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Methylenedioxymethamphetamine (Ecstasy) Decreases Neutrophil Activity and Alters Leukocyte Distribution in Bone Marrow, Spleen and Blood

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Key Words
Methylenedioxymethamphetamine · Oxidative burst · Phagocytosis · Corticosterone · Leukocyte traffic · Neutrophil

Abstract
Objective: Looking for possible neuroimmune relationships, we analyzed the effects of methylenedioxymethamphetamine (MDMA) administration on neuroendocrine, neutrophil activity and leukocyte distribution in mice. ***Methods:*** Five experiments were performed. In the first, mice were treated with MDMA (10 mg/kg) 30, 60 min and 24 h prior to blood sample collection for neutrophil activity analysis. In the second experiment, the blood of naïve mice was collected and incubated with MDMA for neutrophil activity in vitro analysis. In the third and fourth experiments, mice were injected with MDMA (10 mg/kg) and 60 min later, blood and brain were collected to analyze corticosterone serum levels and hypothalamic noradrenaline (NA) levels and turnover. In the last experiment, mice were injected with MDMA 10 mg/kg and 60 min later, blood, bone marrow and spleen were collected for leukocyte distribution analysis. ***Results:*** Results showed an increase in hypothalamic NA turnover and corticosterone serum levels 60 min after MDMA (10 mg/kg) administration, a decrease in peripheral blood neutrophil oxidative burst and a decrease in the percentage and intensity of neutrophil phagocytosis. It was further found that MDMA (10 mg/kg) treatment also altered leukocyte distribution in blood, bone marrow and spleen. In addition, no effects were observed for MDMA after in vitro exposure both in neutrophil oxidative burst and phagocytosis. ***Conclusion:*** The effects of MDMA administration (10 mg/kg) on neutrophil activity and leukocyte distribution might have been induced indirectly through noradrenergic neurons and/or hypothalamic-pituitary-adrenal axis activations.

Introduction

Central nervous system (CNS) regulation of immune activity, including host immunity and resistance to bacterial infection, was initially reported after empirical observations in antiquity. However, Selye [1] reported that animals exposed to a myriad of stressors presented a decrease in thymus and lymph node size. In contrast, Besedovsky et al. [2] reported that antigen exposure increased

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the electrical activity of neurons in the ventromedial area of the rat hypothalamus by more than 100%. These pioneer works clearly showed the existence of a bidirectional relationship between the CNS and the immune system.

Drugs that act on the CNS such as diazepam [3–7], amphetamine [8–10] as well as recreational abuse drugs [11–17] are known to modify some immunological responses in laboratory animals. There have been numerous clinical reports on the association between infectious diseases and the use of illicit drugs [18–20]. Immunomodulation induced by marijuana, cocaine, heroin, opiates and other amphetamines including Ecstasy was reported to be receptor-mediated, either directly by interaction with specific receptors on immune cells or indirectly by reaction with similar receptors present in the nervous system [21–26].

The amphetamine derivative, methylenedioxymethamphetamine (MDMA or Ecstasy) exerts immunosuppressive actions [11–17]. It has been reported [27, 28] that Ecstasy users are often more susceptible to infectious diseases. However, the precise mechanism of this increased susceptibility is not completely clear. In animal models, MDMA decreased neutrophil phagocytosis [11], suppressed the generation of lipopolysaccharide (LPS)-induced proinflammatory cytokines such as tumor necrosis factor- α and interleukin (IL)-1β [29, 30] and increased the LPS-induced anti-inflammatory cytokine IL-10 production [31]. In addition, Boyle and Connor [32] reported that MDMA suppressed interferon-γ (IFN-γ) secretion and signaling in vivo in mice. In humans, the number of circulating natural killer cells was reported to be increased by MDMA as well as tumor growth factor-β and IL-10 levels. A switch was also observed from Th1-type cytokines (IL-2 and IFN-γ) to Th2-type cytokines (IL-4 and IL-10) in vivo after MDMA administration [14–17, 33]. Additionally, it was found that MDMA decreased the number of CD4+ T-helper cells [13] and reduced phytohemagglutinin-stimulated T cell proliferation [33].

