In utero exposure to dexamethasone causes a persistent and age-dependent exacerbation of the neurotoxic effects and glia activation induced by MDMA in dopaminergic brain regions of C57BL6/J mice.

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Abstract

Clinical and preclinical evidence indicates that prenatal exposure to glucocorticoids may induce detrimental effects in the offspring, including reduction in fetal growth and alterations in the CNS. On this basis, the present study investigated whether *in utero* exposure to high levels of glucocorticoids is a risk factor that may lead to an exacerbation of the central noxious effects induced by psychoactive drugs consumed later in life. To this end, pregnant C57BL6/J dams were treated with dexamethasone (DEX, 0.05 mg/kg per day) from gestational day 14 until delivery. Thereafter, the male offspring were evaluated to ascertain the magnitude of dopaminergic damage, astrogliosis and microgliosis elicited in the nigrostriatal tract by the amphetamine-related drug 3,4-methylenedioxymethamphetamine (MDMA, 4×20 mg/kg, 2 h apart, sacrificed 48 h later) administered at either adolescence or adulthood. Immunohistochemistry was performed in the substantia nigra *pars compacta* (SNc) and striatum, to evaluate the levels of tyrosine hydroxylase (TH), as marker of dopaminergic degeneration, as well as of glial fibrillary acidic protein (GFAP) and ionized calcium-binding adapter molecule 1 (IBA-1), as markers of astrogliosis and microgliosis, respectively. Moreover, immunohistochemistry was used to ascertain the co-localization of IBA-1 with either the pro-inflammatory interleukin (IL)-1β or the anti-inflammatory IL IL-10, in order to determine the microglial phenotype. *In utero* administration of DEX induced dopaminergic damage by decreasing the density of TH-positive fibers in the striatum, although only in adult mice. MDMA administration induced dopaminergic damage and glia activation in the nigrostriatal tract of adolescent and adult mice. Mice exposed to DEX *in utero* and then treated with MDMA later in life showed a more pronounced loss of dopaminergic neurons (adolescent mice) and astrogliosis (adolescent and adult mice) in the SNc, compared with control mice. These results suggest that prenatal exposure to glucocorticoids may induce an age-dependent and persistent increase in the susceptibility to central toxicity of amphetamine-related drugs used later in life.

Keywords: adolescence, amphetamine-related drugs, ionized calcium-binding adapter molecule 1, glial fibrillary acidic protein, tyrosine hydroxylase.

1. Introduction

Dexamethasone (DEX) is a synthetic glucocorticoid widely administered in the treatment of pregnancies at risk for preterm delivery, neonatal atelectasis, placenta previa and multiple pregnancy, due to its high capability to penetrate through the placenta into the foetal circulation and to promote the maturation of several organ systems (Kemp et al. 2016; Jobe and Goldenberg, 2018). Nevertheless, clinical studies have shown that the prenatal administration of DEX may cause a series of detrimental alterations at the central and peripheral levels, such as long-term cognitive abnormalities (Alexander et al. 2016) or increased cortisol response to psychosocial stress (Alexander et al., 2012), that can be manifested later in life in both preterm and full-term births (Barker et al. 2002; Seckl and Holmes, 2007; Drozdowicz and Bostwick, 2014).

