caudate putamen and nucleus accumbens. A second group of animals was used to explore the possible role of Ang II AT_1 receptors in the expression of behavioral sensitization. In these animals treated in the same way, bearing intra-cerebral cannula, the locomotor activity was tested 21 days later, after an amphetamine challenge injection and the animals received an AT_1 blocker, losartan, or saline 5 min before the amphetamine challenge. An increase of AT_1 receptor density induced by amphetamine was found in both studied areas and a decrease in angiotensinogen mRNA and protein only in CPu at 21 days after treatment; meanwhile, no changes were established in NAcc. Finally, the increased locomotor activity induced by amphetamine challenge was blunted by losartan administration in CPu. No differences were detected in the behavioral sensitization when the AT_1 blocker was injected in NAcc. Our results support the hypothesis of a key role of brain RAS in the neuroadaptative changes induced by amphetamine.

Key words: angiotensin II, behavioral sensitization, amphetamine, AT_1 receptors, angiotensinogen

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1. INTRODUCTION

The role of brain Angiotensin II (Ang II) is complex and is related with control of the autonomic, hormonal system, sensorial and cognitive processes including regulation of cerebral blood flow [1]. Ang II exerts its principal known actions acting through the AT_1 receptor. In this sense, the AT_1 modulatory action on noradrenergic [2], serotoninergic [3], glutamatergic and gabaergic neurotransmission [4, 5] has been described. In relation to glutamatergic neurotransmission, it has been described that Ang II modulates the neuronal response to glutamate via both AT_1 and AT_2 receptors possibly at postsynaptic level in the superior colliculus, locus coeruleus and dorsal lateral nucleus among other areas [6-8]. In respect to gabaergic neurotransmission a broad range of data supports the view that neuronal angiotensin peptides can modulate the inhibitory responses to GABA and vice versa. Thus, GABA system exerts an inhibitory control on specific neuronal pathways integrated in various central effects of Ang II such as blood pressure responses, drinking and release of vasopressin [9].

Brain Ang II was found to regulate responses induced by drugs of abuse such as cocaine and amphetamine (Amph), among others [10-13]. There is a large body of evidence to support the concept of a relationship between brain Ang II and catecholamine systems [14, 15]. This interaction may participate in some central actions of Ang II such as cardiovascular control, dipsogenesis, and complex behaviors. This observation suggests that drugs which modulate central Ang II may be useful in regulating central dopamine (DA) activity. The presence of Ang II AT_1 receptors has been described in pre- and postsynaptic caudate putamen (CPu) dopaminergic neurons [16], which are involved in the motor and behavioral responses induced by psychostimulants. There is functional evidence that the RAS is involved in modulating DA neurotransmission by increasing DA release in the CPu, an effect mediated by AT_1 receptors [16]. These functional interactions correlate well with anatomical findings that demonstrate high AT_1 receptor density in DA-rich regions, in CPu, hypothalamus, nucleus accumbens (NAcc), ventral pallidum [17, 18]. It was also published that Ang II enhanced the stereotypy induced by apomorphine (DA receptor agonist), and this response was blocked by Ang II AT_1 receptor antagonists [19].

The enhanced response to psychostimulants, a phenomenon termed behavioral sensitization, relies on time-dependent neuroplastic changes in the brain circuitry involved in motivational behavior [20, 21]. These changes are associated with long-lasting hyperactivity of the mesolimbic dopaminergic pathway [22, 23]. The evidence indicates that exposure to a drug of abuse is not needed to be repeated to induce locomotor sensitization; thus, studies in mice and rats showed that a single exposure to psychostimulants

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(Amph or Cocaine) induces behavioral sensitization [24, 25]. The sensitization process encompasses two temporally distinct phases: induction and expression [22, 26]. Neuroadaptive changes in mesotelencephalic dopaminergic projections play a key role in the induction and expression of Amph sensitization. Sensitization can be induced by microinjection of Amph into the ventral tegmental area; meanwhile, its expression is associated with time-dependent adaptations in forebrain DA-innervated areas such as the NAcc and CPu.

In the two-injection protocol, the changes in responsiveness induced by the first psychostimulant administration are revealed by the second administration. Many of the responses induced by repeated exposure to psychostimulants can be achieved by a single exposure [27]; like the increase in extracellular DA [11, 25, 28], activation of NMDA and D_1 receptors, regulation of cAMP-dependent and ERK pathways, among others [29, 30]. For this reason this protocol is useful in order to study the long lasting effects of drugs of abuse allowing to distinguish more precisely the two events in the sensitization phenomenon: induction and expression. However, there is no deep study as regards the two-injection protocol since there is no enough evidence that make it comparable to the repeated administration paradigms.

Our previous findings showed the involvement of the Ang II AT_1 receptors in the development of behavioral and neurochemical sensitization induced by a single exposure to Amph [11, 13]. Based on these results, the following question arose: if this psychostimulant is able to induce long-term changes in brain RAS components and the role of the AT_1 receptors in the expression of behavioral sensitization.

In this study, the determinations were done 7 and 21 days after Amph administration considering our previous results and other authors´ results. The behavioral test was performed after 21 days of psychostimulant injection when the expression of behavioral sensitization was higher [11, 13, 25].

2. EXPERIMENTAL PROCEDURES

2.1 Animals

Adult male Wistar rats (250–330 g) from our own colony (*Facultad de Ciencias Químicas Universidad Nacional de Córdoba, Argentina*) were used. The rats 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. The animals were randomly housed in groups of four per cage 7 days before treatment.

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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.*

2.2 Drugs

D-amphetamine sulphate (Amph, Sigma Chemical Co.) and the AT_1 receptor antagonist Losartan (Los, Sigma-Aldrich) each dissolved in 0.9% saline. The doses were calculated on the basis of the weight of the salt of each drug and each dose was chosen considering previous work [11, 25, 31].

2.3 Experimental Procedures

2.3.1 Experiment 1

The experiment was carried out to study the long-lasting effects of Amph exposure on brain RAS components in a two-injection protocol. For this purpose, a total of 63 animals were randomly divided into two groups and administered once with Amph (5 mg/kg, i.p.) or saline and immediately returned to their home cage. They were left undisturbed until the day of the experiment. Seven or 21 days after pretreatment, the Amph-induced changes were evaluated in both groups by immunohistochemistry, western blotting and PCR analysis (Fig. 1A).

2.3.2 Experiment 2

The experiment was carried out to study the functional role for Ang II AT_1 receptors in the expression of Amph-induced locomotor sensitization in a two-injection protocol. For this purpose a total of 79 animals were randomly divided into two groups and administered once with Amph (5 mg/kg, i.p.) or saline. They were left undisturbed in their home cages and 1 week before the experiments, the animals were implanted with an intracerebral cannulae. Twenty-one days after pretreatment, the locomotor activity was evaluated following a challenge injection of Amph (0.5 mg/kg, i.p.), Fig. 1B. Five minutes before the challenge injection, the animals received a bilateral cerebral microinjection in CPu or NAcc of Los (two doses were evaluated: 4 and 8 μg/side, volume of infusion 0.5 μl) [31].

