Decrease of lymphoproliferative response by amphetamine is mediated by dopamine from the nucleus accumbens: Influence on splenic met-enkephalin levels

María Amparo Assis a, Analía Valdomero a, Constanza García-Keller a, Claudia Sotomayor b, Liliana Marina Cancela a,*

a Departamento de Farmacología (IFEC-CONICET), Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina
b Departamento de Bioquímica Clínica (CIBICI-CONICET), Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina

Abstract

Despite the mesocorticolimbic dopaminergic pathway being one of the main substrates underlying stimulating and reinforcing effects induced by psychostimulant drugs, there is little information regarding its role in their effects at the immune level. We have previously demonstrated that acute exposure to amphetamine (5 mg/kg, i.p.) induced an inhibitory effect on the splenic T-cell proliferative response, along with an increase in the methionine(met)-enkephalin content at limbic and immune levels, 4 days after drug administration. In this study, we investigated if a possible dopamine mechanism underlies these amphetamine-induced effects by administering D1 and D2 dopaminergic antagonists or a dopaminergic terminal neurotoxin before the drug. Pre-treatment with either SCH-23390 (0.1 mg/kg, i.p.) or raclopride (0.1 mg/kg, i.p.), a D1 or D2 dopaminergic receptor antagonist, respectively, abrogated the effects of amphetamine on the lymphoproliferative response and on met-enkephalin levels of the spleen. The amphetamine-induced increase in limbic met-enkephalin content was suppressed by SCH-23390 but not by raclopride pre-treatment. Finally, an intra-accumbens 6-hydroxy-dopamine injection administered 2 weeks previously prevented amphetamine-induced effects on the lymphoproliferative response and on met-enkephalin levels in the prefrontal cortex and spleen. These findings strongly suggest that D1 and D2 dopaminergic receptors are involved in amphetamine-induced effects at immune level as regards the lymphoproliferative response and the changes in spleen met-enkephalin content, whereas limbic met-enkephalin levels were modulated only by the D1 dopaminergic receptors. In addition, this study showed that a mesolimbic component modulated amphetamine-induced effects on the immune response, as previously shown at a behavioral level.

1. Introduction

Dopamine in the mesolimbic pathway is one of the main neurotransmitters associated with the psychostimulant-induced behavioral sensitization (Kalivas and Stewart, 1991). The phenomenon of behavioral sensitization has been described as an adaptive process in addiction to psychostimulants and other drugs of abuse (Robinson and Berridge, 1993). This sensitization process, which shares many of the characteristics of other forms of neuronal plasticity, has been associated with either the early stages or relapse in the cycle of addiction (Robinson and Berridge, 2000; Vezina et al., 2002). Several of the changes that underlie behavioral sensitization occur in the mesocorticolimbic dopamine system, of which the ventral tegmental area (VTA) dopaminergic neurons, projecting to the nucleus accumbens (NAC) (mesolimbic system) and to the prefrontal cortex (PFC) (mesocortical system), are key components (Kalivas and Stewart, 1991). Enkephalin (ENK) and glutamate, among others, interact with dopamine in the motivational circuit underlying sensitization (Pierce and Kalivas, 1997; Wolf, 1998; Kalivas, 2007). The presence of dopamine and met-ENK has also been demonstrated in immune cells, which also express the receptors and the synthetic enzymes for both neurotransmitters (Amenta et al., 2001; Bergquist et al., 1994; Linner et al., 1991). Both dopamine and methionine(met)-ENK, have been found to modulate the immune response (Kavelaars et al., 2005; Stanojevic et al., 2007) and it has been suggested that they might be also involved in psychostimulant-induced immunomodulation. Indeed, evidence from our laboratory demonstrated that dopamine has a modulatory role on chronic amphetamine-induced effects in peripheral lymphocyte subpopulations (Assis et al., 2008), with a single dose of amphetamine inducing an increase of met-ENK
levels in the spleen and thymus, together with a decrease in the lymphoproliferative response (Assis et al., 2006). In addition, following a single exposure to amphetamine, a long-term sensitized response was observed to the effects of a subsequent amphetamine challenge on the lymphoproliferative response and the limbic and splenic met-ENK levels. Interestingly, glutamate is involved in the sensitized responses to the immune response as well as to changes in limbic and splenic met-ENK levels (Assis et al., 2009).

Communication between the CNS and the immune system is important to maintain the body's homeostasis, particularly through the regulatory neuronal and hormonal pathways (Bese dovsky and del Rey, 1996; Madden, 2003). The sympathetic nervous system is the main neuronal pathway, with its noradrenergic fibers being in close contact with the primary lymphoid organs (thymus and bone marrow) and also with the tonsils, spleen and mucosa-associated lymphoid tissues (Haas and Schau enstein, 1997; Steinman, 2004; Tracey, 2002; Nance and Sanders, 2007).

There is evidence that catecholamines and neuro peptides released after sympathetic nervous system activation, as well as the glucocorticoids released by the hypothalamic–pituitary–adrenal (HPA) axis, are responsible for this central modulation (Bryant et al., 1991; Fecho et al., 1996a; Edgar et al., 2003). The final effects of these neurotransmitters and neurohormones, released in response to a central stimulus, depend on the activation of the receptors that each immune cell is able to express (Weigent and Bla lock, 1987). It has also been shown that morphine is able to modulate the immune function through both pathways (Fecho et al., 1996b; Saurer et al., 2004), although information is scarce regarding psychostimulants (Irwin et al., 2007). As mentioned above, psychostimulant drugs mainly affect the mesocorticolimbic dopaminergic system. Within this system, the NAc (Nistico et al., 1994) and the amygdala (Bhatt et al., 2006) have both been implicated in the modulation of some peripheral immune responses, and it may be assumed that this modulation is maintained by a limbic communication with the sympathetic nervous system and the HPA axis. However, it is also possible that autocrine and paracrine actions of neurotransmitters, such as opioid peptides and dopamine produced by immune cells, may be responsible for the effects of drug abuse on the immune system (Gordon and Barnes, 2003; Pellegrino and Bayer, 1998).

