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# A dopamine mechanism is implied in the acquisition and expression of amphetamine and stress-induced effects observed in the lymphocyte subpopulations

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**Abstract**

Drugs of abuse and stress are associated with changes in circulating cell populations and reductions in cell-mediated immune responses. The main goal of this study was to determine the influence of repeated and acute D-amphetamine treatments on the foot-shock stress-induced effects on the peripheral lymphocyte subpopulations, and the involvement of a dopamine mechanism in the development and expression of this phenomenon. Wistar rats received an acute (5 mg/kg/day i.p.) or a repeated (2 mg/kg/day i.p. during 9 days) amphetamine treatment, and were exposed to a foot-shock stress (1 mA, 3 s) 4 days after the last amphetamine injection. Another group was administered with haloperidol (1 mg/kg/day i.p.) 15 min previous to each daily amphetamine injection or previous to the foot-shock stress session. Then, blood cells stained with monoclonal antibodies against CD3-FITC, CD8-PE and CD4-Cy-Chrome, and against CD161a-FITC, CD3-PE, and CD45RA-Cy-Crhome, were analyzed by multiparameter flow cytometry. The exposure to a foot-shock stress induced a decrease in the absolute number of peripheral lymphocytes, as well as in CD4+ and CD8+ T-cells and B-cells in acute and repeatedly amphetamine-treated rats, whereas the NK-cell population remained unchanged. Haloperidol administration previous to each drug administration or the foot-shock stress session reversed these effects. This study provides strong evidence that dopamine can play a more general role in the influence of amphetamine on the stress-induced effects on the lymphocyte subsets.


*Keywords:* Amphetamine; Foot-shock stress; Dopamine sensitization; Haloperidol; CD4+ and CD8+ T-cells; B-cells; NK-cells; Flow cytometry

## 1. Introduction

It is currently known that drugs of abuse clearly perturb immune functions as do stress, mood and emotion in both humans and laboratory animals (Galinowski et al., 1992; Baldwing et al., 1998; Padgett and Glaser, 2003; Barak, 2006). Drug addicts are also known to be highly susceptible to bacterial, viral, and fungal infections, and to have important deficits in the immune function (Vallejo et al., 2004; Islam et al., 2004; Friedman and Eisenstein, 2004).

There is growing evidence demonstrating a regulatory role of the central nervous system (CNS) on the functioning of the immune system through various neuropeptides, neurohormones,

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cytokines and/or neurotransmitters (Oberbeck, 2006; Dantzer, 2006; Ziemssen and Kern, 2007). Behavioral observations during or in response to a novel stressor, as well as studies performed to evaluate the immunological response to an aversive experience, have demonstrated that the effects of stress can be markedly different, depending on the chronic stress or drug paradigms previously applied (Basso et al., 1993, 1994, 1999).

It has been shown that the effects of psychostimulants and stress cross-sensitize each other, as demonstrated not only at behavioral and neurochemical levels (Antelman et al., 1980; Kalivas and Stewart, 1991, Robinson and Berridge, 2000; Saal et al., 2003; Pacchioni et al., 2002) but also in the change of some immune parameters (Basso et al., 1999). It was observed that long-lasting changes in dopaminergic neurons and in the pituitary functions underlie the interchangeability between psychostimulant drugs and stress at behavioral level (Robinson et al., 1987; Diaz-Otañez et al., 1997; Pacchioni et al., 2007); interestingly, a dopamine mechanism was also involved in the influence of repeated amphetamine on the stress-induced effects in the circulating cell populations and reductions in cell-mediated immune responses (Basso et al., 1999).

Several studies have attempted to elucidate the pharmacology of sensitization, using different dopamine receptor antagonists. Pre-treatment with haloperidol, a non-selective dopamine receptor antagonist, prevented the development of psychostimulant sensitization (Mattingly et al., 1996; Weiss et al., 1989). The studies using selective $D_2$ dopamine receptor antagonist have yielded contradictory results. However, most of the experiments have found that $D_2$ dopamine receptor antagonist failed to prevent psychostimulant sensitization (Vezina and Stewart, 1989; White and Wolf, 1991), and there is a general agreement that $D_1$ selective dopamine receptor antagonists, such as SCH-23390, prevent the development of sensitization to amphetamine (Bjijou et al., 1996; Anderson and Pierce, 2005).

It has been shown that an acute dose of a psychostimulant drug such as amphetamine suppresses lymphocyte proliferative response to mitogens, natural killer (NK) cell activity, and the production of cytokines in rodents (Assis et al., 2006; Heilig et al., 1993; Nuñez-Iglesias et al., 1996; Pezzone et al., 1992). Previous findings from our lab have shown that acute exposure to an aversive event (i.e., foot-shock) decreased the percentage of peripheral T-lymphocytes and the delayed type hypersensitivity reaction (Basso et al., 1993, 1994), while no discernible effect was observed in the percentage of B-lymphocytes and in the hemaglutinin titer against sheep red blood cells.