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2.4 Immunohistochemistry for AT_1 receptors

The animals were anesthetized with chloral hydrate 16% (400 mg/kg, i.p.) and perfused transcardially with 250 mL of saline and heparine (200 µL) followed by 400 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed and post fixed in the same fixative overnight at 4 ºC. They were then placed in 30% sucrose in PBS until the brain settled. After this, the brains were sectioned in a cryostat (Leica 1510S) into 20-µm-thick coronal slices and were then used for immunohistochemistry for AT_1 receptors. Section samples were taken from CPu (bregma 2.16 mm) and NAcc (bregma 2.16 mm) according Paxinos & Watson Atlas [32]. In order to inhibit endogenous peroxidase activity, they were placed in a mixture of 10% H_2O_2 and 10% methanol until oxygen bubbles ceased appearing. Blocking of nonspecific binding sites was performed with 10% normal horse serum (NHS) (GIBCO, Auckland, NZ) in 0.1 M Buffer Phosphate (PB). Sections were then incubated overnight, at room temperature, in a mouse monoclonal anti-AT_1 receptor antibody [33], diluted 1:10000 in PB containing 2% NHS and 0.3% Triton X-100 (Flucka Analytical). Followed by incubation with biotin-SP-conjugated Donkey anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, INC), diluted 1:5000 in 2% NHS-PB and the avidin-biotin-peroxidase complex (ABC- Vector Laboratories, Burlingame, CA), diluted 1:200 in 2% NHS-PB, 2 h at room temperature each solution. The peroxidase label was detected with diaminobenzidine hydrochloride (Sigma Chemical Co.); the solution was intensified with 1% cobalt chloride and 1% nickel ammonium sulfate. This method produces a blue-black reaction product. Finally, the free-floating sections were mounted on gelatinized slides, air dried overnight, dehydrated, cleared in xylene and placed under a coverslip with DPX mountant for histology (Flucka Analytical).

The tissue was visualized using a Leica Microscope (DM 4000B). Representative images of at least 5 animals per group were captured with a Leica DFC digital camera. Two slices containing CPu and NAcc were analyzed in both hemispheres for positive immunostaining for AT_1 receptors in a double blind determination. The positive AT_1 immunostaining shows a characteristic diffuse pattern on the tissue.

2.5 Tissue Preparation for WB and PCR.

Animals were decapitated and their brains were quickly removed and placed in an acrylic brain matrix (Stoelting CO., USA) on ice. Coronal brain slices of 2 mm containing CPu and NAcc (bregma: 2.16 mm) were isolated according to Paxinos & Watson Atlas [32] and the areas of interest were bilaterally

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dissected. Each sample consisted of two pooled rat brains. Each structure was weighted and homogenized in 500 µl of TRizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Aqueous and organic phases were separated by addition of chloroform (0.2 mL/ mL of inicial TRizol) followed by centrifugation (12000 xg for 15 min). RNA for PCR was first isolated from the aqueous phase and the organic one was stored at -70 ºC for later protein fraction isolation.

2.6 Western blotting analysis for AT_1 receptors and Angiotensinogen (AOGEN).

2.6.1 Isolation of protein fraction. First DNA was precipitated by ethanol 100% (0.3 mL/ mL TRizol). Proteins were obtained from the supernatant portion by later addition of isopropyl alcohol (1.5 mL/ mL TRizol) and centrifugation at 12000 xg for 10 min. The protein containing pellet was rinsed three times with guanidine hydrochloride (0.3 M in ethanol 95% - 2 mL/ mL TRizol), each one for 5 min centrifugation at 7500 xg. After 30 min incubation with ethanol 100 % (2 mL/pellet) the samples were centrifuged at 7500 xg for 5 min and the proteins precipitated were dried with a vacuum bell. Finally, the proteins were taken to solution with SDS 1% at 50 ºC in a thermostatic bath. After protein concentration determination by Bradford procedure (Bio-Rad Laboratories, Inc., Hercules, CA) the samples were stored at -20 ºC.

2.6.2 Protein quantification. Proteins samples (40µg/line) were separated by 10% SDS-PAGE and transferred to a PVDF membrane (Amersham Hybond- P, GE Healthcare). After blocking with 5% BSA diluted in TTBS buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween) for 1 hour, at room temperature, the membranes were incubated with an appropriate amount of the primary antibody mouse monoclonal anti-AT_1 (1:500; Frei et al.) and rabbit monoclonal anti-AOGEN (1:1500, EPR2931, Abcam) in TTBS, at 4 ºC overnight. After washing the membranes with TTBS for 1 hour, they were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:2000, Jackson) or goat anti-rabbit IgG (1:2500, Millipore) in the same buffer solution for 1 hour at room temperature. After washing for 1 hour, proteins were visualized with X-ray films (Kodak) using a chemiluminescence system (ECL, Amersham Pharmacia Biotech). The resulting film samples were scanned and analyzed with an image analysis program (GelPro32 Analyzer). The amount of each protein was calculated normalizing values of O.D. with values of tubulin control.

2.7 PCR for angiotensinogen

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2.7.1 Isolation of total RNA. For each experimental group, the RNA isolation was performed in triplicate. Following the separation of the aqueous phase, the RNA was precipitated using isopropyl alcohol and then centrifuged at 12000 xg for 10 min. Extracts were diluted 1:100 with nuclease-free water (0.1% diethylpyrocarbonate) and RNA concentration determined by absorption at 260 nm. The total RNAs were treated with TURBO DNA-free kit to remove contaminating DNA.

2.7.2 Expression of mRNA level. Isolation of total RNA from tissue pools and first-strand cDNA synthesis was performed and subjected to RT-PCR analysis. First-strand cDNA synthesis was performed with 1 μL of Oligo-dT20 (50 μM) (Invitrogen), 3 μg of total RNA and 300 U of SuperScript III-RT (Invitrogen) in a 20 μL reaction volume that was incubated at 55 °C for 1 h. Subsequent PCRs used 1 μL of first-strand cDNA as template, 0.2 μM of each specific primer and GADPH primers as positive controls, 0.5 U of Taq Platinum DNA polymerase (Invitrogen), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 2.5 μL of 10X PCR Buffer minus M (Invitrogen) in a 25 μL reaction. The PCR was performed by using a thermocycler Mycycler (Bio-Rad) with an initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 seconds, 58 °C for 1.0 minute, 72 °C for 40 seconds and a final incubation at 72 °C for 10 min. Specific primers were sense: 5′-CATGAGTTCTGGGTGGACAA-3′and antisense: 5′-AAGTTGTTCTGGGCGTCACT-3′. The GADPH–specific universal primer pair was used. The RT-PCR products (10 μL) were separated by electrophoresis on a 10-cm 1.5% agarose gel (Tris-acetate EDTA buffer, pH 8) containing 0.5 μg/mL of ethidium bromide. Digital images were obtained under ultraviolet illumination with a Chemi Doc System (Bio-Rad). Semi-quantification of PCR bands was performed with the IMAGE J Launcher through a graphical method that measures peak areas.