Physical, psychological and chemical stressors were found to modify innate immune activity [34–37]. MDMA was also found to be a stressor agent in laboratory animals [11, 17]. Thus, it may be that the effects of MDMA on innate immunity are due to possible stressor-induced effects. The present experiment was thus designed to search for the effects of MDMA on neutrophil activity and leukocyte distribution in mice, looking for a direct or indirect mechanism of action. Neutrophils were taken as an innate immune target for the present study because only a single paper found in the literature analyzed the effects of MDMA on this cell activity [11]. Mice were chosen as experimental subjects because it is common knowledge that MDMA pharmacokinetics vary with animal species [38–41], and being the parent drug it is the major constituent present in the plasma of mice after MDMA administration [41].

Materials and Methods

Animals

For the study, 112 male BALB/c mice from our own colony, weighing 25–35 g and approximately 10 weeks old, were used. The animals were housed under conditions of controlled temperature (22 ± 2°C), humidity (65–70%) and artificial lighting (12 h light/12 h dark, lights on at 7.00 h) with free access to rodent chow and water. The experiments were performed in a different room with similar housing conditions, to which the animals were transferred and maintained in their home cages 7 days before the beginning of the experiments. The animals were housed and used in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animal Resources of the School of Veterinary Medicine, University of São Paulo, Brazil.

Drug

MDMA used for the experiments was extracted from tablets provided by the Brazilian Federal Police Department (São Paulo, SP, Brazil). Upon arrival at the laboratory, the drug was extracted and purified from the tablets as suggested by Lacava et al. [42]. The obtained MDMA (99% purity level) was dissolved in 0.9% NaCl solution to a concentration of 10 mg/kg. This dose was chosen based on a previous experiment conducted in our laboratory that evaluated 6 different doses (0.2, 1.0, 5.0, 8.0, 10, 20 mg/kg) [unpubl. data]. Vehicle (0.9% NaCl) was administered alone to the mice of the control group. Solutions were intraperitoneally administered in a volume of 0.1 ml/10 g.

Hypothalamic Noradrenalin Determinations

Stressors are known to increase noradrenalin (NA) activity within the CNS [34–37]. Because of this, NA activity was analyzed after MDMA administration. For the analysis, animals were decapitated after euthanasia under deep CO_2 anesthesia. The brains were immediately removed and dissected, with the hypothalamus excised as rapidly as possible (not more than 3 min) and then weighed and frozen on dry ice. Samples were homogenized by sonication in 0.1 M perchloric acid containing 1.1 mM sodium metabisulfite and 0.54 mM disodium EDTA and centrifuged for 30 min at 11,200 *g*. The supernatants were stored frozen at –80°C until analysis. NA and its metabolite vanillylmandelic acid (VMA) were measured using a high performance liquid chromatography system (model 6A, Shimadzu, Kyoto, Japan) with an electrochemical detector (model 6A, Shimadzu, Kyoto, Japan) as described in detail elsewhere [43]. Briefly, samples (20 μl) were loaded into a sample injector and the mobile phase was delivered at a constant rate of 1 ml/min through a C18 analytical column (Shimpak, ODS, Kyoto, Japan) placed inside a column heater (35°C). The mobile phase (pH 2.9) consisted of 0.02 M disodium phosphate, 0.02 M citrate, 0.11 mM disodium EDTA, 2.75 mM heptanesul-

fonic acid and 10% methanol. The signal from the detector was recorded with an integrator (Chromatopac, Shimatzu, Kyoto, Japan); the runtime for each sample was 25 min. The quantification limits were 0.02 ng for both NA and VMA. Inter- and intra-assay variation coefficients were smaller than 15% and the recuperation indices higher than 80% for both substances. Concentrations of NA and VMA were expressed as nanograms per gram of tissue.

Serum Corticosterone Determination

Corticosterone is the most abundant circulating steroid secreted by rodents and is considered a good indicator of adrenocortical function in this order [44, 45]. This hormone was determined in serum using commercially available kits (Coat-a-Count, DPC). This procedure is based on a solid-phase radioimmunoassay in which ^{125}I-labeled corticosterone competes for a fixed time with the corticosterone present in the murine sample for antibody sites. Serum samples were assayed directly without extraction or purification. To decrease data variability of serum corticosterone levels, the mice were handled daily for a period of 7 days for habituation to the experimental conditions of euthanasia and blood collection. Animals were euthanized for decapitation after deep CO_2 anesthesia. To minimize the reported circadian variations of serum glucocorticoid levels [46], control and experimental animals were intermixed for euthanasia and their blood was taken at the same time of the day, i.e. between 8.00 and 9.00 h, 60 min after MDMA and vehicle administration.