Dopamine exerts its effect by activation of second messengers through the interaction with protein G-coupled transmembrane receptors. The dopaminergic receptors are classified into two main families: D1-dopaminergic receptors (D1R), which include two different subtypes (D1 and D1A) and are coupled to Gs; and D2 dopaminergic receptors (D2R), which include three different subtypes (D2, D2L, and D2A) and are coupled to Gi/Go (Lachowicz and Sibley, 1997; Gingrich and Caron, 1993; Seeman and Van Tol, 1994; Sibley and Monsma, 1992). In the CNS, the D1R are expressed mainly in post-synaptic membranes, while the D2R are expressed not only in post- but also in pre-synaptic membranes (Callier et al., 2003; Palermo-Neto, 1997). In the immune system, only D1R from the D1R family was detected, while all D2R were identified. T-lymphocytes and monocytes have a low expression of dopamine receptors, whereas neutrophils and eosinophils have moderate expression, with B cells and natural killer (NK) cells having a higher and more consistent expression (McKenna et al., 2002). The D1- and D2-dopaminergic transmissions in the CNS have both been associated with the long-term expression of amphetamine-induced behavioral sensitization (Vanderschuren et al., 1999) and the modulation of the immune response (Basu and Daga Gupta, 2000; Boldyrev et al., 2005).

Given all this evidence, our first goal was to determine the influence of D1R and D2R on the changes in the lymphoproliferative response and the met-ENK levels in the NAc and spleen after a single dose of amphetamine. The availability of selective blockers for each receptor type provides a pharmacological tool to understand the action of dopamine more thoroughly. Therefore, the highly selective D1R and D2R antagonists SCH-23390 and Raclopride, respectively, both able to cross the blood–brain barrier and widely employed as antipsychotic drugs (Iorio et al., 1983; Höberg et al., 1987; Andersen, 1988; Seeman and Van Tol, 1994), were used for this purpose. As an involvement of dopamine was shown, the second aim of this study was to determine the CNS contribution (specifically, the mesolimbic dopaminergic pathway) in amphetamine-induced effects on the T-lymphoproliferative response. Since amphetamine is able to increase extracellular dopamine levels in the NAc, which is a key brain area in the modulation exerted by the limbic system in numerous functions (Di Chiara, 2002; Di Chiara and Bassareo, 2007; Kalivas, 2007), we proposed that this nucleus may be a critical neural substrate in amphetamine-induced immunomodulation. Thus, we investigated the influence of a single dose of amphetamine on the lymphoproliferative response and splenic met-ENK levels in animals previously submitted to a 6-hydroxy-dopamine (6-OHDA)-induced lesion in the dopaminergic terminals of the NAc. Given that this injury made the NAc inappropriate for determining met-ENK, and that the PIC is another limbic structure also innervated by VTA dopaminergic neurons, and as we previously described the influence of amphetamine on met-ENK content in the PIC (Assis et al., 2008, 2009), the met-ENK content was studied in the PIC from rats submitted to this procedure. We demonstrated that a dopaminergic mechanism is involved in the amphetamine-induced effects at the T-lymphoproliferative response and in those changes occurring in the limbic and splenic enkephalinergic system. These data highlight the importance of mesolimbic dopamine transmission in amphetamine-induced immunomodulation and in the related changes in met-ENK levels of the central nervous and immune systems.

2. Methods

2.1. Animals

Adult male Wistar rats (250–330 g) from the Facultad de Ciencias Veterinarias of the Universidad Nacional de La Plata (Buenos Aires, Argentina) were maintained at 20–24 °C under a 12 h light–dark cycle (lights on at 07:00 a.m.) with free access to food and water. Rats were collectively housed (2 rats per cage) in the experimental room for at least 7 days before starting the treatments, with 6 rats per group being used in the experiments. Every attempt was made to minimize the pain and discomfort of the experimental animals, with all procedures being conducted 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.

2.2. Drugs

For all experiments, D-amphetamine sulfate, SCH-23390, raclopride (Sigma Co., St. Louis, MO) and desipramine (Droguería Prest, Buenos Aires, Argentina) were dissolved in an isotonic saline solution (0.9% NaCl), which was also used for vehicle control injections. To induce anesthesia, a ketamine/xylazine (Kensol, Lab König, Buenos Aires, Argentina) mixture in distilled water (55 and 11 mg/kg, respectively) was used. All these drugs were injected intraperitoneally (i.p.) at a volume of 1 ml/kg. For lesion of dopaminergic neurons, 6-OHDA (Sigma Co., St. Louis, MO) was dissolved in an isotonic saline solution (0.9% NaCl) with 1 mg/ml ascorbic acid, which was also used for vehicle control injections. 6-OHDA, as well as its vehicle, was injected intra-accumbens. All the treatments were performed at 11 a.m. (Zeitgeber 4) to avoid
the influence of the circadian rhythm on the immune response (Haus and Smolensky, 1999) or on behavioral sensitization to psychostimulants (Abarca et al., 2002).

2.3. Drug treatments

The following treatments were performed, with an independent control group being used for each one.