2.8 Surgery for cannulae implantation

Animals were anesthetized i.p. with ketamine (55 mg/kg, Holliday)/ xylazine (11 mg/kg, Köing). In aseptic conditions, rats' skull were exposed and using a stereotaxic device (Stoelting) were implanted with bilateral stainless-steel cannulae (22 gauge) fixed with dental cement (Subiton, Argentina). Also one stainless-steel screw was anchored to the skull. Cannulae were placed 2 mm above the final place of injection. According to Paxinos & Watson Atlas [32] coordinates in respect to bregma were: **CPu:** AP = +2.16 mm; L = ±1.6 mm; DV = –2.5 mm. **NAcc:** AP = +2.16 mm; L = ±1.5 mm; DV = –4.5 mm. Immediately after surgery and on the next day, the animals received Norciciline (5000000 U.I., i.p. Laboratorios NORT) and were maintained undisturbed during a week for recovery.

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2.9 Intracerebral infusion of Losartan

The day of the experiment, animals were injected with Los or saline inserting a stainless steel injection cannula (30 gauges) into the guide cannula. This cannula was attached through a polyethylene catheter (P10) to a 10 µl microsyringe (Hamilton). Volumes of 0.5 µl of saline or Los solution were gradually injected over 1 minute periods into left and right sides using an infusion bomb (HARVARD, model 22). The injection cannula was left in place for an additional 30 sec. to allow complete liquid diffusion.

2.10 Locomotor activity

The testing apparatus consisted of rectangular cages (30.5 x 19.5 x 46.5 cm^3) equipped with two parallel infrared photocell beams located 3 cm above the floor. Interruption of either beam resulted in a photocell count. The testing apparatus was placed in a different room from the one where the animals were housed. All the rats were tested once between 09 a.m. and 06 p.m. under white light in a quiet room. They were placed individually in the testing apparatus for a 2-hour habituation period before being injected with saline and tested for 1 hour. Finally, they were injected with Amph (0.5 mg/kg i.p), 5 minutes after intracerebral infusion of Los or saline. Motor activity counts were monitored at 10-minutes intervals for 2 hours following the injection.

2.11 Histology

After locomotor activity was recorded, animals were euthanatized by an overdose of chloral hydrate 16%; their brains were removed and immersion-fixed in a 4% formol solution for one week. Coronal sections of 60 µm were obtained using a cryostat (Leica CM1510S) and analyzed under magnifying glass in order to confirm the guide cannula location. Only animals with correct cannula position were considered for statistical analysis.

2.12 Statistical analysis

Experimental data were reported as means ± SEM. Molecular experiments were analyzed by one-way ANOVA, followed by post hoc analysis Newman-Keuls multiple comparison test. For the locomotor activity 10-min block analysis, the study design used three-way ANOVA considering the following factors: Drug (saline/ Amph), treatment (saline/ Los) and time (analyzed as a repeated measure). If an interaction and/or main effect were observed, pair-wise comparisons following ANOVA were made

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using Bonferroni post test. The total photocell counts during the 2 hours after Amph challenge were analyzed by two-way ANOVA using the Bonferroni post test with Amph and saline as treatment factor and Los and saline as drug factor. A value of $p < 0.05$ was considered significant.

3. RESULTS

3.1 Localization and quantification of AT_1 receptors

Representative microphotographs exhibited a great increase of AT_1 receptors immunostaining induced by Amph pretreatment in both studied areas, CPu (Fig. 2A) and NAcc (Fig. 2C), 7 and 21 days after the pretreatment.

The increase of AT_1 receptors was corroborated using the western blot technique (Fig. 2B and 2D). The one-way ANOVA of CPu data showed a significant effect of the treatment $F_{(2, 15)} = 8.60$, $p < 0.05$. Bonferroni *post hoc* comparisons indicated significant differences between animals treated with Amph, 7 ($p < 0.05$) or 21 ($p < 0.01$) days before and the control animals (Fig. 2B).

The one-way ANOVA of NAcc data revealed a significant effect of the treatment $F_{(2, 14)} = 12.75$, $p<0.001$. Bonferroni *post hoc* comparisons noted significant differences between animals treated with Amph, 7 ($p < 0.05$) or 21 ($p < 0.01$) days before and the control animals (Fig. 2D).

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3.2 Expression and quantification of angiotensinogen.

The expression of the mRNA and the protein for the precursor of Ang II, AOGEN, were studied. The results showed a decrease in the expression of the mRNA of AOGEN in CPu 21 days after Amph pretreatment (Fig. 3A). The one-way ANOVA indicated a significant effect of treatment in CPu, $F_{(2, 24)} = 6.88$, $p < 0.05$. Bonferroni *post hoc* indicated significant differences between Amph pretreated animals 21 days before and the control animals ($p < 0.01$). No significant differences were observed in CPu of animals pretreated with Amph 7 days before vs. saline group (Fig. 3A), neither in NAcc of animals pretreated with Amph at none of the studied times respect to control group (Fig. 3C).

The results of the western blot analysis also showed a decrease in the protein density of AOGEN in CPu, induced by Amph 21 days before (Fig. 3B). The one-way ANOVA revealed a significant effect of treatment in CPu, $F_{(2, 15)} = 4.04$, $p < 0.05$. Bonferroni *post hoc* indicated significant differences between Amph pretreated animals 21 days before and the control animals ($p < 0.05$). No significant differences

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were observed in CPu of animals pretreated with Amph 7 days before vs. saline group (Fig. 3B), or in NAcc of animals pretreated with Amph at any of the times with respect to control group (Fig. 3D).

3.3 Expression of amphetamine-induced locomotor sensitization

Fig. 4 and 5 shows the register of locomotor activity after a challenge injection of Amph (0.5 mg/kg, i.p.) of animals pretreated with Amph 21 days before, which received AT_1 receptor blocker, Los (8 µg/µL), in CPu or NAcc, 5 minutes before the challenge injection of Amph. In another group of animals, a lower dose of Los (4 µg/µL) was tested and no significant differences were found between the groups (data not shown).

The results show that the increase of Amph-induced locomotor activity was blunted by AT_1 receptors blockade in CPu. No effects in the expression of Amph-induced sensitization were observed by blocking AT_1 receptors in NAcc.

The sal-sal, sal-Los, Amph-sal, Amph-Los groups were statistically analyzed together, but are shown in two graphs for better visualization. See below the statistical data analysis of each studied areas.

3.3.1 CPu: Three-way ANOVA analysis after an Amph challenge revealed a significant effect of drug (sal/Amph) $F_{(1, 26)} = 10.49$ $p < 0.01$, treatment (sal/Los) $F_{(1, 26)} = 7.64$ $p < 0.05$, time $F_{(11, 286)} = 12.46$ $p < 0.0001$ (analyzed as a repeated measure), interaction drug*treatment $F_{(1, 26)} = 8.67$ $p < 0.01$ and interaction between the three factors (drug*treatment*time) $F_{(11, 286)} = 2.36$ $p < 0.01$. No significant effect was observed for interaction time*drug $F_{(11, 286)} = 1.29$ $p = 0.22$ and interaction time*treatment $F_{(11, 286)} = 1.65$ $p = 0.085$. Bonferroni *post hoc* comparisons on the triple interaction indicated that photocell counts at 90, 100 and 110 min in Amph-sal group were significantly different from sal-sal and sal-Los groups ($p < 0.05$). In addition, Bonferroni *post hoc* comparisons indicated that photocell counts at 90, 100 and 110 min in Amph-sal group were significantly higher than Amph-Los group ($p < 0.05$) (Fig. 4B-C). No significant differences were found between sal-sal and sal-Los groups (Fig. 4B). Three-way ANOVA analysis of saline challenge indicated no significant effect for drug $F_{(1, 26)} = 0.01$ $p = 0.91$ and treatment $F_{(1, 26)} = 0.20$ $p = 0.66$, a significant effect was found for time $F_{(5, 130)} = 5.09$ $p < 0.001$.