Drugs of abuse and stress are associated with changes observed in circulating cell populations and reductions in cell-mediated immune responses (Dhabhar et al., 1995; Dhabhar and McEwen, 1996; Islam et al., 2004). We have previously observed that a repeated amphetamine treatment led to a decrease in both circulating cell populations and cell-mediated immune responses, after a subsequent acute stress exposure, although each one had no effects on its own. This facilitation was reversed by a pre-treatment with haloperidol, a non-selective $D_1/D_2$ dopamine receptor antagonist (Basso et al., 1999). The main goal of this study was to determine the influence of repeated and acute amphetamine treatments on the stress-induced effects on the lymphocyte subsets, and the involvement of a dopamine mechanism in the development and the expression of this phenomenon.

## 2. Materials and 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 in cages in the experimental room for at least 7 days before starting the experiments. All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals as approved by Animal Care and Use Committee of the Facultad de Ciencias Químicas, Universidad Nacional de Córdoba.

### 2.2. Drugs

For all experiments, amphetamine sulfate (Sigma Co, St. Louis, MO) was dissolved in an isotonic saline solution (0.9% NaCl), which was also used for vehicle control injections. Haloperidol (Droguería Prest, Yugoslavia) was dissolved in a 1% V/V acetic acid solution, and the pH was then adjusted to 5.5–5.7 with addition of a 0.1 NaOH solution. All injections were administered intraperitoneally in a volume of 1 ml/kg and the treatments were made at 11 a.m. (ZT 4) to avoid the influence of the circadian rhythm on the immune response (Haus and Smolensky, 1999) and on the behavioral sensitization to psychostimulants (Abarca et al., 2002).

### 2.3. Monoclonal antibodies

The following antibodies were used: Fluorescein Isothiocyanate (FITC)-conjugated mouse anti-rat CD3 monoclonal antibody (MoAb), R-Phycoerythrin (PE)-conjugated mouse anti-rat CD8a MoAb, Cy-Chrome™-conjugated mouse anti-rat CD4 MoAb, FITC-conjugated mouse anti-rat CD161a MoAb, R-PE-conjugated mouse anti-rat CD3 MoAb and Cy-Chrome™-conjugated mouse anti-rat CD45RA MoAb (BD Bioscience, NJ, U.S.A.).

### 2.4. Foot-shock stress

Rats were randomly exposed to a regimen of foot-shock stress as was previously described by Basso et al. (1999). Briefly, a chamber measuring 25 × 23 × 20 cm served as the shock apparatus. It had a grid floor of stainless steel rods through which scrambled electric shocks could be delivered via a shock generator. The stress protocol consisted of 15 min of foot-shock exposure. The amplitude of the shock was 1 mA, and the shock duration was 3 s. Shocks were presented according to a variable interval schedule, with an average of one shock/min. Immediately after stress, peripheral blood cells were obtained as mentioned below. Control animals were left undisturbed in their home cages.

### 2.5. Acute amphetamine treatment

Rats were randomly assigned to one of two acute treatments: Vehicle group or Amphetamine 1×5 (5 mg/kg i.p.) group. The amphetamine or vehicle treatment was administered during day 1, and on day 5 (four days following the last drug injection) animals were placed in the foot-shock chamber and were stressed or not.

### 2.6. Repeated amphetamine treatment

Rats were randomly assigned to one of two repeated treatments: Vehicle group and Amphetamine 9×2 (2 mg/kg/day i.p.) group. The amphetamine or vehicle treatment was administered during days 1–9, and on day 13 (four days following the last drug injection) animals were placed in the foot-shock chamber and were stressed or not.

### 2.7. Haloperidol pre-treatment

Rats were randomly assigned to one of two pre-treatments: vehicle group and Haloperidol (1 mg/kg/day, i.p.) group. In order to study the dopaminergic participation in the development of cross-sensitization, animals were pre-treated daily with haloperidol or vehicle, 15 min prior to each daily amphetamine or vehicle treatment throughout the entire acute and repeated drug regimen, and on day 5 or 13, respectively, were stressed or not. In order to study the dopaminergic participation in the expression of cross-sensitization following the acute amphetamine treatment, animals were pre-treated with haloperidol or vehicle, 15 min prior to foot-shock chamber exposure, and were stressed or not.

### 2.8. Flow-cytometry studies

Animals were bled by cardiac puncture under ketamine/xylazine anesthesia and the blood was collected into EDTA anticoagulated syringes. To analyze the total count of T- and B- and NK-cells in peripheral blood, three-colour immunofluorescence staining was performed and the intensity of stained cells was analyzed by flow cytometry (Cytoron Absolute flow cytometer; Ortho Diagnostic System, Raritan, NJ, U.S.A.). For three-colour staining, the samples were processed in two tubes. To determine T-lymphocytes (CD3+ cells), T-lymphocytes CD4+ (CD3+ CD4+ cells) and T-lymphocytes CD8+ (CD3+ CD8a+), 100 μl of peripheral blood were incubated with anti-CD3-FITC, anti-CD8a-PE and anti-CD4-Cy-Chrome MoAb in the first tube, and to determine NK-cells (CD161a+ CD3-cells) and B-lymphocytes (CD45RA+ CD3-cells), an equal volume of peripheral blood was incubated with CD161a-FITC, CD3-PE and CD45RA-Cy-Chrome MoAb. The incubation was for 30 min in the dark, followed by 15 min with $NH_4Cl$ lysis buffer and three washes in PBS (pH 7.2). After that, the cells were fixed with 2% formaldehyde, washed three times in PBS and finally resuspended in isoton II buffer prior to analysis in the cytometer.