In experimental animals, *in utero* exposure to DEX is an established model to induce intrauterine growth retardation (IUGR), a condition associated with detrimental consequences in humans, including neuropsychiatric disorders. Consistent with this, persistent brain abnormalities have been demonstrated in rodents that were exposed to DEX during prenatal life, and previous studies have shed light on the neuronal circuits affected by *in utero* exposure to DEX. Studies in rats have demonstrated that prenatal exposure to DEX induces gender-specific alterations in the nigrostriatal dopaminergic system, with a notable feminization of the 3D cytoarchitecture and organization of tyrosine hydroxylase (TH)-immunoreactive neurons in the substantia nigra *pars compacta* (SNc) and ventral tegmental area (VTA) of males (McArthur et al. 2007). Studies in rats have also demonstrated that prenatal exposure to DEX, besides altering the dopaminergic system, affected the serotonergic system, as it caused modifications in synaptic signaling even at doses below those used therapeutically in preterm infants, and in the absence of somatic growth inhibition (Slotkin et al. 2006 and 2016). In the same species, prenatal exposure to DEX has also been found to affect glial cells, since it induced a reduction in the length of primary processes of astrocytes in the hippocampus (HPC) (Shende et al. 2015), as well as long-lasting changes in microglia morphology in adult male rats born from dams treated with DEX during gestation. Collectively, these results appear of interest, since the presence of neuronal and glial alterations in the brain of the offspring born from DEX-treated dams may lead to the speculation that *in utero* exposure to DEX could render the brain more vulnerable to the detrimental effects that can be elicited by noxious stimuli experienced later in life. This hypothesis may acquire particular relevance with regard to the abuse of psychoactive substances, many of which have been shown to possess neurotoxic and neuroinflammatory potential (Costa et al. 2020b). However, no information is currently available as to whether *in utero* exposure to DEX may somehow exacerbate the neurotoxic and/or neuroinflammatory effects that are elicited by drugs consumed later in life. In this regard, a relevant experimental approach may be the study of the neurotoxic and neuroinflammatory effects of 3,4-methylenedioxymethamphetamine (MDMA, or “ecstasy”) in mice born from dams treated with DEX during pregnancy, according to a protocol that has

been demonstrated to induce behavioral and molecular abnormalities later in life (Bose et al., 2010; Spulber et al. 2015; Conti et al. 2017; Raciti and Ceccatelli, 2018).

Studying MDMA is of interest because this amphetamine-related drug is consumed by adolescents and young adults (Strote et al. 2002; Barrett et al. 2006; Costa et al. 2020b) and it not only has abuse properties but may also elicit neurotoxic effects and glia activation in several animal species, although the systems affected vary with the species considered (Stone et al. 1987, 1989; Parrott 2001; Easton and Marsden, 2006; Baumann et al. 2007; Gudelsky and Yamamoto, 2008). For example, in rats MDMA elicits neurotoxic effects at the level of the serotonergic system (Ricaurte et al. 1988; Adori et al. 2006; Kovács et al. 2007), although dopaminergic damage in rats treated with MDMA has also been described (Cadoni et al., 2017). Conversely, MDMA administration to mice elicits a peculiar profile of neurotoxicity and glia activation that involves the dopaminergic nigrostriatal and mesolimbic systems (Colado et al. 2001; Green et al. 2003; Cadet et al. 2007; Granado et al. 2008; Frau et al. 2013, 2016a; Moratalla et al. 2017; Costa et al. 2017, 2020b), both of which may be affected by *in utero* exposure to DEX in experimental rodents. Moreover, rodent studies have previously demonstrated that MDMA can interact with other drugs and toxins, which may lead to an exacerbation of neurotoxicity and glia activation (Frau et al. 2016a; Gòrska et al. 2018), and that the neurotoxic effects of MDMA on dopaminergic systems are age-dependent (Costa et al. 2019b). Interestingly, a previous study in rats has demonstrated that the effects of MDMA on serotonin receptors number and density in the HPC and brainstem may be modulated by adrenalectomy and administration of DEX, supporting a role for glucocorticoid-mediated signaling in the effects of MDMA in the brain (Aguirre et al. 1997). Based on these considerations, it appears of interest to study the dopaminergic damage and glia activation induced by MDMA in mice born from DEX-treated dams, in order to clarify whether *in utero* exposure to a glucocorticoid may exert a detrimental influence on the neurotoxic and neuroinflammatory effects of drugs that are consumed at later stages of life.

To address this issue, adolescent and adult C57BL/6J male mice born from dams treated with either DEX or vehicle during gestation received the administration of MDMA, according to a regimen that has previously been demonstrated to induce neurotoxicity and glia activation in the dopaminergic nigrostriatal tract (Frau et al. 2016a,b). In these mice, the presence of degeneration of dopaminergic neurons and fibers, astrogliosis and microgliosis was evaluated in the SNc and striatum by means of immunohistochemistry for TH, glial fibrillary acidic protein (GFAP) and ionized calcium-binding adapter molecule 1 (IBA-1). Moreover, in the same brain regions we looked at immunohistochemical co-localization of IBA-1 with either interleukin (IL)-1β or IL-10, in order to determine the two microglial phenotypes: M1, characterized by the production of pro-inflammatory cytokines such as IL-1β, chemokines, as well as reactive oxygen species, and M2, characterized by the production of anti-inflammatory cytokines such as IL-10.