When analyzed the total locomotor activity after Amph challenge, it was found that Los was able to blunt the expression of Amph-induced locomotor activity. The results obtained from the two-way ANOVA analysis showed a significant effect of treatment $F_{(1,27)} = 10.26$ $p < 0.001$ and interaction $F_{(1,27)} = 4.65$ $p < 0.05$. Bonferroni *post hoc* indicated that Amph-sal was significantly higher than the control group, sal-sal ($p < 0.01$). The Amph-induced sensitization was significantly decreased in the Amph-Los group

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(Amph-sal vs. Amph-Los, $p < 0.05$). Amph-Los did was not statistically different from sal-Los ($p > 0.05$). No differences were found between control groups (sal-sal vs. sal-los, Fig. 4D).

3.3.1 NAcc: Three-way ANOVA analysis after an Amph challenge revealed a significant effect of drug (sal/Amph) $F_{(1, 31)} = 21.99$ $p < 0.0001$ and time $F_{(11, 341)} = 2.90$ $p < 0.005$ (analyzed as a repeated measure). No significant effect was observed for treatment (sal/Los) $F_{(1, 31)} = 0.002$ $p = 0.96$, interaction drug*treatment $F_{(1, 31)} = 0.89$ $p = 0.35$, time*drug $F_{(11, 341)} = 1.25$ $p = 0.25$, time*treatment $F_{(11, 341)} = 0.75$ $p = 0.69$ and drug*treatment*time $F_{(11, 341)} = 1.67$ $p = 0.08$. Three-way ANOVA analysis of saline challenge indicated no significant effect for drug $F_{(1, 31)} = 3.15$ $p = 0.08$ and treatment $F_{(1, 31)} = 3.84$ $p = 0.06$, a significant effect was found for time $F_{(5, 155)} = 2.39$ $p < 0.05$ (Fig. 5B-C).

When analyzed the total locomotor activity after Amph challenge, it was found that Los was able to blunt the expression of Amph-induced locomotor activity. The results obtained from the two-way ANOVA analysis showed a significant effect of drug $F_{(1, 31)} = 21.99$ $p < 0.0001$. No significant effect was found for treatment $F_{(1, 31)} = 0.002$ $p = 0.96$ and interaction $F_{(1, 31)} = 0.89$ $p = 0.35$ (Fig. 5D).

4. DISCUSSION

The main findings of this work are the long-lasting changes in brain RAS components induced by Amph within CPu and NAcc. Moreover, a functional role for the AT_1 receptors in CPu in the expression of behavioral sensitization to Amph was found.

It has been shown that repeated and single Amph administration increases the reactivity of DA release in CPu and NAcc [11, 25]. Evidence suggests that DA and Ang II systems directly counter regulate each other in the striatum and substantia nigra of rodents [34]. In this sense, the depletion of DA with reserpine induced a significant increase in the expression of AT_1 and AT_2 receptors which decreased as the DA function was restored. The same phenomenon on Ang II receptors was observed after the dopaminergic denervation with 6-hydroxydopamine and the administration of L-Dopa decreased the augmented AT_1 and AT_2 receptor density [34]. An up-regulation of Ang II AT_1 receptors in NAcc in mouse induced by chronic haloperidol treatment has been described using in vitro autoradiography [35]. To this respect, Labandeira-Garcia group suggests that the AT_1 receptor expression is closely related to DA levels through direct (DA and AT_1 receptors) and indirect (changes in Ang II levels) mechanisms [36]. Moreover, chronic blockade of AT_1 receptors induced an increase of D_1 together with a decrease in D_2 receptors in CPu [37]. Furthermore, there is evidence that supports the existence of an

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intracellular/intracrine RAS in DA neurons in particular which has also been suggested for several cell types [38, 39].

The increase in AT_1 receptors in CPu and NAcc shown in this study indicate that exposure to Amph is able to induce persistent alterations in a RAS component in brain regions which are target for the actions of drugs of abuse. Based on the evidence describing interactions between DA system and AT_1 receptors, it is possible to suggest that the long-lasting changes induced by Amph exposure could be due to dopaminergic system influences.

There is also evidence showing that changes in Ang II levels may also affect Ang II receptors density. In this sense, transgenic rats with very low levels of brain Ang II showed increased AT_1 receptors expression [34]. In contrast, repeated Ang II i.c.v. administration increased the expression of the AT_1 receptors in different brain areas associated with a decreased response to Ang II [40]. Given the techniques applied in the present study, it is not possible to assure that the augmented AT_1 receptors are functional. Moreover, the staining obtained in the present work has been previously described to be located intracellularly and in the neuron membrane [41].

It is well known that the astroglia is the main site of AOGEN synthesis [42, 43] although it is probably produced at low levels in neurons [44, 45]. In the present study, both studied regions CPu and NAcc presented long-lasting changes in AT_1 receptors density after Amph exposure, but only in CPu a significant decrease in the Ang II precursor, AOGEN, was found. There is evidence supporting a relation between the AT_1 receptors and the Ang II precursor in the brain. To this respect, using two different AT_1 receptor antagonists (losartan and irbesartan), there was a widespread up-regulation of AOGEN mRNA with low doses and down-regulation with higher doses [46]. Other authors found that systemic administration of candesartan, an AT_1 receptor antagonist, decreased Ang II, AOGEN mRNA and angiotensin converting enzyme (ACE) in basal ganglia [47]. Despite the effects of the different receptor antagonists it is clear that manipulations of the AT_1 receptors resulted in changes of AOGEN levels. One possible explanation for our results is that the decrease of AOGEN in CPu is related to an overstimulation of AT_1 receptors, showing that these receptors are functional, in contrast to those in NAcc.

There is evidence showing changes in RAS components in response to ethanol, another drug of abuse. In this sense, ethanol dependent rats showed a decrease on AT_1 receptor density and an increase of AOGEN mRNA expression in medial prefrontal cortex after 7 weeks of alcohol abstinence [48]. Moreover, Maul et al. [49], found an altered DA concentration in ventral tegmental area in transgenic rats expressing a specific AOGEN antisense RNA in the brain (TGR(ASrAOGEN)680). Also, these rats exhibit reduced

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AOGEN levels in the brain, resulting in a reduction in central Ang II formation and increased AT_1 expression, associated with a lower ethanol consumption [48, 49]. Additional studies, using gene microarrays reported an increased AOGEN expression in high ethanol preference rats [50]. These studies further support the relation between brain RAS components and neuroadaptive responses to a drug of abuse as well as a counter regulation between AT_1 receptors and Ang II synthesis.