Lymphocytes were gated on the basis of their characteristic light-scatter. Fluorescence intensity was depicted on a three-decade logarithmic scale and in single-parameter analysis as histograms. Absolute numbers of lymphocytes were calculated according to lymphocyte subset percentages and the absolute value of leukocytes (analyzed in a Coulter T-540 hematology analyzer).

### 2.9. Statistical analysis

Data from the acute or repeated amphetamine treatments were analyzed with a two-way ANOVA (drug treatment × shock status). There were two levels for the drug treatment factor (amphetamine or vehicle) and two levels for the shock status factor (shock or no shock). Data from the experiments with pre-treatments were analyzed with three-way ANOVA (haloperidol or vehicle), with two levels for the repeated drug treatment factor (amphetamine or vehicle), and two levels for the shock status factor (shock or no shock). Data represent means ± S.D., and corresponded to quadruplicate values of five different rats. Following significance in the overall ANOVA, post-hoc comparisons among means were performed with the Newman–Keul's test (the level of significance was set at $P<0.05$).

## 3. Results

### 3.1. Effects of foot-shock exposure on peripheral lymphocyte subpopulation number in animals previously submitted to acute amphetamine treatment

Exposure to foot-shock resulted in an immunosuppressive effect in animals previously subjected to an acute amphetamine

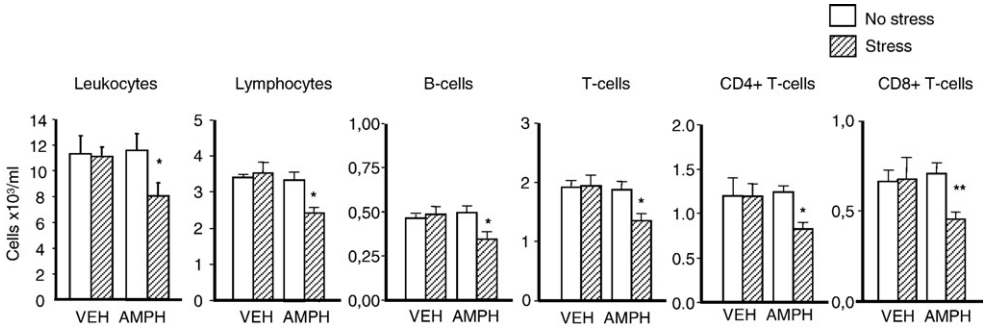


Fig. 1. Effect of foot-shock on the absolute number of peripheral leukocytes, lymphocytes, B-cells, T-cells, CD-4+ and CD8+ T-cells, in animals previously subjected to vehicle (VEH) or acute amphetamine (AMPH) treatments. Data show the mean +/− SD of five rats per group. *$P<0.01$ and **$P<0.05$. These data are representative of at least two independent experiments.

Table 1
Effect of foot-shock stress on blood NK CD161a+ CD3-cell population ($\times 10^3$ cell/ml) after four days of a repeated (2 mg/kg/day i.p.) or an acute (5 mg/kg i.p.) amphetamine treatment

| | Acute | | Repeated | |
|---|---|---|---|---|
| | Vehicle | Amphetamine | Vehicle | Amphetamine |
| No stress | 0.048±0.021 | 0.047±0.016 | 0.045±0.026 | 0.045±0.026 |
| Stress | 0.043±0.022 | 0.021±0.019 | 0.045±0.023 | 0.015±0.019 |

The results are the means±S.D. of five rats.

treatment, relative to their vehicle-treated controls. This effect was seen by a significant decrease in the absolute number of total peripheral leukocytes, and this decrease was supported by a significant decrease in peripheral lymphocytes number as well as in the absolute values of their subpopulations (Fig. 1).

Thus, the absolute number of peripheral B-lymphocytes, T-lymphocytes, CD4+ T-cells and CD8+ T-cells decreased significantly (Fig. 1) in rats previously exposed to an acute amphetamine administration and a subsequent foot-shock, compared to the remaining experimental groups. It is important to address that the relative percentages of B- and T-cells (18±6%, and 71±11% of total lymphocytes, respectively), as well as of CD4+ and CD8+ T-cells (61±9%, and 32±7% of T-lymphocytes, respectively), remained unchanged in all experimental groups.