Flow Cytometry

A flow cytometer (FACSCalibur®, Becton Dickinson Immunocytometry Systems, San Jose, Calif., USA), interfaced with a Macintosh G4 computer, was used. Data from 7,000 events were collected in list mode and analyzed using Cell Quest software (Becton Dickinson Immunocytometry Systems). Cell populations were identified based on their properties on forward scatter versus side scatter (FSC/SSC) plots, as described elsewhere [47]. Fluorescence data were collected on log scale. Green fluorescence from 2′,7′-dichlorofluorescein diacetate (DCF; Molecular Probes, Eugene, Oreg., USA) was measured at 530 ± 30 nm (FL1 detector). Red fluorescence from propidium iodide (PI)-labeled *Staphylococcus aureus* (SAPI; Sigma, St. Louis, Calif., USA) was measured at 585 ± 42 nm (FL2 detector). PI and DCF fluorescence were analyzed after compensation to correct for possible crossover between PI and DCF signals.

Oxidative Burst and Phagocytosis: MDMA in vivo

Treatment

Blood was collected after deep CO_2 anesthesia for decapitation. The substances used for triggering the oxidative burst were phorbol myristate acetate (PMA/100 ng) and *S. aureus* (2.4×10^9 bacteria/ml). Briefly, 100 μl of whole blood (2×10^5 cells/100 μl) was mixed with 200 μl of DCF (0.3 mM) in phosphate-buffered saline (PBS) and 100 μl of either SAPI or PMA in different polypropylene tubes. Samples were incubated at 37°C during 30 min. Reactions were stopped by adding 2 ml of cold EDTA solution (3 mM) in order to stop phagocytosis reaction. After centrifugation (250 *g* for 10 min), erythrocytes were lysed from all samples with sterile 0.2% NaCl (2 ml per tube) for 20 s. Immediately thereafter, 1.6% NaCl sterile solution (2 ml) was added to each sample to restore isotonicity. These procedures were repeated twice for complete erythrocyte lysis. Samples were then centrifuged (250 *g* for 10 min) and the cell pellets resuspended in 200 μl of cold PBS. Direct measurements of mean fluorescence of green and red channels were recorded as oxidative burst and phagocytosis, respectively, as proposed elsewhere [48].

Quantification of oxidative burst was estimated by the geometric mean of DCF fluorescence/cell. The intensity of phagocytosis was expressed by the geometric of SAPI fluorescence ingested by neutrophils. Percent phagocytosis (percentage of neutrophils ingesting bacteria) was expressed by the number of neutrophils with red fluorescence, divided by the total number of cells, multiplied by 100 [47].

Oxidative Burst and Phagocytosis: MDMA in vitro Assay

Blood was collected after decapitation under deep CO_2 anesthesia. Briefly, 100 μl of blood was incubated with MDMA solutions to give a final concentration of 66.7, 667, and 6,670 ng/ml at 37°C during 60 min. After that, oxidative burst and phagocytosis were assessed as described above. These concentrations were selected based on the concentration found in blood analyses performed 60 min after MDMA (10 mg/kg) administration [42]. Additional tubes were used for each sample in order to verify cell viability by adding PI at the time of analysis.

MDMA Effects on Leukocyte Traffic

Mice were under deep CO_2 anesthesia and blood samples were taken by cardiac puncture with plastic syringes containing 50 μl of sodium heparin. Immediately afterwards, samples were transferred to microtubes until analysis. The spleen and right femurs from each mouse were then removed aseptically and transferred to tubes containing cold RPMI-1640 solution. The spleens were weighed prior to grinding, to determine the relative spleen weight.

Leukocyte Counts and Blood Parameters

White blood cell and red blood cell counts and hemoglobin were determined with a CC530 automatic cell counter (CELM®, São Paulo, SP, Brazil). The hematocrit was determined in capillary tubes by the microhematocrit method. Differential cell count was carried out on blood smears using standard morphological criteria after staining with Rosenfeld's dye to establish the percentages of each cell type.