There is indirect evidence of RAS involvement in neuroadaptative changes induced by psychostimulants. In this sense, sodium depletion which activates RAS and Ang II synthesis was found to develop cross-sensitization effects leading to enhanced locomotor activity responses to Amph [51]. Recently, it was demonstrated in rats that repeated administration of the mineralocorticoid agonist, deoxycorticosterone acetate (DOCA), induced progressive increase in daily hypertonic saline consumption (i.e., sensitization of sodium appetite) and a greater locomotor response to cocaine [52]. These experiments indicate that treatments implying RAS activation show reciprocal behavioral cross-sensitization with psychostimulants.

Direct relationship between RAS and behavioral sensitization was found in our laboratory. It was shown that Ang II AT_1 receptors are involved in the development of behavioral and neurochemical sensitization induced by a single exposure to Amph [11, 13]. Based on these previous results, the following question arose: Do the AT_1 receptors have a functional role in the expression of behavioral sensitization? In this sense, the expression of behavioral sensitization was attenuated by AT_1 receptor blockade only in CPu. The two brain areas evaluated are rich in dopaminergic terminals and are strongly related to psychostimulants sensitized responses. However, there is evidence showing differential DA release in response to electrical stimulation between CPu and NAcc. Moreover, the DA release in the CPu is not regulated by dopamine autoreceptor activation in contrast to NAcc [53]. To this respect, Grace has suggested two mechanisms regulating DA release in the projections of the dopaminergic systems. A phasic release regulated by depolarization of dopaminergic nerve cell bodies and a tonic regulation of DA release independent of electrical activity of these neurons [54]. In this context, it has been suggested that tonic influences may be more significant in the CPu than in the NAcc [53]. This last is in agreement with the evidences showing the tonic influence of Ang II over DA synthesis and release in CPu through AT_1 receptors [16, 55].

With respect to psychostimulant effects, these two brain areas are believed to have different roles in the process of drug abuse. The NAcc has been repeatedly shown to be important for reward and motivation of drug-seeking behavior [56, 57]. This area is hypothesized to be involved in the initial stages of the

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addiction process. The CPu, on the other hand, is seen to be important for habituation and involved in the long-term reinforcement of drug abuse [56]. Bjorklund et al, showed different activities and possibly molecular or regulatory changes in the neuronal DA transporter in CPu versus NAcc accompanying methamphetamine sensitization [58]. However, the localization of AT_1 receptors in DA neurons in CPu and NAcc, and the DA release in both brain areas through AT_1 receptor stimulation have been described. These similarities were observed in basal conditions, but the same studies were not performed after psychostimulants drug exposure or after stress or salt deprivation. In addition, Ang II not only interacts with dopaminergic but also interacts with glutamatergic or GABAergic neurotransmission [59], giving a more complex scenario.

In summary, this is the first study -to our knowledge- showing that the long-lasting changes in brain RAS should be considered among all psychostimulant-induced neuroadaptations previously described. It is important to highlight that pharmacological treatment or environmental conditions altering RAS components could lead to dysfunctional responses to endogenous Ang II. Moreover, these results suggest a functional role for the AT_1 receptors in a brain area strongly related to drug abuse disorders. More studies characterizing the cell type location of AT_1 receptors could help to better understand the brain RAS role in this phenomenon.

AUTHORS DISCLOSURES

This study was supported by grants from CONICET 11220090100852-KB1, SECyT, FONCyT and MINCyT. The authors declare no conflicts of interest.

ACKNOWLEDGEMENTS

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LEGEND TO THE FIGURES

Figure 1. Experimental protocols

A) Experimental design for molecular analyses. **B)** Experimental design for locomotor activity measurements.

Figure 2. Localization and quantification of AT_1 receptors

Microphotographs: representative microphotographs showing AT_1 receptors immunostaining in CPu **(A)** and NAcc **(C)** in basal condition, 7 and 21 days after a single dose of Amph (5 mg/kg i.p.). Upper panel 200x magnification, lower panel 400x magnification, the circles indicate representative positive immunostaining for AT_1 receptors (n= 9-14). **Graphs:** optic density (O.D.) of AT_1/tubulin ratio of CPu **(B)** and NAcc **(D)** samples in basal condition, 7 and 21 days after a single dose of Amph (5 mg/kg i.p.), *p< 0.05 different from saline group, (n= 4-8). **Immunoblots**: representative immunoblots of AT_1 receptors (41 kDa) and tubulin (55 kDa).

Figure 3. Expression and quantification of angiotensinogen

Left panel graphs: optic density (O.D.) of AOGEN/GADPH ratio of CPu **(A)** and NAcc **(C)** samples in basal condition, 7 and 21 days after a single dose of Amph (5 mg/kg i.p.), *p< 0.01 different from saline group, (n= 8-10). **Gels:** Representative gels of amplification products of AOGEN (1389 pb) and the constitutive expression of GADPH (307 pb). **Right panel graphs:** optic density (O.D.) of AOGEN/tubulin ratio of CPu **(B)** and NAcc **(D)** samples in basal condition, 7 and 21 days after a single dose of Amph (5 mg/kg i.p.), *p< 0.05 different from saline group, (n= 4-8). **Immunoblots**: representative immunoblots of AOGEN (55 kDa) and tubulin (55 kDa).

Figure 4. Expression of amphetamine-induced locomotor sensitization. Losartan intra-CPu microinjection.

Locomotor activity recorded in response to an Amph challenge 21 days after Amph administration in sal/Los intra-CPu microinjected animals. Brain coronal section schemes showing representative disposition of cannulae implanted in CPu for microinjection of Los in animals tested for locomotor activity 21 days after the single dose of Amph (closed circle) or saline (open circle) (**A).** Counts per 10 min bin of sal-sal and sal-Los groups (n= 8) (**B).** Counts per 10 min bin of Amph-sal and Amph-Los

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groups, *p< 0.05 (n= 6-8) (**C**). Total counts (2 h) after an Amph challenge, *p< 0.05 different from all groups (**D**).

Figure 5. Expression of amphetamine-induced locomotor sensitization. Losartan intra-NAcc microinjection.

Locomotor activity recorded in response to an Amph challenge 21 days after Amph administration in sal/Los intra-NAcc microinjected animals. Brain coronal section schemes showing representative disposition of cannulae implanted in NAcc for microinjection of Los in animals tested for locomotor activity 21 days after the single dose of Amph (closed circle) or saline (open circle) (**A**). Counts per 10 min bin of sal-sal and sal-Los groups (n= 6-7) (**B**). Counts per 10 min bin of Amph-sal and Amph-Los groups, *p< 0.05 (n= 9-12) (**C**). Total counts (2 h) after an Amph challenge, *p< 0.05 different from all groups (**D**).

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Highlights

1. A single dose of amphetamine increases AT_1 receptors in CPu and NAcc, 7 or 21 days later.
2. A single dose of amphetamine decreases Angiotensinogen in CPu 21 days later.
3. Expression of behavioral sensitization to amphetamine is prevented by AT_1 receptor blockade.