A two-way ANOVA (acute drug treatment × shock) indicated statistically significant differences for peripheral leukocytes (acute drug: $F(1,12)=26.04$ $P<0.01$, shock: $F(1,12)=7.66$ $P<0.05$, acute drug × shock: $F(1,12)=12.67$ $P<0.01$), lymphocytes (acute drug: $F(1,12)=15.23$ $P<0.01$, shock: $F(1,12)=21.32$ $P<0.01$, acute drug × shock: $F(1,12)=6.18$ $P<0.05$), B-lymphocytes (acute drug: $F(1,12)=16.37$ $P<0.01$, shock: $F(1,12)=5.30$ $P<0.05$, acute drug × shock: $F(1,12)=15.19$ $P<0.01$), T-lymphocytes (acute drug: $F(1,12)=12.33$ $P<0.01$, shock: $F(1,12)=51.77$ $P<0.01$, acute drug × shock: $F(1,12)=8.92$ $P<0.01$), CD4+ T-cells (acute drug: $F(1,12)=23.46$ $P<0.01$, acute drug × shock $F(1,12)=10.96$ $P<0.01$), and CD8+ T-cells (acute drug: $F(1,12)=16.59$ $P<0.01$, acute drug × shock: $F(1,12)=10.98$ $P<0.01$). Fisher's post-hoc comparisons among means revealed that exposure to foot-shock reduced the absolute number of peripheral leukocytes ($P<0.01$), lymphocytes ($P<0.01$), B-lymphocytes ($P<0.01$), T-lymphocytes ($P<0.01$), CD4+ T-cells ($P<0.01$), and CD8+ T-cells ($P<0.05$) in peripheral blood of rats previously exposed to a acute amphetamine administration, compared to the remaining experimental groups.

In the case of peripheral NK-cells, although a decrease in the number of these cells was observed in the amphetamine-treated group compared with the remaining experimental groups, it did not reach statistical significance (Table 1).

### *3.2. Effects of foot-shock exposure on peripheral lymphocyte subpopulation number in animals previously submitted to repeated amphetamine treatment*

The results obtained after foot-shock in animals previously exposed to a repeated amphetamine treatment were similar to those seen after acute drug treatment. The statistical analysis revealed that exposure to foot-shock stress reduced the absolute numbers of peripheral leukocytes, lymphocytes, B-lymphocytes, T-lymphocytes, CD4+ T-cells and CD8+ T-cells for rats previously exposed to a repeated amphetamine administration, compared to the remaining experimental groups (Fig. 2).

A two-way ANOVA (repeated drug treatment × shock) indicated statistically significant differences for peripheral leukocytes (repeated drug: $F(1,12)=38.43$ $P<0.01$, shock: $F(1,12)=12.85$ $P<0.01$, repeated drug × shock: $F(1,12)=21.03$ $P<0.01$), lymphocytes (repeated drug: $F(1,12)=19.05$ $P<0.01$, shock: $F(1,12)=10.40$ $P<0.01$, repeated drug × shock: $F(1,12)=18.11$ $P<0.01$), B-lymphocytes (repeated drug: $F(1,12)=9.46$ $P<0.01$, shock: $F(1,12)=19.30$ $P<0.01$, repeated drug × shock: $F(1,12)=25.15$ $P<0.01$), T-lymphocytes (repeated drug: $F(1,12)=12.54$ $P<0.01$, shock: $F(1,12)=13.75$ $P<0.01$, repeated drug × shock: $F(1,12)=13.40$ $P<0.01$), CD4+ T-cells (repeated drug: $F(1,12)=20.01$ $P<0.01$, shock: $F(1,12)=19.13$ $P<0.01$, repeated drug × shock: $F(1,12)=18.76$ $P<0.01$), and CD8+ T-cells (repeated drug: $F(1,12)=6.77$ $P<0.05$, shock: $F(1,12)=6.60$ $P<0.05$, repeated drug × shock: $F(1,12)=7.24$ $P<0.05$). Fisher's post-hoc comparisons among means revealed that exposure to foot-shock reduced the absolute number of peripheral leukocytes ($P<0.01$), lymphocytes ($P<0.01$), B-lymphocytes ($P<0.01$), T-lymphocytes ($P<0.01$), CD4+ T-cells ($P<0.01$), and CD8+ T-cells ($P<0.05$) in peripheral blood of rats previously exposed

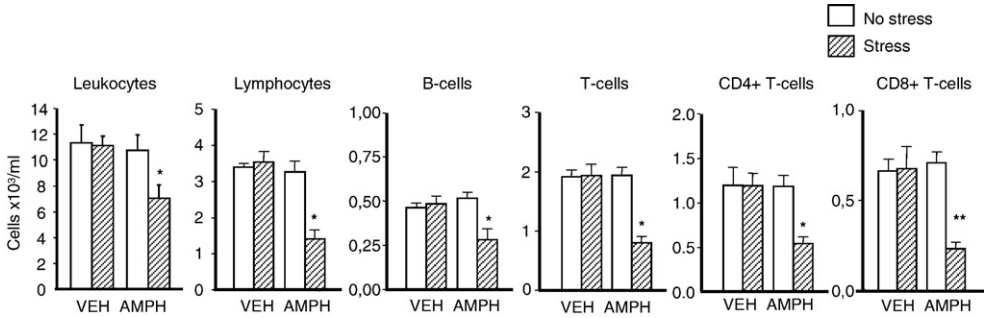


Fig. 2. Effect of foot-shock on the absolute number of peripheral leukocytes, lymphocytes, B-cells, T-cells, CD-4+ and CD8+ T-cells, in animals previously subjected to vehicle (VEH) or repeated amphetamine (AMPH) treatments. Data show the mean+/−SD of five rats per group. *$P<0.01$ and **$P<0.05$. These data are representative of at least two independent experiments.