2.3.1. Single dose treatment with amphetamine: antagonist pre-treatments

In order to assess the participation of dopaminergic mechanisms in the effects of a single dose of amphetamine on lymphoproliferative response and met-ENK levels, we used selective SCH-23390, D1R (experiments 1 and 2) and raclopride, D2R (experiments 3 and 4) antagonist pre-treatments to block these receptors during the presence of amphetamine. Thus, 15 min before the amphetamine (5 mg/kg, i.p.) or vehicle injection, the animals were pre-treated with SCH-23390 (0.1 mg/kg, i.p.), raclopride (0.1 mg/kg, i.p.) or vehicle. On day 5 (4 days after the amphetamine injection), the animals were killed by decapitation and the spleen and brains were removed (Fig. 1A). The NAc of both hemispheres were dissected, and splenic mononuclear cells were isolated. For each experiment, the group that received an i.p. vehicle injection as pre-treatment on day 1, and another i.p. vehicle injection 15 min after as treatment, was considered the control group.

2.3.2. Single dose treatment with amphetamine: dopaminergic neuronal lesion

In order to assess the participation of the mesolimbic dopaminergic pathway in the effects of a single dose of amphetamine on the lymphoproliferative response, and to measure the immune and limbic met-ENK levels, we used a selective toxin to lesion the dopaminergic terminals in the NAc (experiments 5 and 6). 6-OHDA affects dopaminergic and noradrenergic nerve terminals because it is taken up through the monoaminergic transporters. Cell bodies are more resistant to the neurotoxin, but retrograde degeneration of cell bodies after destruction of the terminals may be slight (Thoenen and Tranzer, 1973). Thus, on day 1, a freshly prepared solution of 6-OHDA (8 µg in 2 µl saline containing 1 mg/ml ascorbic acid) or vehicle was stereotaxically administered in the NAc of both hemispheres. To avoid neurotoxin uptake in noradrenergic neurons, 60 min prior to the 6-OHDA or vehicle intra-accumbens injection, animals were pre-treated with desipramine (15 mg/kg i.p.). As described by Kelly et al. (1975), the neuronal lesion reaches its peak 14 days after the surgery (40–60% lesioned neurons). Thus, on day 15, animals were administered amphetamine (5 mg/kg, i.p.) or vehicle, and on day 19 (4 days following the drug injection), the animals were killed by decapitation, and spleens and brains were removed (Fig. 1B). The NAc and PFC of both hemispheres were then dissected and the splenic mononuclear cells were isolated. The animals that received an intra-accumbens vehicle injection as pre-treatment on day 1 were considered as sham animals (see Table 1). Thus, the sham animals receiving an i.p. vehicle injection as treatment on day 15 were considered as the control group.

2.4. Surgery

Animals under ketamine/xylazine anesthesia were placed in a stereotaxic instrument (Stoelting, Wood Dale, IL), with the incisor bar being set at −3.3 mm. The scalp was incised and retracted, and small bore holes (2 mm diameter) were drilled into the skull using a dental drill. The infusion cannula (30 gauge; length, 20 mm) was stereotaxically lowered into the NAc using the following coordinates: anterior, +1.5 mm; lateral, ±1.0 mm; ventral, −7.0 mm, according to the atlas of Paxinos and Watson (1997). The infusion cannulae were connected via polyethylene tubing (PE 10; Becton Dickinson, Sparks, MD) to 10 µl microsyringes (Hamilton, Reno, NV) mounted on a microinfusion pump (Harvard Apparatus, Holliston, MA). Each rat was injected with 2 µl/side at a flow rate of 1 µl/min. This volume was selected according to the size and structure of these nuclei. Immediately after the micro-injection, the cannulae were retracted, the holes were covered with wax and the skin was sutured with surgical thread. Healing

![Fig. 1. (A and B) Schematic diagrams of drug treatments: on day 1, the pre-treatment with antagonist or vehicle was administered 15 min prior to the amphetamine (5 mg/kg, i.p.) or vehicle injection and the tests were carried out 4 days after drug exposure (A). In order to lesion mesolimbic dopaminergic neurons, 6-OHDA was stereotaxically injected in the NAc of both hemispheres on day 1 in animals previously treated with desipramine. Fourteen days after, acute amphetamine (5 mg/kg, i.p.) or vehicle was administered, and on day 19, tests were carried out (B). (C) Schematic section of the rat brain, adapted from the stereotaxic atlas of Paxinos and Watson (1997), showing the location of micro-injector in the NAc (n = 6). For details and exact coordinates, see Section 4. Amph: amphetamine; Veh: vehicle; DMI: desipramine; Ket/xyl: ketamine/xylazine.]
powder was used to improve the wound recovery, and gentamicin (10 mg/kg, s.c.) was injected near the wound to minimize the risk of infection. After surgery, the animals were removed from the stereotaxic instrument and recovered under a heat lamp with close supervision.

2.5. Isolation of mononuclear cells

Spleen cell suspensions were obtained by gently grinding tissue into RPMI 1640 culture medium (Sigma–Aldrich, Steinheim, Germany) under sterile conditions as was previously described (Assis et al., 2006). Splenic mononuclear cells (SMC) were separated by Ficoll–Hypaque density gradient (1.083 g/ml) centrifugation, and those concentrated at the interface were collected and washed twice in RPMI 1640 medium. After cell counting, SMC were suspended at a final concentration of 2 \times 10^6 cells/ml in RPMI 1640 medium supplemented with 10% of inactivated fetal bovine serum, 2 mM glutamine, 10 mM sodium bicarbonate, 100 UI/ml penicillin and 100 \mu g/ml streptomycin (denominated complete RPMI 1640 medium).