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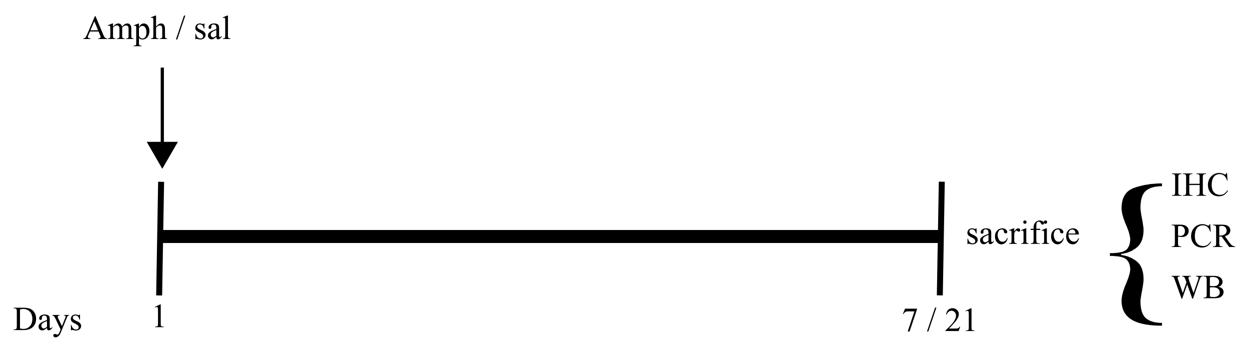
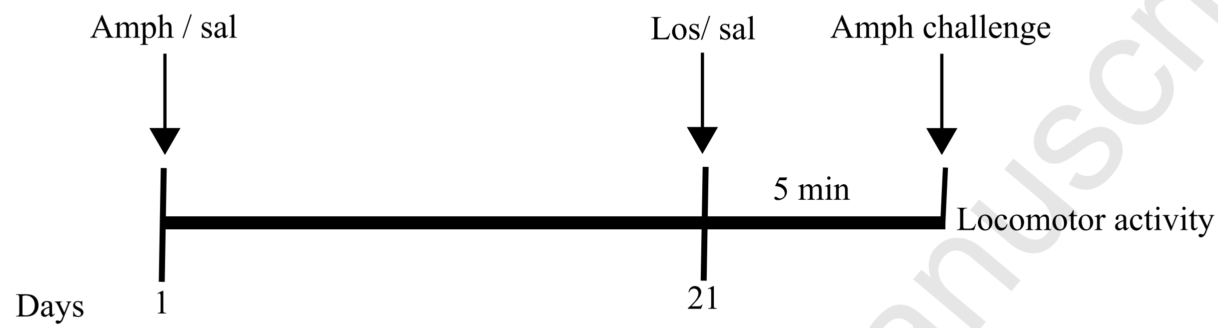
A) Experiment 1



B) Experiment 2



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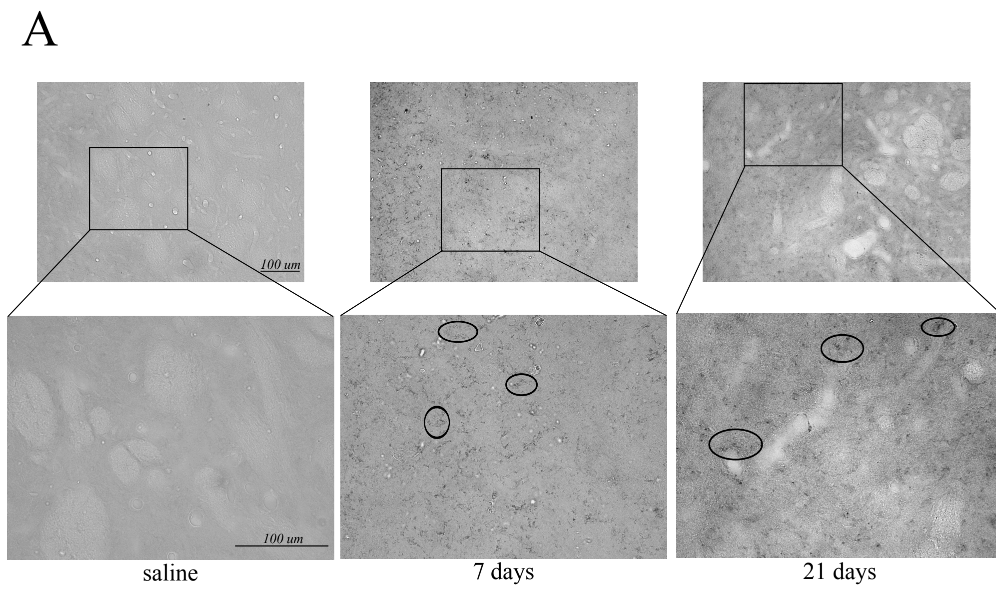

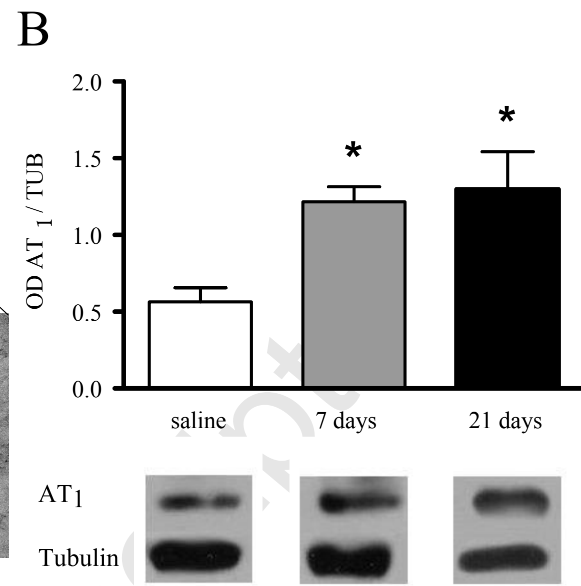

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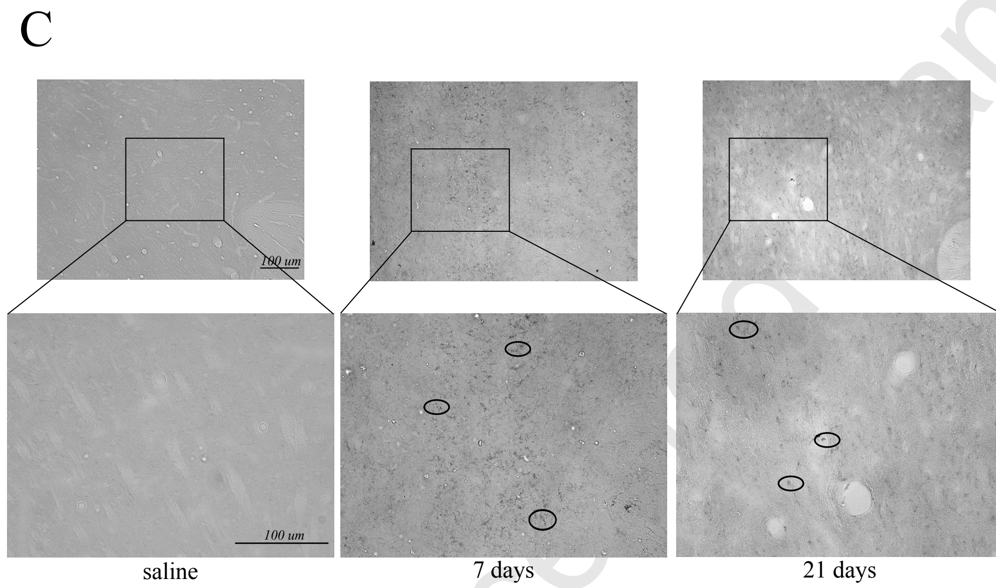