Bone Marrow and Spleen Cells Counts

The epiphyses of the femurs were cut off on both ends and the bone marrow was flushed out with 5 ml of ice-cold RPMI-1640. Splenocyte suspension was obtained by grinding the tissue between two frosted microscope slides. Red blood cells were lysed with lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM Na_2EDTA, pH 7.2–7.4). Cells were washed with PBS and total cell numbers of bone marrow and spleen were determined under a light microscope in Neubauer chambers. The viable cells were determined by Tripan blue dye.

Experimental Design

Five experiments were done in accordance with Good Laboratory Practice protocols and quality assurance methods. In the first experiment, 40 mice were divided randomly and equally into 4 groups: 3 experimental groups (E1, E2 and E3) and 1 control group (C). To eliminate any confounding effect of injection stress, all animals used in this study received 3 injections as suggested

elsewhere [31] and presented in table 1. Thus, mice of group C were treated 3 times with MDMA vehicle and analyzed 30 min after the last administration. Mice of groups E1, E2 and E3 were treated twice with MDMA vehicle and once with 10 mg/kg of MDMA and were analyzed 30, 60 min and 24 h after MDMA administration, respectively. The animals were sacrificed immediately after the treatments and blood samples were collected for neutrophil oxidative burst and phagocytosis determinations as described above.

MDMA in vitro effects were studied in the second experiment. The blood samples of 32 naïve mice were collected into sodium heparin microtubes. Then, 100 μl of blood were incubated with 0.9% NaCl (control group) or different MDMA concentrations (66.7, 667, and 6,670 ng/ml) for 60 min at 37°C. After incubation, the samples were processed and neutrophil oxidative burst and phagocytosis were measured as described above. At the time of the measurements, 40 μl PI (100 ng/ml) was added into an additional tube (containing only cells), previously incubated with 0.9% NaCl or the different MDMA concentrations as described above, to analyze cell viability.

The 3rd and 4th experiments used 20 mice, divided randomly and equally into 2 groups: 1 control (C) and 1 experimental (E1) group. Group C received 0.9% NaCl and group E1 received MDMA (10 mg/kg). At 60 min after the injections, the animals were sacrificed for blood and brain collection for serum corticosterone levels and NA determinations, respectively.

In the 5th and final experiment, 20 mice were divided randomly and equally into 2 groups: 1 control group (C) and 1 experimental (E1). Group C received 0.9% NaCl and group E1 received MDMA (10 mg/kg). At 60 min after the injections, the animals were anesthetized with CO_2 and euthanized. Blood, spleen and bone marrow were then collected and processed to assess the effects of MDMA on leukocyte traffic.

Statistical Analysis

Bartlet's test showed that the data obtained in all experiments were parametric. Thus, they were analyzed by the Student's t test for independent pairs or by one-way analysis of variance followed by Tukey's test for multiple comparisons. The level of significance was set at $p < 0.05$. All statistics were done using GraphPad InStat version 3.01 for Windows. Data were expressed as means ± SD.

Table 1. Scheme of treatment used to eliminate the confounding effects of stress induced by injection in the first experiment

Groups	Time prior to sacrifice		
	24 h	60 min	30 min
Control	vehicle	vehicle	vehicle
E1	vehicle	vehicle	MDMA
E2	vehicle	MDMA	vehicle
E3	MDMA	vehicle	vehicle

All animals received 3 injections: E1, E2, and E3 groups were treated twice with vehicle and once with MDMA and the samples were analyzed 30 min, 60 min, and 24 h after MDMA (10 mg/kg) injection, respectively. The control group was treated 3 times with vehicle and was analyzed 30 min after the last injection.

Results

MDMA in vivo Treatment Decreases Neutrophil Activity

We first analyzed the MDMA effects on neutrophil oxidative burst and phagocytosis 30, 60 min and 24 h after injections. As can be seen in figure 1a, 30 and 60 min after administration, mice that received MDMA (10 mg/kg) presented a decrease in neutrophil oxidative burst induced by SAPI [$F(3, 34) = 3.288$; $p < 0.05$]. Sixty minutes after MDMA (10 mg/kg), a decrease in neutrophil oxidative burst induced by PMA [$F(3, 36) = 4.912$; $p < 0.05$] was also observed (fig. 1b). Furthermore, a decrease in both intensity [$F(3, 36) = 29.432$; $p < 0.05$] (fig. 1c) and percentage of neutrophil phagocytosis [$F(3, 36) = 22.904$; $p < 0.05$] (fig. 1d) was detected 60 min after MDMA administration.