2.6. Mitogenic assay

The mitogen concanavalin A (Con A) (Sigma–Aldrich, Steinheim, Germany) was used to evaluate the splenic T-cell response. We chose a functional parameter of the immune system, as this would be able to provide information regarding the immune competence of T-cell populations. The Con A-induced lymphoproliferative response was utilized as it is the most widely used test to reflect the in vitro T-cell functional response (Coligan et al., 1999). Thus, 150 \mu l of the splenic mononuclear cells suspension were added in quadruplicate to each well of 96-well flatbottom tissue culture plates (TPP, Switzerland) with 5 \mu g/ml of Con A. Optimization of cell number and mitogen concentration was conducted in previous studies (Rabinovich et al., 1997; Iglesias et al., 1998; Assis et al., 2006). The plates were incubated at 37 °C in a humidified atmosphere with 5% CO2 for 48 h. Then, 18 h before harvesting, cells were pulsed with 1 \mu Ci [3H]-thymidine (PerkinElmer Life Sciences, Wellesley, MA). Finally, quadruplicates were collected onto glass fiber filter paper (Whatman, UK) using a Scatron micro cell harvester (SIEM, Córdoba, Argentina). A 1205 Betaplate liquid scintillation counter (PerkinElmer Life Sciences, Wellesley, MA) was used to detect the incorporation of radioactive thymidine in a Packard Tri-Carb Liquid Scintillation Analyzer. The radioactivity was expressed as counts per million (c.p.m.), the percentage of responses was calculated relative to the control group, and the data shown is representative of 2–3 independent experiments (Assis et al., 2006).

2.7. Free met-ENK radioimmunoassay (RIA)

Frozen immune tissues and brain areas were suspended in 1 M acetic acid containing 50 mM HCl, then boiled for 15 min, homogenized with a Polytron, and centrifuged at 50,000 g for 1 h. An aliquot of the supernatant was lyophilized and reconstituted in 50 mM Tris–HCl buffer, pH 8.4, and 2 mM CaCl2. Met-ENK and polyclonal met-ENK antibody were provided by Peninsula Lab-Bachem (San Carlos, USA). Free immunoreactive met-ENK was determined by RIA as described in Assis et al. (2006). Briefly, 100 \mu l of standards or samples were incubated at room temperature with 100 \mu l of rehydrated antiserum, 15,000 c.p.m/50 \mu l of 125I-met-ENK and 50 \mu l of RIA buffer, 0.1% BSA and 0.25% normal rabbit serum. Twenty-four hours later, 100 \mu l of diluted normal rabbit serum and 500 \mu l of goat anti-rabbit IgG serum (diluted 1:900 in PBS, 8% PEG 6000) were added, and, following 2 h at room temperature and 1 h at 4 °C, the samples were centrifuged at 1700 g for 30 min at 4 °C. The supernatants were carefully aspirated and the pellets were counted for 125I in a gamma counter.

2.8. Dopamine and noradrenaline determination by high performance liquid chromatography (HPLC)

In order to confirm the efficacy of 6-OHDA treatment in lesioning the dopaminergic terminals, as well as the efficacy of desipramine pre-treatment in conserving the integrity of noradrenergic neurons after 6-OHDA administration, dopamine and noradrenaline levels in the NAc were evaluated. The NACs were weighed, homogenized in 0.2 N HClO4 and centrifuged at 14,000 r.p.m. for 10 min at 4 °C. The supernatant was filtered and the dopamine and noradrenaline content measured by HPLC and electrochemical quantification. Thus, a reverse-phase column (ultrasphere C 18, Beckman, Germany) was used, connected to a BAS LCD-4 electrochemical detector with a glass–carbon electrode and to a Spectra Series P200 pump. The potential was set at 650 mV versus an Ag/AgCl reference electrode. The mobile phase was composed of 50 mM NaH2PO4, 0.1 mM Na2HPO4, 0.1 mM EDTA-Na2, 0.5 mM n-octyl sodium sulfate and 12% methanol (pH = 5.5) for dopamine determination, and 75 mM NaH2PO4, 0.5 mM sodium dodecyl sulfate, 0.02 mM EDTA, 0.04% triethylamine, 15% methanol and 3% acetonitrile (pH = 5.7) for noradrenaline determination, and delivered at a flow rate of 1 ml/min. Peaks from the HPLC system were displayed, integrated and stored using Peak Analysis II Data System (SRI Inst., CA, USA). Quantification was made by comparing the peak heights of the samples with those of a standard curve.

2.9. Histological analysis

The cannulae placement was histologically verified in another group of lesioned rats following the Paxinos and Watson (1997) coordinates by cresyl violet staining (Figs. 1C and 5). Experimental animals were anesthetized with chloral hydrate (400 mg/kg i.p.), perfused transcardially with 250 ml of saline, and fixed with 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4). The brains were removed and postfixed in the same fixative overnight at 4 °C. Then, they were placed in 30% sucrose in PBS.
until the brains sedimented. After this, the brains were sectioned in a cryostat (Leica CM1510S Germany) into 20-µm-thick coronal slices and were then stained with cresyl violet. The histological sections were examined under high magnification microscope to determine the accuracy and size of the bilateral lesions produced by 6-OHDA injections to NAc similar to Wanchoo et al. (2010).

2.10. Statistical analysis

Cell proliferation was measured in quadruplicate by the [3H]-thymidine incorporation assay and results were plotted as the percentage of changes between amphetamine- and vehicle-treated rats. Data were analyzed with a two-way ANOVA (drug/lesion pre-treatment × drug treatment). There were two levels for the drug treatment factor (amphetamine or vehicle), and also two levels for the drug pre-treatment factor (SCH-23390 or vehicle, raclopride or vehicle, 6-OHDA or vehicle). Following significance in the two-way ANOVA, post hoc comparisons among means were performed with the Bonferroni test. Data represented means ± SEM. For all the statistical tests, the level of significance was set at \( p < 0.05 \).