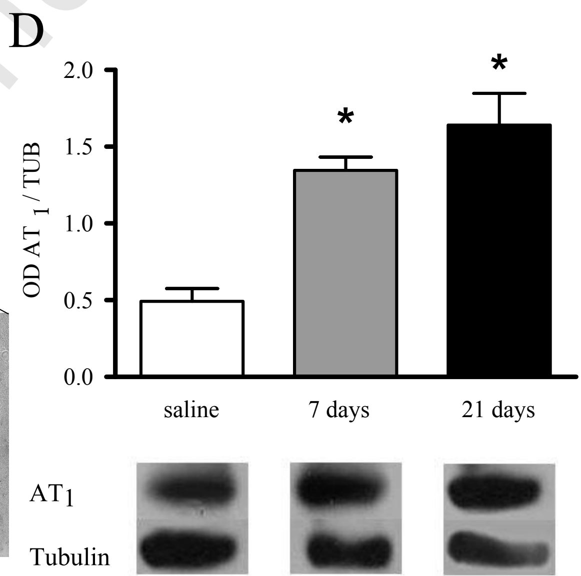

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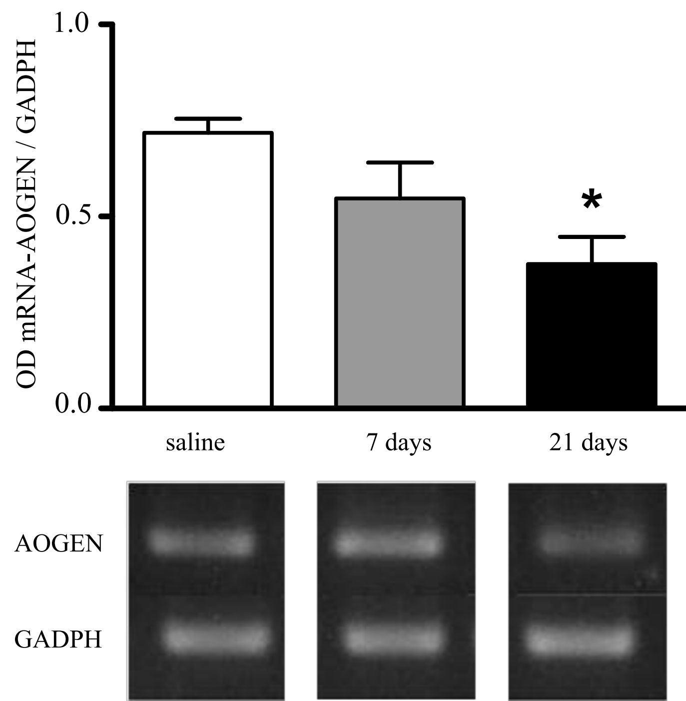

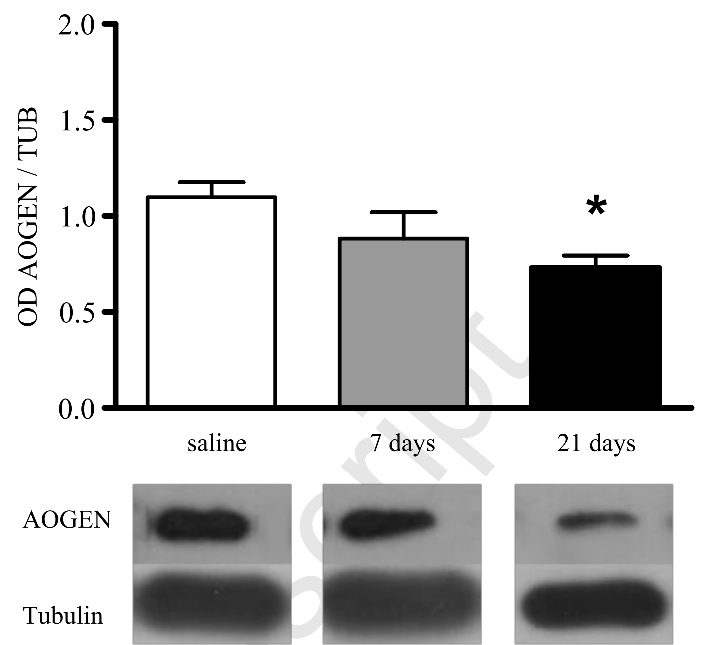

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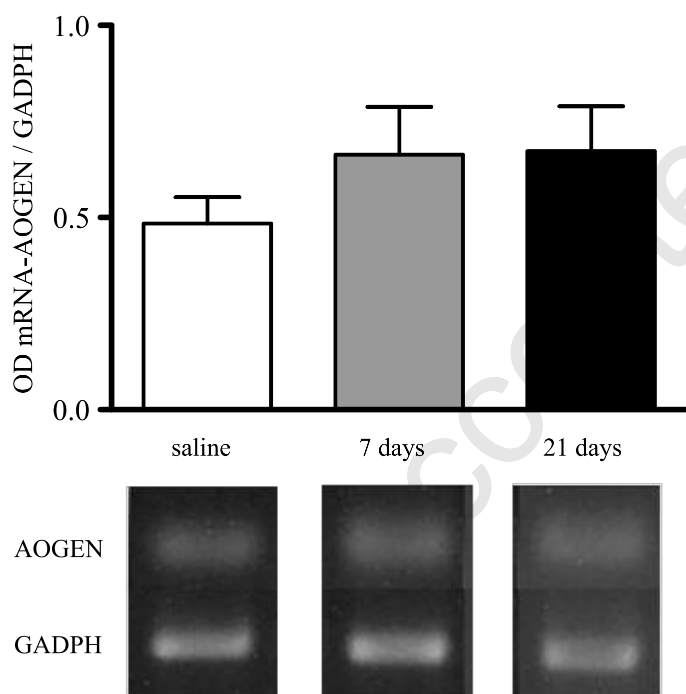