MDMA in vitro Treatment Does Not Decrease Neutrophil Activity and Viability

A decrease in neutrophil activity was observed 60 min after MDMA (10 mg/kg) in vivo treatment. Thus, we designed an experiment to verify whether this effect was mediated via a direct action on neutrophils. Furthermore, we also verified whether MDMA induces cell death. As can be seen in table 2, in vitro MDMA treatment induced no effects on neutrophil activity and viability.

MDMA Injection Increases Corticosterone Serum Levels and Hypothalamic NA Turnover

Our previous results showed that the effects of MDMA on neutrophil activity were not the consequence of a direct drug action on immune cells. Hence, we hypothesize that the observed decrease in neutrophil activity could have been induced indirectly via activation of the hypothalamic-pituitary-adrenal axis. As can be seen in table 3, MDMA (10 mg/kg) treatment increased corticosterone serum levels [$t(16) = 13.665$; $p < 0.0001$], decreased hypothalamic NA concentration [$t(17) = 2.564$; $p = 0.0201$] and increased hypothalamic NA turnover [$t(17) = 2.220$; $p = 0.0403$].

MDMA in vivo Treatment Modifies Leukocyte Traffic

Finally, we thought it would be interesting to analyze the effects of MDMA (10 mg/kg) on leukocyte traffic