3. Results

3.1. SCH-23390 pre-treatment: influence of a single dose of amphetamine

3.1.1. Experiment 1. SCH-23390 prior to amphetamine: effects on lymphoproliferative response

Rats were exposed to a single dose amphetamine treatment (5 mg/kg, i.p.) and after 4 days, the function of the T-cell compartment was evaluated by proliferative assay against a mitogenic stimulus. Thus, a significant decrease of 66% \( ( p < 0.05) \) was observed in the [3H]-thymidine incorporation in Con A-stimulated splenocytes from amphetamine-treated rats. However, this effect was not evident in rats administered SCH-23390 prior to amphetamine or observed following SCH-23390 in vehicle treated animals (Fig. 2A). The proliferative response to Con A for the control group (vehicle-pre-treated and -treated rats) in this experiment represented the maximum percentage of proliferation, with its absolute value being 6.2 ± 1.0 \( \times 10^5 \) c.p.m.

A two-way ANOVA (drug pre-treatment × drug treatment) indicated statistically significant differences in lymphoproliferative response (drug pre-treatment: \( F(1, 20) = 4.93, p < 0.05 \), drug pre-treatment × drug treatment: \( F(1, 20) = 6.91, p < 0.05 \)). Bonferroni’s post hoc comparisons among means revealed that treatment with amphetamine decreased the lymphoproliferative response \( ( p < 0.05) \) of vehicle-pre-treated rats, compared to the remaining experimental groups.

3.1.2. Experiment 2. SCH-23390 prior to amphetamine: effects on limbic (NAc) and immune (spleen) met-ENK levels

A single dose of amphetamine increased the met-ENK levels in the NAc (84%, \( p < 0.01 \)) and spleen (57%, \( p < 0.01 \)) 4 days after drug exposure. SCH-23390 administration suppressed this amphetamine-induced effect and had no effect on its own on the met-ENK levels in the NAc and spleen (Fig. 2B). The absolute values (pg/mg protein) of met-ENK levels for the control group (vehicle-pre-treated and -treated rats) in this experiment were: 1611 ± 45 for the NAc and 2294 ± 178 for the spleen.

A two-way ANOVA (drug pre-treatment × drug treatment) indicated statistically significant differences for met-ENK in NAc (drug pre-treatment: \( F(1, 20) = 87.54, p < 0.01 \), drug treatment: \( F(1, 20) = 134.51, p < 0.01 \), drug pre-treatment × drug treatment: \( F(1, 20) = 121.24, p < 0.01 \)) and spleen (drug pre-treatment: \( F(1, 20) = 18.45, p < 0.01 \), drug treatment: \( F(1, 20) = 13.24, p < 0.01 \), drug pre-treat-

Fig. 2. Influence of SCH-23390 pre-treatment on the acute amphetamine-induced effects on lymphoproliferative response (A) and on met-ENK levels (B). Animals were pre-treated with SCH-23390 (0.1 mg/kg, i.p.) or vehicle 15 min prior to a treatment with amphetamine (5 mg/kg, i.p.) or vehicle on day 1. Then, on day 5, the Con A-induced lymphoproliferative response (A) and the met-ENK levels in the NAc and spleen (B) were evaluated. Data are expressed as a percentage related to the control group and show the means ± SEM of 6 rats per group. \( * p < 0.01 \), \( * * p < 0.05 \), Bonferroni’s test, different from all the remaining groups. The lymphoproliferative data are representative of at least two independent experiments. Amph: amphetamine; Veh: vehicle; Pre-treat: pre-treatment; Treat: treatment.
ment × drug treatment: $F(1, 20) = 14.65$, $p < 0.01$). Bonferroni’s post hoc comparisons among means revealed that treatment with amphetamine increased the met-ENK levels in NAc ($p < 0.01$) and spleen ($p < 0.01$) of vehicle-pre-treated rats, compared to the remaining experimental groups.

3.2. Raclopride pre-treatment: influence on a single dose of amphetamine

3.2.1. Experiment 3. Raclopride prior to amphetamine: effects on lymphoproliferative response

The effect of amphetamine (5 mg/kg, i.p.), administered 4 days previously, on $^3$H-thymidine incorporation in Con A-stimulated splenocytes (a decrease of 59%, $p < 0.05$) was suppressed by pre-treatment with raclopride. However, no effect was observed following raclopride in vehicle-pre-treated animals (Fig. 3A). The proliferative response to Con A for the control group (vehicle-pre-treated and -treated rats) in this experiment represented the maximum percentage of proliferation, with its absolute value being $5.8 \pm 1.2 \times 10^4$ c.p.m.

A two-way ANOVA (drug pre-treatment × drug treatment) indicated statistically significant differences for met-ENK in the NAc (drug pre-treatment: $F(1, 20) = 7.35$, $p < 0.05$). Bonferroni’s post hoc comparisons among means revealed that treatment with raclopride decreased the lymphoproliferative response ($p < 0.05$) of vehicle-pre-treated rats, compared to the remaining experimental groups.

3.2.2. Experiment 4. Raclopride prior to amphetamine: effects on limbic (NAc) and immune (spleen) met-ENK levels

Amphetamine increased the met-ENK content in the NAc (80%, $p < 0.01$) and spleen (45%, $p < 0.01$), 4 days after drug exposure. Raclopride pre-treatment suppressed the amphetamine effect on met-ENK of the spleen but not of the NAc. Consequently, animals pre-treated with raclopride showed an increase of 85% ($p < 0.01$) in the met-ENK of the NAc. Raclopride had no effect on its own on the met-ENK level in the NAc or spleen (Fig. 3B). The absolute values (pg/mg protein) of met-ENK levels for the control group (pre-treated and treated with vehicle) in this experiment were: 1745 ± 42 for the NAc and 2647 ± 182 for the spleen.