2. Materials and methods

2.1 Animals

Pregnant C57BL/6J dams (Charles River, Calco, Italy) were housed individually in Plexiglas cages (length, 42 cm; width, 24 cm; height, 15 cm) in a room under controlled temperature (21 ± 1°C) humidity (55±10%) and light cycle, with food and water available *ad libitum* throughout the pregnancy. Once weaned, mice from different litters were housed according to the prenatal and postnatal treatment, so that mice in a given cage received identical prenatal (vehicle or DEX) and postnatal (vehicle or MDMA) treatment. All mice were housed in groups of 5, in temperature- and humidity-controlled rooms under a 12-h light/dark cycle (lights on 7:00 am) and fed an *ad libitum* diet of standard mouse chow. All experiments were conducted in accordance with the guidelines for animal experimentation of the EU directives (2010/63/EU; L.276; 22/09/2010) and with the guidelines approved by the Ethics Committee of the University of Cagliari. Experiments were designed to minimize animal discomfort to the least possible extent and to reduce the number of animals used.

2.2 Drugs

DEX (Sigma-Aldrich, Milan, Italy) was solubilized as described elsewhere (Spulber et al., 2015) and administered subcutaneously (s.c.) in a volume of 10 ml/kg. MDMA was synthesized and solubilized as described elsewhere (Frau et al. 2013). MDMA was administered by the intraperitoneal (i.p.) route in a volume of 10 ml/kg. Mice in the control groups were administered s.c. or i.p. with vehicle, at the same time of the day as drugs-treated mice.

2.3 DEX treatment

Pregnant C57BL/6J dams were treated with DEX (0.05 mg/kg per day) or vehicle from gestational day (GD) 14 until delivery, recorded as postnatal day (PND) 0. The dose of DEX used in this study was selected because studies in rodents have shown that it induces a moderate fetal growth retardation without affecting litter size, gestational length, and maternal behavior (Celsi et al., 1998; Spulber et al., 2015).

2.4 MDMA treatment

Male offspring from DEX-treated and vehicle-treated dams were used. Adolescent (28 PND) or adult (84 PND) mice received 4 administrations of MDMA in one day (20 mg/kg i.p., 2 h apart). Control mice were treated with vehicle according to the same schedule.

Forty-eight hours after the last MDMA or vehicle administration, mice were deeply anesthetized and sacrificed by transcardial perfusion with 4% paraformaldehyde in phosphate buffer (PB, 0.1 M, pH 7.4). Brains were then removed, post-fixed for 2 h, and processed for immunohistochemical studies as reported below, according to the procedures previously described (Costa et al. 2014). The time of sacrifice was selected based on our previous experiments showing that repeated treatment with MDMA followed by sacrifice 48 h after discontinuation reduced the levels of TH-positive terminals and neurons in the nigrostriatal dopaminergic system of mice (Frau et al. 2016a,b).

2.6.1 Tissue preparation

Coronal brain sections were cut at 50 μm on a vibratome and stored in a cryoprotectant solution at −20°C until use. For each mouse, three sections were collected from each of the two brain regions analyzed at the following coordinates: from −2.92 to −3.52 mm (SNc) and from 1.34 to 0.74 mm (striatum), relative to bregma, according to the mouse brain atlas of Paxinos and Franklin (2008).

2.6.2 Reaction protocols

Free-floating sections were rinsed in 0.1 M PB, blocked in a solution containing 3% normal donkey serum (Jackson ImmunoResearch Europe, Suffolk, UK) and 0.3% Triton X-100 in 0.1 M PB at room temperature for 2 h, and then incubated in the same solution with the primary antibody for two nights. After the incubation with the primary antibody was completed, sections were rinsed three times in 0.1 M PB and then incubated with the secondary antibody in 0.1 M PB at room temperature for 2 h. Table 1 summarizes the features and dilutions of the primary and secondary antibodies used in this study. After the incubation with the secondary antibody was completed, sections were rinsed, incubated for 10 min with DAPI (4′,6-diamidine-2′-phenylindole dihydrochloride, 1:10000, Sigma-Aldrich, Milan, Italy), to allow visualization of cell nuclei, and then mounted onto glass slides. Omission of either the primary or secondary antibodies served as negative control and yielded no labeling (data not shown).