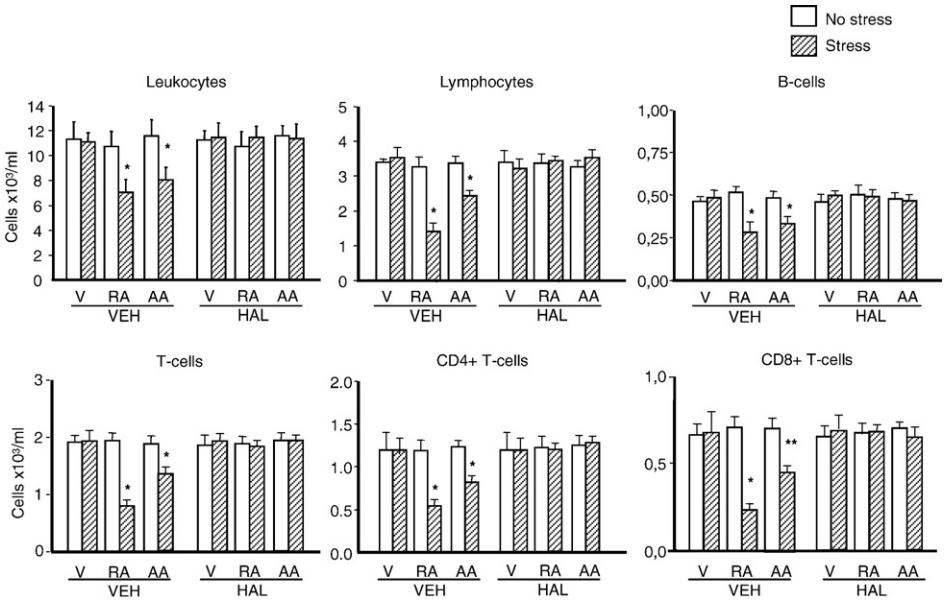


Fig. 3. Acquisition of amphetamine-sensitization: effect of foot-shock on the absolute number of peripheral leukocytes, lymphocytes, B-cells, T-cells, CD-4+ and CD8+ T-cells, in animals previously subjected to vehicle (VEH), repeated (RA) or acute (AA) amphetamine treatments with or without haloperidol (HAL) injections previous to each daily drug administration. Data show the mean +/− SD of five rats per group. *$P<0.01$ and **$P<0.05$. These data are representative of at least two independent experiments. The values of vehicle pre-treated rats were repeated in order to compare with those of haloperidol pre-treated rats.

to a repeated amphetamine administration, compared to the remaining experimental groups.

Also similar to those observed after acute amphetamine treatment, the absolute number of NK-cells in peripheral blood (Table 1), as well as the percentages of circulating lymphocytes subpopulations (data not show) were not modified by the repeated amphetamine treatment and/or foot-shock exposure in any experimental group.

### *3.3. Effect of haloperidol pre-treatment on the acquisition of amphetamine-induced sensitization on foot-shock-evoked changes in the peripheral lymphocyte subpopulations*

In order to evaluate the participation of dopaminergic mechanisms in the acquisition of cross-sensitization, we used a non-selective dopamine receptor antagonist pre-treatment to block dopamine receptors during the presence of amphetamine. Thus, the animals received an haloperidol pre-treatment 15 min before to each daily injection of amphetamine.

As described previously, animals treated to acute or repeated amphetamine displayed a decrease in the absolute number of different peripheral leukocytes populations (Figs. 1 and 2) following exposure to foot-shock stress. This effect was not evident in rats that received haloperidol injections prior to their daily amphetamine injections. No difference was observed among animals submitted only to haloperidol injections (acute or repeatedly) and then exposed or not to the shock stimulus and its appropriated controls (Fig. 3).

The absolute number of NK-cells in peripheral blood was not modified by haloperidol pre-treatment in any experimental group (Table 2).

### *3.4. Effect of haloperidol pre-treatment on the expression of amphetamine-induced sensitization on foot-shock-evoked changes in the peripheral lymphocyte subpopulations*

In order to evaluate the participation of dopaminergic mechanisms in the expression of cross-sensitization, we used a non-selective dopamine receptor antagonist to block dopamine receptors during the stress exposure, only after an acute amphetamine treatment. Thus, the animals received an haloperidol injection 15 min before the acute foot-shock stress exposure.

In this experiment, we showed that haloperidol administered prior to foot-shock prevented the acute amphetamine-induced decrease in the absolute number of peripheral leukocyte

Table 2
Acquisition of amphetamine-sensitization: effect of haloperidol pre-treatment on foot-shock stress on blood NK CD161a+ CD3-cell population ($\times 10^3$ cell/ml) after four days of an acute (5 mg/kg i.p.) or a repeated (2 mg/kg/day i.p.) amphetamine treatment

| Pre-treatment | Vehicle | | Repeated amphetamine | | Acute amphetamine | |
|---|---|---|---|---|---|---|
| | No stress | Stress | No stress | Stress | No stress | Stress |
| Vehicle | 0.044±0.027 | 0.040±0.019 | 0.047±0.036 | 0.039±0.018 | 0.051±0.029 | 0.042±0.011 |
| Haloperidol | 0.038±0.025 | 0.045±0.035 | 0.056±0.036 | 0.053±0.009 | 0.044±0.038 | 0.045±0.021 |

The results are the means±S.D. of five rats.