A two-way ANOVA (drug pre-treatment × drug treatment) indicated statistically significant differences for met-ENK in the NAc (drug pre-treatment: $F(1, 20) = 280.44$, $p < 0.01$) and spleen (drug pre-treatment: $F(1, 20) = 21.81$, $p < 0.01$; drug treatment: $F(1, 20) = 15.58$, $p < 0.01$; drug pre-treatment × drug treatment: $F(1, 20) = 11.21$, $p < 0.01$). Bonferroni’s post hoc comparisons among means revealed that treatment with amphetamine increased the met-ENK level in the NAc of rats previously pre-treated with vehicle ($p < 0.01$) or raclopride ($p < 0.01$), and in the spleen of rats pre-treated with vehicle ($p < 0.01$), compared to the remaining experimental groups.

3.3. Lesion of mesolimbic dopaminergic neurons: influence on amphetamine treatment

3.3.1. Experiment 5. 6-OHDA intra-accumbens prior to amphetamine: effects on lymphoproliferative response

The effect of amphetamine (5 mg/kg, i.p.) administered 4 days previously, on $^3$H-thymidine incorporation in Con A-stimulated splenocytes (resulting in a decrease of 70%, $p < 0.01$), was abrogated in animals previously submitted to a 6-OHDA intra-accumbens pre-treatment. The depletion of dopamine in the NAc, with no alteration in noradrenergic neurotransmission (Table 1), was comparable with that previously reported by Kelly et al. (1975),

![Fig. 3](image_url)

**Fig. 3.** Influence of raclopride pre-treatment on the acute amphetamine-induced effects on lymphoproliferative response (A) and on met-ENK levels (B). Animals were pre-treated with raclopride (0.1 mg/kg, i.p.) or vehicle 15 min prior to a treatment with amphetamine (5 mg/kg, i.p.) or vehicle on day 1. Then, on day 5, the Con A-induced lymphoproliferative response (A) and the met-ENK levels in the NAc and spleen (B) were evaluated. Data are expressed as a percentage related to the control group and show the means ± SEM of 6 rats per group. **$p < 0.01$, Bonferroni’s test, different from the control group. *$p < 0.01$,* $p < 0.05$, Bonferroni’s test, different from all the remaining groups. The lymphoproliferative data are representative of at least two independent experiments. Amph: amphetamine; Veh: vehicle; Pre-treat: pre-treatment; Treat: treatment.
and confirmed correct cannulae placement, without any effect on its own on the lymphoproliferative response (Fig. 4A). The proliferative response to Con A for the control group (sham rats administered with vehicle i.p. 14 days after surgery) in this experiment represented the maximum percentage of proliferation, with its absolute value being 5.6 ± 1.0 × 10^5 c.p.m.

A two-way ANOVA (lesion pre-treatment × drug treatment) indicated statistically significant differences in lymphoproliferative response (lesion pre-treatment: \( F(1, 20) = 12.42, p < 0.01 \), lesion pre-treatment × drug treatment: \( F(1, 20) = 9.88, p < 0.01 \)). Bonferroni’s post hoc comparisons among means revealed that treatment with amphetamine decreased the lymphoproliferative response \( (p < 0.01) \) of rats previously pre-treated with vehicle, compared to the remaining experimental groups.

### 3.3.2. Experiment 6. 6-OHDA intra-accumbens prior to amphetamine: effects on limbic (PfC) and immune (spleen) met-ENK levels

Amphetamine increased the met-ENK content in the PfC (31%, \( p < 0.05 \)) and spleen (39%, \( p < 0.01 \)), 4 days after drug exposure, in sham rats. The administration of the 6-OHDA neurotoxin in the NAc, 2 weeks previously, suppressed the amphetamine effect on met-ENK content, not only in the spleen but also in the PfC. The lesion of the mesolimbic pathway had no effect on its own on the met-ENK levels in the PfC or spleen (Fig. 4B). The absolute values (pg/mg protein) of met-ENK levels for the control group (sham rats administered with vehicle i.p. 14 days after surgery) in this experiment were: 491 ± 40 for the PfC and 2613 ± 213 for the spleen.

A two-way ANOVA (lesion pre-treatment × drug treatment) indicated statistically significant differences for met-ENK content in PfC (drug treatment: \( F(1, 20) = 12.94, p < 0.01 \)) and spleen (drug pre-treatment: \( F(1, 20) = 9.61, p < 0.01 \), drug pre-treatment × drug treatment: \( F(1, 20) = 7.97, p < 0.01 \)). Bonferroni’s post hoc comparisons among means revealed that treatment with amphetamine increased the met-ENK levels in the PfC \( (p < 0.05) \) and spleen \( (p < 0.01) \) of sham rats, compared to the remaining experimental groups.

### 4. Discussion

In a previous study, we demonstrated that a single dose treatment with amphetamine (5 mg/kg, i.p.) was able to induce, 4 days after the drug injection, a decrease in the lymphoproliferative response together with an increase of met-ENK levels in the NAc, PfC, spleen and thymus (Assis et al., 2006). In the current study, we showed that the activation of D1R and D2R, as well as the mesolimbic dopaminergic pathway, are needed to mediate the immunosuppressive effect induced by amphetamine on the lymphoproliferative response, and also to induce the increase of met-ENK content in spleen. However, only D1R was able to mediate the increase of met-ENK levels in the NAc following amphetamine. Furthermore, the lesion of the mesolimbic dopaminergic neurons also blocked the effects of amphetamine on met-ENK content in the PfC, another key limbic area associated with psychostimulant behavioral sensitization. These results provide the first evidence of a central dopaminergic mechanism underlying psychostimulant-induced effects on the T-lymphoproliferative response and on the splenic met-ENK content.