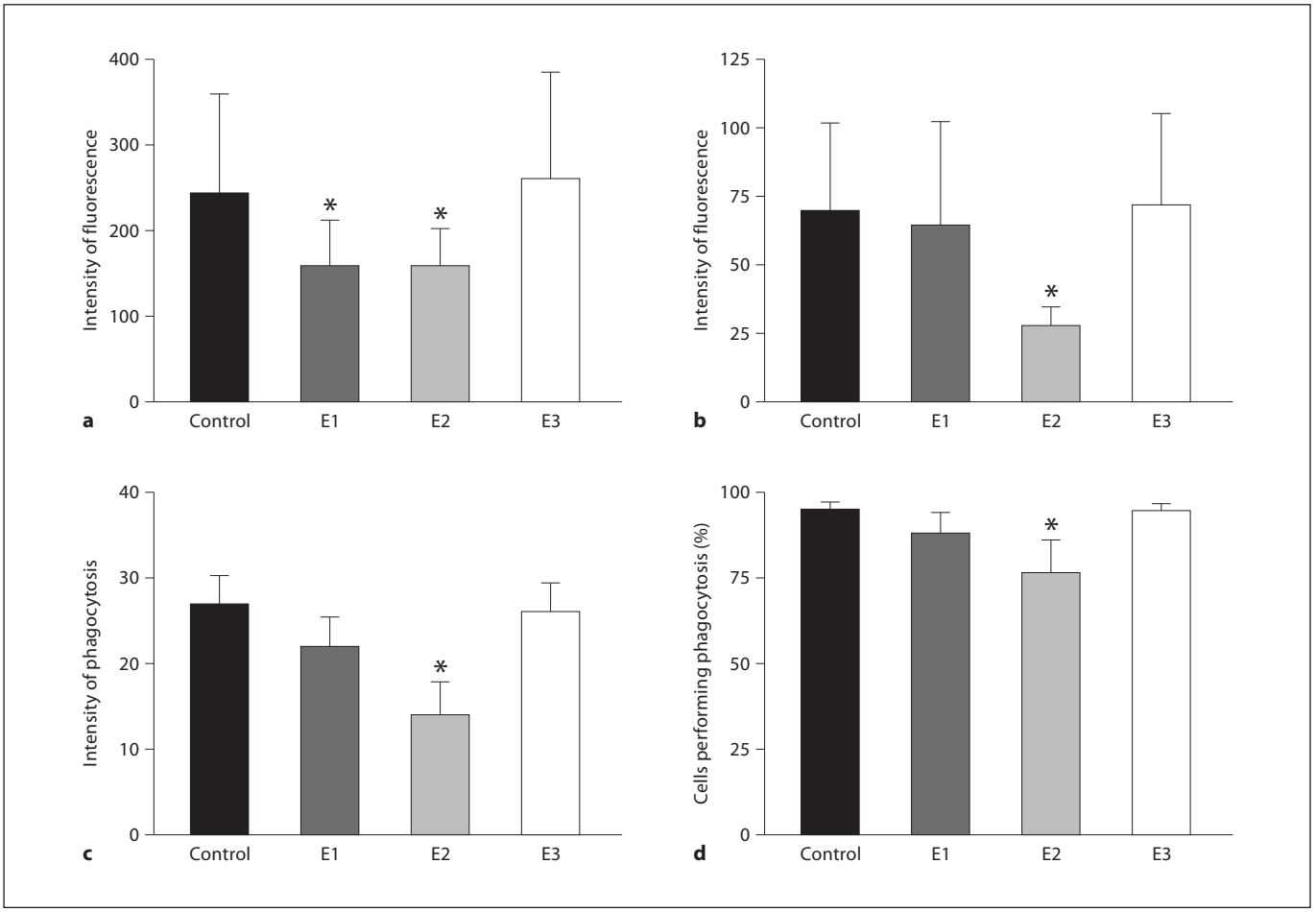

Fig. 1. MDMA (10 mg/kg) effects on neutrophil oxidative burst and phagocytosis measured 30, 60 min and 24 h after drug administration. The figure shows the neutrophil oxidative burst induced by SAPI (**a**) and PMA (**b**) and the intensity (**c**) and the percentage of phagocytosing neutrophils (**d**). Data are means ± SD of 10 mice per group. * $p < 0.05$ compared to control; ANOVA followed by Tukey's test.

Table 2. Effects of MDMA (10 mg/kg) on neutrophil activity and cell viability in vitro

Parameters	Groups			
	control	MDMA (67 ng/ml)	MDMA (670 ng/ml)	MDMA (6,700 ng/ml)
Oxidative burst induced by SAPI	90.17 ± 6.13	68.16 ± 21.21	81.83 ± 22.50	82.01 ± 24.11
Oxidative burst induced by PMA	23.82 ± 7.82	22.08 ± 6.36	25.70 ± 7.13	23.72 ± 8.20
Intensity of phagocytosis	46.40 ± 5.17	47.91 ± 8.93	50.76 ± 7.70	48.87 ± 3.39
Percentage of phagocytosis	93.92 ± 1.53	92.24 ± 4.34	92.35 ± 3.29	92.72 ± 1.94
Cell viability	12.39 ± 3.50	12.62 ± 24.54	11.36 ± 3.64	10.56 ± 5.26

Data are means ± SD of 8 mice per group. $p > 0.05$ compared to control; ANOVA followed by Tukey's test.

Table 3. Effects of MDMA (10 mg/kg) on corticosterone serum levels and hypothalamic NA levels and turnover measured 60 min after drug administration

Levels	Groups	
	control	MDMA
NA, ng/g	1,290.20 ± 248.40	1,073.90 ± 93.80*
VMA, ng/g	1,840.00 ± 237.1	1,655.10 ± 182.40
VMA/NA	1.44 ± 0.10	1.53 ± 0.07*
Corticosterone, ng/ml	71.66 ± 21.89	451.31 ± 80.42**

Data are means ± SD of 10 mice per group.
* $p < 0.05$, ** $p < 0.0001$ compared to control. Student's t test.

Table 4. Effects of MDMA (10 mg/kg) on leukocyte traffic measured 60 min after drug administration

Parameters analyzed	Groups	
	control	MDMA
Blood		
Leukocytes, $\times 10^6$/mm^3	3.94 ± 0.57	4.48 ± 0.52
Lymphocytes, %	60.50 ± 6.04	50.80 ± 3.73**
Monocytes, %	2.00 ± 0.66	2.10 ± 1.10
Neutrophils, %	38.40 ± 7.58	47.40 ± 4.88**
Erytrocytes, $\times 10^6$/mm^3	8.29 ± 0.71	9.32 ± 1.01*
Hematocrit, %	47.80 ± 1.13	51.60 ± 1.43***
Hemoglobin, g/dl	13.43 ± 0.53	14.85 ± 0.49***
Spleen		
Total number of cells, $\times 10^6$	197.77 ± 31.95	238.05 ± 42.31*
Relative weight, g/100 g	0.48 ± 0.02	0.45 ± 0.02*
Bone marrow		
Total number of cells, $\times 10^6$	150.00 ± 20.67	132.51 ± 6.96*

Data are means ± SD of 10 mice per group.
* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$ compared to control, Student's t test.