Antibody	Species	Type	Diluition	Supplier
TH	Rabbit	Polyclonal	1:1000	Millipore, Temecula, CA, USA
GFAP	Mouse	Monoclonal	1:1000	Sigma-Aldrich, Milan, Italy
IBA-1	Rabbit	Polyclonal	1:300	Wako Chemicals, Neuss, Germany
IL-1β	Goat	Polyclonal	1:50	Santa Cruz

IL-10				Biotechnology, Santa Cruz, CA, USA
anti rabbit^{594} **anti mouse^{488}** **anti goat^{488}**	Donkey		1:500	Jackson ImmunoResearch Europe, Suffolk, UK

Table 1. List of primary and secondary antibodies used in this study.

2.6.3 Stereological counting of TH-immunoreactive neurons in the SNc.

Stereological analysis of the total number of TH-positive neurons in the SNc was carried out blind in both hemispheres, using a software (Stereologer) connected to a motorized stage on a light microscope (Pinna et al. 2016). The SNc region was outlined at low magnification (2×), and quantification of cells was achieved using automatically randomized sampling and an optical dissector (50×50×15 µm). Cells were sampled with a 40× objective through a defined depth with a guard zone of 2 µm. Coefficients of error ranged from 0.05 to 0.1 (Costa et al. 2019a).

2.6.4 Analysis of TH immunoreactivity in the striatum.

Images were acquired with an epifluorescence microscope (Axio Scope A1, Zeiss, Oberkochen, Germany) connected to a digital camera (1.4 MPixels, Infinity 3-1, Lumenera, Nepean, Canada), digitized in grey scale and captured at 5× magnification. Analysis was performed blind in the three sections. The density of immunoreacted fibers was determined quantitatively using the ImageJ software (U.S. National Institutes of Health, USA). The final values are expressed as a percentage of the groups of mice born from vehicle-treated dams and treated as adolescents or adults with vehicle. No significant differences in the densities of immunoreactive fibers were observed among the three sections of each brain area evaluated (data not shown); accordingly, values from different levels were averaged (Costa et al. 2013).

2.6.5 Analysis of GFAP- and IBA-1-positive cells in the SNc and striatum.

In each of the three brain sections, the whole SNc and two portions of the striatum (dorsolateral and ventromedial), left and right, were acquired with the same epifluorescence microscope cited above. Sections were captured at 10× magnification for analysis of the SNc, or at 20× magnification for analysis of the striatum (Costa et al. 2014). The number of cells labeled with the nuclear marker DAPI was counted manually for each level of the SNc and striatum using the ImageJ software. Cells were counted when a cell body with branching processes was observed, as well as when processes were detected that converged onto a central point likely corresponding to a cell body located deeper in

the tissue. Fibers that expressed GFAP or IBA-1 without a clear indication of associated cell bodies were not considered. To assure that the quantification of the number of GFAP-/IBA-1-positive cells accurately reflected the total number of GFAP-/IBA-1-positive cells in a single section, we analyzed only those cells labeled with the nuclear marker DAPI (Costa et al. 2020a).

2.6.6 Analysis of IBA-1+IL-1β and IBA-1+IL-10 co-localization in the SNc and striatum.

Each of the three brain sections, (i.e. whole SNc, dorsolateral and ventromedial striatum, left and right) were acquired at high magnification (40×) using the same epifluorescence microscope cited above. Quantitative analysis of co-localization of IBA-1 with either IL-1β or IL-10 was performed with the ImageJ plugin JACoP (Just Another Co-localisation Plugin) (Bolte and Cordelières, 2006). The correlation of signal intensity was calculated as a Pearson correlation coefficient (Rr), which quantifies the degree of overlapping between the fluorescence signals obtained from two channels (Dunn et al. 2011).

2.7 Data collection, analysis, and statistics

Statistical differences between MDMA- and vehicle-treated mice were evaluated by means of three-way ANOVA (treatment × exposure × age), followed by Newman–Keuls post-hoc test. Significance threshold was always set at $p < 0.05$. Results are expressed as mean ± SEM for every analysis performed.