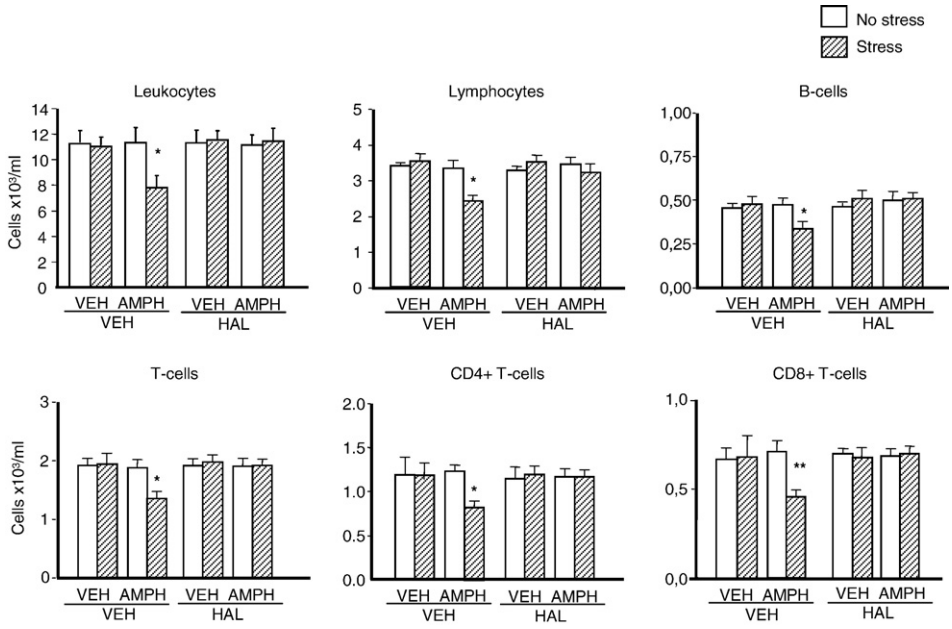


Fig. 4. Expression of amphetamine-sensitization: effect of foot-shock on the absolute number of peripheral leukocytes, lymphocytes, B-cells, T-cells, CD-4+ and CD8+ T-cells, in animals previously subjected to vehicle (VEH) or acute amphetamine (AMPH) treatments, and an haloperidol (HAL) or VEH injection previous to foot-shock stress. Data show the mean +/− SD of five rats per group. These data are representative of at least two independent experiments.

populations (Fig. 4). There is not any influence of haloperidol treated animals, exposed or not to the foot-shock stimulus, on the peripheral leukocyte population.

The absolute number of NK-cells in peripheral blood was not modified by haloperidol pre-treatment in any experimental group (Table 3).

## 4. Discussion

The present studies showed some interesting findings related to the influence of the psychostimulant treatment and stress on the immunosurveillance state and the possible mechanisms underlying this influence. Firstly, they show that following four days of an acute as well as a repeated amphetamine treatment, the exposure to a foot-shock stress session resulted in a clear decrease in the absolute numbers of total blood leukocytes, lymphocytes, B-cells, T-cells, CD4+ and CD8+ T-cells, while the NK population remained unchanged. Secondly, these studies show that a pre-treatment with a mixed $D_1/D_2$ receptor antagonist, such as haloperidol, prevented the stress-induced decrease in the lymphocyte subpopulations following amphetamine. Thirdly, they show that the reversion of haloperidol on the changes in the different lymphocyte subpopulations was evident when the receptor antagonist was administered before either the drug or the foot-shock stress (i.e. either during the acquisition or the expression of the cross-sensitization phenomenon, respectively). The biological changes induced by a repeated drug treatment could be different for those observed after a single drug exposition; thus, it is important to point out that in this study a single amphetamine dose, which had been previously shown to induce behavioral, neuroendocrine and neurochemical sensitization (Vanderschuren et al., 1999a), was also able to sensitize the foot-shock stress effects on the immune system The current findings indicate that, it is highly probable that the amphetamine-induced dopamine release from central sites may induce increases in the plasmatic circulating dopamine, which could underlie the drug effects on the immune system during the stress response. The present findings extend previous evidence from our lab showing that haloperidol reversed the foot-shock stress-induced decrease in the circulating T-cells and the reduction in delayed hypersensitivity response, after a repeated amphetamine treatment (Basso et al., 1999).