60 min after its injection. Table 4 shows that MDMA increased not only the circulating erythrocyte counts [$t(14) = 2.338$; $p = 0.0347$] but also the hematocrit [$t(18) = 6.582$; $p < 0.0001$] and hemoglobin [$t(18) = 6.171$; $p < 0.0001$] values. We also observed a decrease in the number of circulating lymphocytes [$t(18) = 4.318$; $p = 0.0004$] and an increase in that of circulating neutrophils [$t(18) = 3.154$; $p = 0.0055$]. Additionally, we observed a decrease in bone marrow cellularity [$t(16) = 2.405$; $p = 0.0286$] and an increase in spleen cellularity [$t(16) = 2.279$; $p = 0.0367$]. A decrease in spleen/body relative weight [$t(14) = 2.395$; $p = 0.03$] was also observed.

Discussion

The results reported here showed neurochemical, endocrine and immunological effects 60 min after MDMA (10 mg/kg) administration in mice. These conclusions were based on the following observed data: (1) decreased hypothalamic NA concentration; (2) increased hypothalamic NA turnover; (3) increased corticosterone serum levels; (4) decreased neutrophil oxidative burst induced by SAPI and PMA; (5) decreased neutrophil percentage and intensity of phagocytosis; (6) increased hematocrit and hemoglobin values and increased number of circulating erythrocytes; (7) decreased number of circulating lymphocytes; (8) increased number of circulating neutrophils; (9) decreased bone marrow cellularity; (10) increased spleen cellularity and (11) decreased spleen relative weight.

Stressors are known to increase NA activity in the CNS as indicated by NA levels and turnover [34 35]. In particular, areas of the limbic system such as the hypothalamus, hippocampus and amygdala were shown to present a rapid and significant increase in NA turnover after stressors [36, 37]. These stress-induced effects were reported to be the consequence of an increase in tyrosine hydroxilase activity [49]. A positive correlation was found between the decrease in NA levels following stressors and the stress responses in laboratory animals [10, 50]. Exposure to virtually any stressor activates the HPA axis and results in a readily discernible elevation in plasma corticosterone levels. Assessment of plasma corticosterone has been used as a dependent measure of stress response [35, 51]. In this work, we showed that MDMA (10 mg/kg) administration decreased hypothalamic NA concentration, increased hypothalamic NA turnover and increased corticosterone serum levels. Thus, the neuroendocrine results described here strongly suggest that MDMA (10 mg/kg) acts as a chemical stressor in mice, a hypothesis supported by data reported elsewhere in animals and humans [11, 17].

Stressors are known to alter innate immune cell function [51–54]. Glucocorticoids are important neuroendocrine mediators of immune responses, particularly of monocytes and neutrophils [55–58]. A decrease in circulating neutrophil oxidative burst (induced by SAPI and PMA) and phagocytosis, two measures indicative of neutrophil activity, is now reported 60 min after MDMA (10

mg/kg) administration. Connor [11] showed that a similar MDMA dosing schedule suppressed the neutrophil oxidative burst in response to opsonized zymosan (a phagocytic stimulus) but not in response to PMA in rats.