The current findings are not only an example of the interaction between both neurotransmitter systems, met-ENK and dopamine, which has been shown previously (Katoh et al., 1991; Nikoshkov et al., 2008), but also suggest that the change in the splenic met-ENK might be related to the immunomodulatory actions of amphetamine. This was further supported by previous evidence from our own laboratory that a N-methyl-D-aspartate (NMDA)

![Fig. 4. Influence of 6-OHDA-induced lesion in the NAc on the acute amphetamine-induced effects on lymphoproliferative response (A) and on met-ENK levels (B). Animals were pre-treated with 6-OHDA (8 μg/side, intra-accumbens) or vehicle 14 days prior to a treatment with amphetamine (5 mg/kg, i.p.) or vehicle. Then, on day 19, the Con A-induced lymphoproliferative response (A) and the met-ENK levels in PfC and spleen (B) were evaluated. Data are expressed as a percentage related to the control group and show the means ± SEM of 6 rats per group. *p < 0.01, ‡p < 0.05, Bonferroni’s test, different from all the remaining groups. The lymphoproliferative data are representative of at least two independent experiments. Amph: amphetamine; Veh: vehicle; Pre-treat: pre-treatment; Treat: treatment.

![Graph A: Lymphoproliferative Response](image)

![Graph B: Prefrontal Cortex](image)
glutamatergic receptor antagonist, abrogated both the decrease of T-lymphoproliferative response and the increase of splenic met-ENK following a single dose of amphetamine (Assis et al., 2009). It should be borne in mind that met-ENK has been consistently associated with a decrease in the adaptive immune response, particularly in T-cell functionality (Fulford et al., 2000; Saravia et al., 1998), although a biphasic modulation that depends on met-ENK levels has also been suggested (Sizemore et al., 2004). Piva et al. (2005) showed that met-ENK and their derived peptides, at low or high doses \textit{in vitro}, were able to induce or inhibit, respectively, interferon (IFN)-\(\gamma\) production. High concentrations of met-ENK and YG, a related peptide, also suppress interleukine (IL)-2 and IL-4 production, and the previous administration of naloxone (a \(\mu,\delta\)-opioid receptor antagonist) blocks the met-ENK effect, but only on IFN-\(\gamma\) production. Thus, it is reasonable to assume that the enhancement of splenic met-ENK levels could modulate the cytokine production and/or other immune mechanisms responsible for the decrease in the lymphoproliferative response.

4.1. Dopaminergic receptors in amphetamine effects on lymphoproliferative response

In order to study the role of D1R and D2R in the amphetamine-induced effect on the lymphoproliferative response, it is important to consider the peripheral function of dopamine in neuroimmune communication. Although dopamine present in sympathetic neurons serves basically as a precursor for the synthesis of norepinephrine, some controversial evidence indicates that dopamine might be released as a transmitter from the sympathetic nerve terminal in the spleen (Bencsics et al., 1997). Dopamine also performs numerous autocrine and paracrine functions when released by lymphocytes (Cosentino et al., 2002, 2003). Since the systemic administration of both D1R and D2R antagonists blocks the amphetamine-induced effect on the lymphoproliferative response, it is possible to conclude that the simultaneous activation of cellular pathways triggered by both types of dopaminergic receptors is necessary to induce this effect. The concomitant activation of D1R and D2R is also crucial in the control of other physiological functions regulated by dopamine (Aizman et al., 2000) and, although some authors have proposed an interrelationship between D1R and D2R-mediated control of distinct central functions (Kobayashi et al., 2004), there is no firm conclusion to explain these effects. Although it is important that future studies should try to untangle the central and peripheral contribution of these two dopamine receptor subtypes, this study provides a platform from which their contributions in amphetamine-induced immunomodulation can be further investigated.

It is highly probable that at least some amphetamine effects may be also mediated by blocking the uptake of noradrenaline from sympathetic nerves that innervate the spleen, and thus, the activation of \(\beta\) receptors, and mainly j2 receptors (Fig. 6), may also contribute to the drug-induced proliferative alterations of immune cells after amphetamine (Nance and Sanders, 2007).

4.2. Dopaminergic receptors in amphetamine effects on central and splenic met-ENK levels

The current data show the participation of D1R and D2R in the amphetamine-induced increase in met-ENK levels in spleen, but only for the D1R in the NAc. Dopamine, acting on its specific receptors, modulates the expression of some transcription factors, such as cAMP response element-binding (CREB) and c-fos (Das et al., 1997), with both of these being capable of modulating the proENK gene (Bacher et al., 1996; Konradi et al., 1994; Monnier and Loefler, 1998). The activation of D1R increases CREB phosphorylation and c-fos expression, and there is also a synergistic mechanism if D2R is also activated (Cho et al., 2007; Kashihara et al., 1999).

4.2.1. Spleen

The activation of D1R and D2R, and the concomitantly induced intracellular events at central and/or immune level, are necessary in order to increase the splenic met-ENK content following amphetamine administration (Fig. 6). Thus, it is possible that the central dopaminergic receptors may mediate the message to the immune system and/or, by a direct action of dopaminergic receptors expressed on immune cells, finally modulate proENK gene expression. It is important to stress that the nuclear factor-kB activates the proENK gene in T-cells (Rattner et al., 1991), which is involved in dopamine-induced immunosuppression (Bergquist et al., 2000), being modulated by D2 dopamine receptors (Zhen et al., 2001).