In this study, neither the stress exposure, nor the repeated or single amphetamine treatment, were sufficient stimuli by themselves to induce any change in the lymphocytes subpopulations, compared to the control group (vehicle—no stress group). However, when both variables, stress and drug, were associated (amphetamine—stress group) differences appeared. Thus, it is likely that the exposure to amphetamine sensitizes the animal to a subsequent event (i.e. foot-shock stress) that had no effect on its own at blood leukocyte levels. It should be addressed that the exposure of a novel environment (stress chamber) without foot-shock delivery also has no effect on the immune parameters measured, compared to control animals that were not exposed to this novelty (data not shown). The facilitatory effect following

Table 3
Expression of amphetamine-sensitization: effect of haloperidol pre-treatment on foot-shock stress on blood NK CD161a+ CD3-cell population ($\times 10^3$ cell/ml) after four days of an acute amphetamine (5 mg/kg i.p.) treatment

| Pre-treatment | Vehicle | | Acute amphetamine | |
|---|---|---|---|---|
| | No stress | Stress | No stress | Stress |
| Vehicle | 0.050±0.016 | 0.042±0.021 | 0.043±0.028 | 0.049±0.019 |
| Haloperidol | 0.043±0.018 | 0.050±0.025 | 0.042±0.021 | 0.051±0.023 |

The results are the means±S.D. of five rats.

drug and stress can be associated to the fact that common mechanisms at the CNS and/or immune system are triggered by both stimuli. Stressors as well as drugs of abuse acutely activate the mesocorticolimbic dopaminergic systems (Imperato et al., 1992; Kalivas and Duffy, 1995; Pontieri et al., 1995; Di Chiara and Imperato, 1988). In the context of the long-lasting effects of stress and drug exposure, the dopaminergic neurotransmission has also been implicated in the development and expression of sensitization to psychostimulants (Kalivas and Stewart, 1991; Robinson and Berridge, 2000; Vanderschuren et al., 1999b), and there is evidence of an immunomodulatory role for it (Bergquist et al., 1994, Bergman and Sautner, 2002). Dopaminergic immunomodulation is dominated by immunosuppressive effects, such as the induction of IL-6 and the inhibition of TNF-α via $D_2$ dopaminergic receptors (Ritchie et al., 1996), as well as the attenuation of the chemoatractant effect of IL-8 and the inhibition of endothelial adhesion (Sookhai et al., 2000). The immunosuppressive function of this monoamine is also supported by the downregulation of the proinflammatory hormone prolactin in the pituitary gland (Ben-Jonathan and Hnasko, 2001). Dopamine has been involved in the regulation of apoptosis of hematopoietic cells and can induce lymphocyte apoptotic cell death (Josefsson et al., 1996; Cosentino et al., 2002). Concerning the possibility that amphetamine-stimulated corticosterone release may mediate the effect referred to as cross-sensitization, it is likely that the psychostimulant-sensitized animals exhibit elevated levels of corticoids in response to a mild stressor such as the foot-shock stress applied in the present work (Barr et al., 2002). Since it is known that glucocorticoids also exhibit a well-known immunosuppressive effect (De Bosscher et al., 2000), a role for these hormones in the stress-induced immunosuppressive effects following either a single or a repeated amphetamine could be also considered. Furthermore, we have recently shown that another neurotransmitter, met-enkephaline, closely related with sensitization to psychostimulants, is similarly modified at both CNS and immune system (Assis et al., 2006).

It is well known that, depending on the nature and duration of the stressor and the immunological parameter under investigation, stress response can enhance, have no effect, or suppress these immunological parameters (Pruett, 2001). Our findings indicate that following 15 (1 mA, 3 s) inescapable foot-shocks, there were no changes in any of the blood subpopulations levels evaluated. Related to this stress protocol, for a functional parameter, Shurin et al. (1994) showed a decrease on the mitogenic response of blood and splenic lymphocytes in Lewis rats after only a single foot-shock exposition (1.6 mA, 5 s). Without paying attention to the differences between both protocols and the strains used, these results could indicate that the stress-induced changes at a functional level have a lower threshold than those necessary to modify the quantitative parameters of the immune response. It is important to remark that our foot-shock protocol can induce changes by itself on blood leukocytes levels if the number of expositions is increased to 45 foot-shocks (data not shown). In fact, we could see decrease in this immunological parameter after 15 foot-shocks only in those animals previously treated with amphetamine, which might have acted as a previous chronic stress. This change in circulating lymphocyte patterns after stress was also observed by Dhabhar et al. (1995) following 1 h restraint-stress, and was evidenced for a transient lymphopenia as lymphocytes migrate from the blood to tissues. By flow cytometry, they observed a decrease of 40–60% relative to the pre-stress baseline in lymphocyte (T-, B- and NK-cells) numbers after stress. It is debatable whether this kind of stress-induced decrease in blood leukocyte numbers could either enhance or suppress the subsequent immune response. It is possible that this transient lymphopenia represents a redistribution of immune cells from the blood to other body compartments, and that such a redistribution may serve to enhance immune tissue-surveillance (i.e. skin) (Dhabhar and McEwen, 1996; Viswanathan and Dhabhar, 2005), ultimately resulting in an realignment of cellular duties in response to an anticipated immunological challenge that could accompany injury. On the other hand, this transient blood cell depletion may lead to an increase in the susceptibility of the organism to an immune challenge in the blood, which is even more likely considering the reduction in the blood lymphoproliferative response and in the NK-cell cytotoxicity reported after stress (Shurin et al., 1994; Irwin et al., 1990). Thus, Ben-Eliyahu et al. (1991) have shown that acute stress results in immunosuppression, as measured by increased tumor burden when tumor cells were injected into the bloodstream following stress.