Intracerebroventricular corticotropin-releasing factor infusion is known to increase the concentrations of NA in the hypothalamus as well as corticosterone in serum [59]. These results clearly suggested that corticotropin-releasing factor administration decreased neutrophil phagocytosis, most probably via an increase in corticosterone serum level [59]. Neutrophils incubated with corticosterone showed a significant reduction in superoxide production [47]. Thus, it may be suggested that the decreased neutrophil activity observed here 60 min after MDMA treatment might have been induced indirectly via hypothalamic NA neurons and/or HPA axis activations. As presently shown, MDMA increased both hypothalamic NA activity and corticosterone serum levels in mice. Our data showed no effects of MDMA administration on neutrophil activity 24 h after its administration, most probably because corticosterone serum levels had already returned to their basal levels. Indeed, the increase in corticosterone levels was observed 30 min, but not 6 h, after MDMA administration [61]. Futhermore, in the present experiment, in vitro MDMA exposure was unable to change neutrophil activity (oxidative burst and phagocytosis), a fact that strongly suggests this drug has no direct effects on neutrophils. Several works measuring the in vitro effects of MDMA on cytokine production and network found no effects [11, 30, 62]. Together, these data reinforce our previous statement of an indirect action of MDMA on neutrophil activity.

It is generally accepted that the number and proportion of leukocytes in blood provide a good representation of the state of activation of the immune system as well as of the pattern of immune cell distribution in the body [66]. Glucocorticoids are known to modify the leukocyte traffic and cell distribution in peripheral blood, bone marrow and spleen [8, 9, 63–65]. In the present work, we showed a decrease in the number of peripheral blood lymphocytes and an increase in that of neutrophils. We also observed a decrease in bone marrow cellularity, an increase in spleen cellularity and an increase in corticosterone serum levels. Thus, and as stated above, it seems legitimate to suggest that the effects of MDMA administration on immune cell traffic reported here also depend on corticosterone serum levels. Some reports [66] (a) showed the relevance of adrenal hormones for leukocyte distribution; (b) adrenalectomy abrogated the effects of an acute stressor on leukocyte distribution, and (c) administration of RU-486 (a glucocorticoid receptor antagonist) to adrenalectomized animals changed leukocyte distribution in a manner similar to that observed in intact animals during stress. Together, these results [66] suggest that corticosterone, acting on type II steroid receptors, changes blood leukocyte distribution. According to Sendo et al. [67], stress-induced increase in endogenous glucocorticoids in peripheral blood affected neutrophil survival and thus neutrophil accumulation in peripheral blood. Glucocorticoids are known to delay neutrophil apoptosis, and chronic glucocorticoid treatment increased neutrophil number in peripheral blood and the spleen [68–71].

However, it should not be forgotten that primary and secondary lymphoid organs such as the spleen are extensively innervated by noradrenergic sympathetic nerve fibers [72, 73] and also that NA acting on β-adrenoreceptors modifies neutrophil activity [74]. We showed increased blood erythrocyte counts, hematocrit and hemoglobin and a concomitant decrease in spleen relative weight. It is known that the sympathetic nervous system as well as sympathomimetic drugs produce splenic capsule constriction, increasing peripheral blood erythrocyte and hematocrit values [75–78]. These effects are in the same direction as those reported here for MDMA.

MDMA was shown to release dopamine and norepinephrine [79] and to act directly as an agonist on serotonin 5-HT_2, α_2-adrenergic, dopaminergic, and muscarinic M-1 receptors. The presence of neurotransmitter transporters in human and murine leukocytes further supports the involvement of neurotransmitters in the regulation of the immune function [80–83]. Pacifici et al. [13] showed a strong serotonergic activity component in the effects of MDMA on immune system modulation in humans. Specifically, they reported that an acute dose of MDMA (100 mg) produced a time-dependent decrease in CD4 T-helper cells, a decrease in the functional responsiveness of lymphocytes to mitogenic stimulation, a simultaneous increase in natural killer cells and a simultaneous increase in cortisol and prolactin stimulation kinetics. Thus, the MDMA effects reported here may also depend on the sympathetic nervous system and/or on other neurotransmitter activations. To clarify our results, further work is now in progress using 6-hydroxydopamine-induced chemical sympathectomy and inhibitors of corticosterone synthesis (metyrapone).

Taken together, the present data might suggest that a neuroimmune mechanism is responsible for the effects of MDMA on neutrophil activity. Neutrophils are the first

line of cell defense against infection [84]. Thus, the present data are in line with those that suggest that MDMA might decrease human and animal resistance to infectious disease. However, it seems relevant to remember that the effects of MDMA depend on the animal species selected [41], so that data generated in mice cannot be simply extended to other animal species including humans. Furthermore, humans are usually exposed to repeated and not single MDMA doses.

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