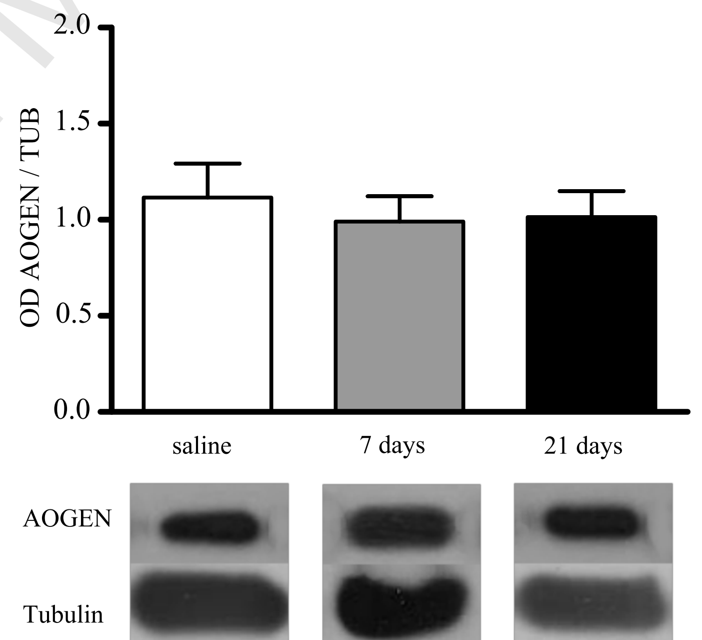

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Figure 4

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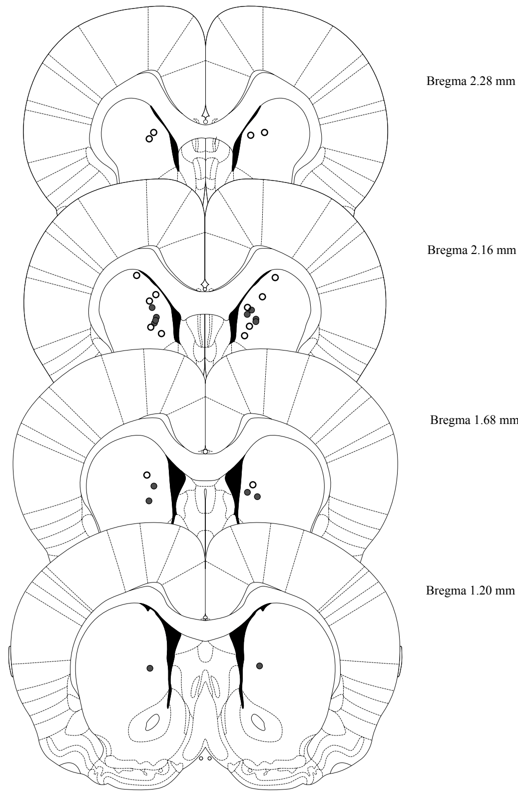
A



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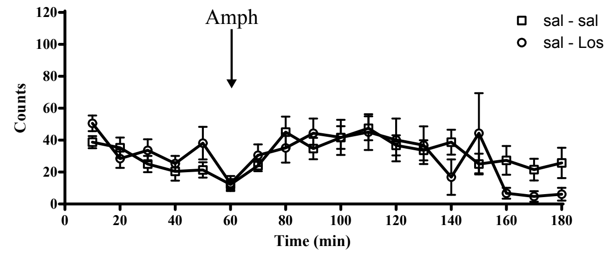

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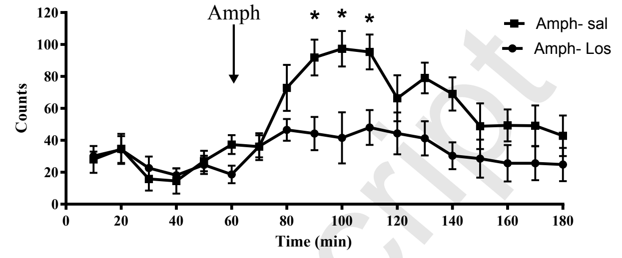

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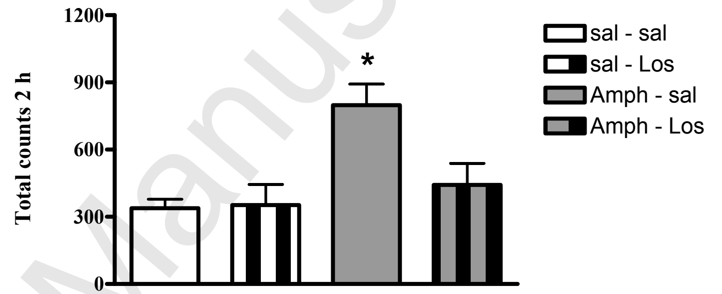

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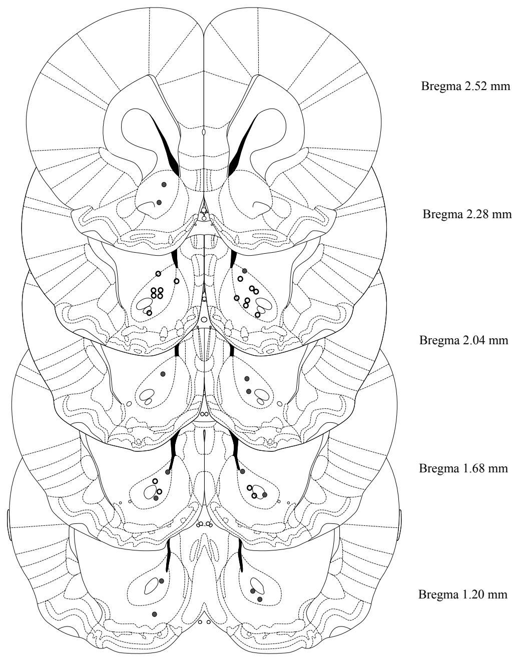
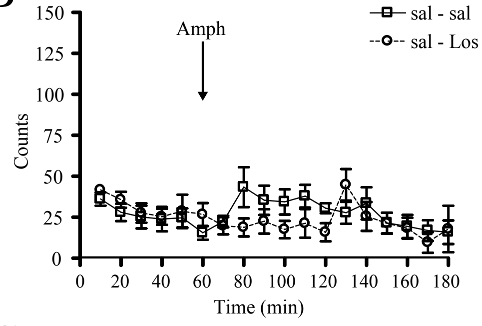
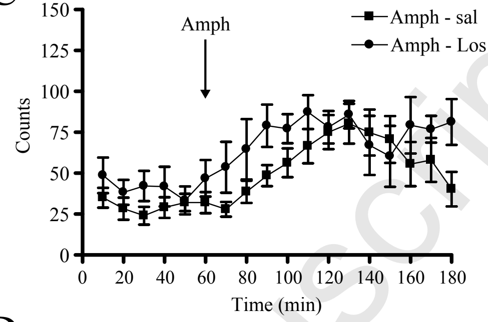
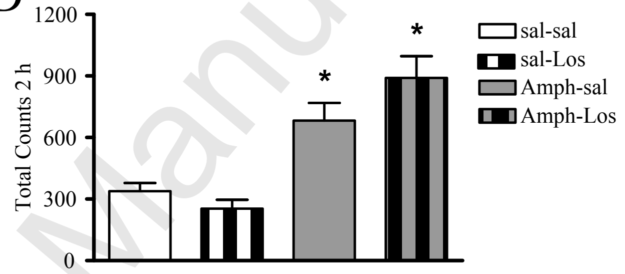
Nucleus Accumbens









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