The lymphocyte migration, circulation and traffic are under the influence of CNS, with the sympathetic nervous system also playing a significant role in this process (Elenkov et al., 2000; Madden, 2003). Although the mechanisms by which catecholamines modulate lymphocyte distribution are not well established, one possible mechanism is that the sympathetic nervous system, which directly innervates the vascular smooth muscle, regulates the regional blood flow thereby changing the delivery of lymphocytes to post-capillary venules of tissues, and the opportunity for lymphocytes to enter tissues (Elenkov et al., 2000). The existence of dopamine receptors on several cell lines of the immune system was demonstrated (Bondy et al., 1996; Amenta et al., 1999; McKenna et al., 2002), and in view of the lack of dopamine innervation, plasma dopamine (Van Loon, 1983) or DOPA (Kvetnansky et al., 1992) could be a candidate to activate these receptors. It was shown (Bencsics et al., 1997) that the noradrenergic axon terminals in the spleen are able to take up dopamine from the circulation, convert it in part into noradrenaline, and release it as both dopamine and noradrenaline in response to neural activity. Since the exposure of an organism to any of a variety of stressors that increase sympathetic tone is accompanied by an increase in plasma concentrations of dopamine (Van Loon, 1983), it is highly probable that in our study the stress given to amphetamine-treated animals could induce an increase in the circulating dopamine which, as noted by Bencsics et al. (1997), could be taken up by noradrenergic terminals. Similar to noradrenaline, dopamine can be synthesized in the immune cells, which also express the transporters, receptors and synthesis enzymes for this neurotransmitter (Josefsson et al., 1996; Amenta et al., 2001; McKenna et al., 2002; Cosentino et al., 2002). Since, in

this study, we administered the dopamine $D_1/D_2$ receptor antagonist systemically, we cannot separate the effects of amphetamine on dopamine of CNS from any that might target immune cells directly. We can neither discard an influence of haloperidol, attributed at least in part to the alpha-1 receptor antagonist properties of this drug (Arnt and Skarsfeldt, 1998; Amargos-Bosch et al., 2003). Further studies are necessary to clarify these points.

In addition, it has been hypothesized that stress may induce changes in cellular trafficking by altering expression of cell adhesion molecules on lymphocytes or endothelial cells. Indeed, there is evidence suggesting that acute stress in humans (Mills and Dimsdale, 1996), mice (Tarcic et al., 1995), and rats (Bauer et al., 2001) promotes changes in cell adhesion molecules on lymphocytes and this phenomenon may mediate changes in the cell distribution associated with stress (Bauer et al., 2001), which could help to explain the present results. It has been shown that dopamine interacts directly with dopaminergic receptors in normal human T-cells and triggers β1 integrin-mediated T-cell adhesion to a major extracellular matrix component, fibronectin, while the dopamine receptor antagonists butaclamol and haloperidol suppress it (Levite et al., 2001). Although we did not evaluate the kinetics of the stress response, it is well known that many of the changes in circulating lymphocyte numbers are observed within 30 min and complete recovery occurs within 1–3 h after the cessation of the stress (Bauer et al., 2001). Thus, it is highly probable that the changes observed in the present study are mainly associated with trafficking and extravasation of lymphocytes across blood vessels and tissue barriers, rather than through a cell destruction process. Future experiments should focus on these aspects.

In conclusion, this work has demonstrated that an acute, as well as a repeated, treatment with a psychostimulant drug such as amphetamine, facilitates the occurrence of a decrease in the blood leukocytes, T-lymphocyte subpopulations (CD4+ and CD8+) and B-lymphocytes following a subsequent exposure to a stressor (i.e. foot-shock stress) without *per se* effect. This facilitation of the stress-induced effects following the psychostimulant treatment could be attributed to the fact that both the stress and drug trigger common biological mechanisms at the CNS which can influence the immune system. It has been described that either acute administration of amphetamine or stress induces an increase of the synaptic strength at excitatory synapses on midbrain dopaminergic neurons (Saal et al., 2003), as well as an augmented dopamine release in these mesolimbic terminals (Pacchioni et al., 2007). It is highly probable that in the present study a dopamine-enhanced release to foot-shock stress can occur as a result of amphetamine effects on excitatory synapses on the midbrain dopamine neurons, rendering neurons more vulnerable to the subsequent stress exposure. Since a pretreatment with haloperidol abolished the stress-induced decrease in the lymphocyte subpopulations following amphetamine, it is likely that this effect could be associated with the dopamine $D_1/D_2$ receptor antagonist properties of haloperidol, by preventing the central dopamine systems influencing the immune system during the stress response. Considering that stress-induced changes in leukocyte redistribution may not only enhance the tissue immunosurveillance but also exacerbate immunopathology during inflammatory or autoimmune diseases, the present results obtained with haloperidol might have a potential therapeutic relevance. Future experiments should clarify the specific contribution of distinct dopamine receptor populations.

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