4.2.2. NAc

Our data indicate that D1R, but not D2R, is involved in the amphetamine-induced intracellular changes that modulate met-ENK content in this brain area. As previously mentioned, D1R and D2R act synergistically to induce an increase in CREB and c-fos activity (Cho et al., 2007; Kashihara et al., 1999), which in turn can modulate the proENK gene. Nevertheless, the present results show that D2R blockade is not sufficient to prevent the

![Fig. 5. Photomicrographs (40×) showing the effect of the vehicle or 6-OHDA injection into NAc obtained from sham (no lesion) and 6-OHDA lesioned animals, respectively. The arrow indicates the location of the injection sites, whereas the dark shaded area indicates the spread of 6-OHDA in the NAc. aca: anterior commissure; CPU: caudate putamen.](image-url)
amphetamine-induced effect on met-ENK modulation. Thus, our data suggest that D2R is not indispensable for this amphetamine-induced effect.

4.3. A role for NAc dopamine in amphetamine effects on the lymphoproliferative response and splenic met-ENK levels

The data obtained in the NAc 6-OHDA lesion experiment indicate that the amphetamine-induced effects on the lymphoproliferative response and the content of splenic met-ENK are modulated by a central message that involves, at least in part, dopaminergic neurotransmission in the NAc. In agreement, previous findings have shown that the NAc is a key brain area in the effects on NK-cell activity following morphine (Saurer et al., 2006). Related to this, Nistico et al. (1994) demonstrated that the D1R antagonist, micro-injected in the NAc but not in the VTA or hippocampus, induced a decrease in the lymphoproliferative response to Con A.

Since the lesion induced in the NAc by retrograde transport involves the VTA neurons, it is possible that the lesioned dopaminergic neurons may be projected not only to the NAc, but also to other brain areas, such as the amygdala or the PFC, which, in turn, send glutamatergic and GABAergic projections, respectively, to the VTA (Wolf, 2002). Thus, the lesion of VTA dopaminergic neurons may be projected not only to the NAc, but also to other brain areas, such as the amygdala or the PFC, which, in turn, send glutamatergic and GABAergic projections, respectively, to the VTA (Wolf, 2002). Thus, the lesion of VTA dopaminergic neurons could also affect these other pathways, which might also be involved in the amphetamine effect. This factor could explain the results observed following amphetamine administration in the met-ENK content of the PFC in animals pre-treated with 6-OHDA.

In the NAc two sub-regions have been identified, the core and the shell, based on their anatomical connections and their neurochemical and histological characteristics (Zahm and Heimer, 1990). The NAc core is associated with sensorimotor integration, and is anatomically and functionally related with the dopaminergic nigrostriatal pathway in motor control (Zahm and Brog, 1992), and the NAc shell has been included in the extended amygdala (Heimer et al., 1997) and could be involved in the regulation of motivation and reward (Di Chiara, 2002). Interestingly, a differential modulation between the core and shell has been shown in morphine-induced effects on NK-cell activity. Thus, the administration of a D1R antagonist in the NAc shell, but not in the core, was able to reverse the suppressor effect of morphine on these cells (Saurer et al., 2006). From these findings, it is possible that the NAc shell connections, related to the extended amygdala and emotional control, might also modulate the immune function in response to emotional regulation. It is also feasible that the specific activation of this NAc sub-region, the shell, more than the core, could be the mediator of the amphetamine-induced effect observed in this work.

As previously described, the CNS message to the immune system could be mediated by neural and/or hormonal pathways. In this study, the pathway involved in the amphetamine-induced message from the mesolimbic system to the immune system has not been assessed. However, it is possible that the modulation exerted by the amygdala (and hence by the NAc) on the sympathetic nervous system could reach the spleen and the adrenal glands (Nistico et al., 1994; Bhatt et al., 2006). The catecholamine release induced by sympathetic nervous stimulation, not only in the spleen but also in the blood due to adrenal activation, could be the main cause of the amphetamine-induced effects observed here (the immunosuppressive effect and splenic met-ENK content increase) (Fig. 6). In addition, the sympathetic catecholaminergic transmission induced by amphetamine at the central level might be potentiated at peripheral level if this drug also induces catecholamine uptake blockade in the immune cells. Regarding hormonal modulation, HPA activation is possibly involved, with corticosterone playing a specific role as an immunosuppressive hormone (Pruett, 2001). Thus, in a similar way, another central dopaminergic pathway, the tuberoinfundibular, may also participate as a secondary mechanism in the amphetamine-induced effect, due to its activation inhibiting prolactin release, which has a well-known pro-inflammatory effect (Orbach and Shoenfeld, 2007).
Summing up, the amphetamine-induced effects on the lympho-proliferative response and on the splenic met-ENK levels seem to be primarily modulated by the NAc dopamine, which may exert its influence on the immune system by activating the sympathetic nervous system (through its connection with the amygdala) and/or the HPA axis. The reversal of the amphetamine effect on splenic met-ENK content after the lesion induced in the NAc, in parallel with the reversal of the effect on the lymphoproliferative response, is more evidence regarding the possible role of met-ENK in the immunosuppressive effect observed following amphetamine. Future studies of gene disruption of proENK in specific cell populations in vivo, using conditional gene expression systems, will be helpful to elucidate the specific contribution of the increase in splenic met-ENK to the immunosuppressive effects of amphetamine.

Acknowledgments

This study was supported by grants from FONCYT, Ministerio de Ciencia y Tecnología de Córdoba, CONICET and SeCyT (Argentina). The authors wish to express their sincere gratitude to Ms. Estela Salde, Ms. Paula Icely and Ms. Elsa R. Pereyra for their excellent technical assistance. We are grateful to Joss Heywood, native speaker, for revision of the manuscript.

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