3. Results

Adolescent and adult mice born from vehicle-treated or DEX-treated dams received either vehicle (VV or DV, respectively) or MDMA (VM or DM, respectively). Regarding mice in the DV groups, the only modification in the markers analyzed consisted in a decrease in the density of TH-positive fibers in the striatum of adult mice , compared with adult mice in the VV group.

3.1 Immunoreactivity for TH in the SNc

Treatment with MDMA reduced the numbers of TH-positive neurons in the SNc of adolescent and adult mice, and this effect was influenced by exposure to DEX in adolescent mice only. Three-way ANOVA revealed significant effects of treatment with MDMA ($F_{1,28} = 117.03$, $p < 0.001$) and exposure to DEX ($F_{1,28} = 5.61$, $p = 0.025$), as well as a significant interaction age × exposure to DEX ($F_{1,28} = 7.54$, $p = 0.01$).

In both adolescent and adult mice, Newman–Keuls post-hoc test revealed that the total number of TH-positive neurons was reduced in the VM groups compared with the VV groups ($p < 0.001$ for both adolescent and adult mice, Fig. 1). Moreover, adolescent mice in the DM group displayed a reduction in the total number of TH-positive neurons compared with adolescent mice in the VM group ($p < 0.05$, Fig. 1), indicating that prenatal treatment with DEX exacerbated the neurotoxic effects of MDMA administered at adolescence.

3.2 Immunoreactivity for TH in the striatum

Treatment with MDMA reduced the density of TH-positive fibers in the striatum of adult mice, and this effect was not influenced by exposure to DEX. Three-way ANOVA revealed significant effects of treatment with MDMA ($F_{1,44} = 5.61$, $p = 0.025$) and age ($F_{1,44} = 38.42$, $p < 0.01$), as well as significant interactions treatment with MDMA × age ($F_{1,44} = 13.34$, $p < 0.01$) and treatment with MDMA × exposure to DEX × age ($F_{1,44} = 3.81$, $p < 0.05$).

In adolescent mice, Newman–Keuls post-hoc test indicated that the VM and DM groups did not display any significant modifications in the mean density of TH-positive fibers, compared with the VV and DV groups, respectively ($p > 0.05$; Fig. 2).

In adult mice, Newman–Keuls post-hoc test indicated that the VM ($p < 0.001$), DV ($p < 0.05$) and DM ($p < 0.05$) groups showed a reduced mean density of TH-positive fibers, compared with the VV group (Fig. 2). Newman–Keuls post-hoc test revealed no significant differences in the mean density of TH-positive fibers between mice in the VM and DM groups ($p > 0.05$).

3.3 Immunoreactivity for GFAP in the SNc

Treatment with MDMA increased the numbers of GFAP-positive cells in the SNc of adolescent and adult mice, and this effect was influenced by exposure to DEX. Three-way ANOVA revealed significant effects of treatment with MDMA

($F_{1,45}$ = 12.08, p <0.01), exposure to DEX ($F_{1,45}$ = 12.3, p <0.01) and age ($F_{1,45}$ = 5.78, p =0.02), as well as a significant interaction treatment with MDMA × exposure to DEX ($F_{1,45}$ = 6.03, p <0.05).

In adolescent mice, Newman–Keuls post-hoc test indicated that the number of GFAP-positive cells was significantly increased in the DM group compared with the VV (p < 0.001), VM (p < 0.05) and DV (p < 0.01) groups.

In adult mice, Newman–Keuls post-hoc test indicated that the number of GFAP-positive cells was significantly increased in the DM group compared with the VM (p < 0.05) and DV (p < 0.05) groups, but not the VV group, albeit this effect was close to statistical significance (p = 0.07).

Finally, Newman–Keuls post-hoc test indicated that in both adolescent and adult mice the VM group did not show any modifications in the number of GFAP-positive cells, compared with the VV group (Fig. 3).

3.4 Immunoreactivity for GFAP in the striatum

Treatment with MDMA increased the numbers of GFAP-positive cells in the striatum of adolescent and adult mice, and this effect was not influenced by exposure to DEX. Three-way ANOVA revealed significant effects of treatment with MDMA ($F_{1,44}$ = 317.49, p <0.01) and age ($F_{1,44}$ = 198.06, p <0.01), as well as a significant interaction treatment with MDMA × age ($F_{1,44}$ = 208.3, p <0.01).

In both adolescent and adult mice, Newman–Keuls post-hoc test revealed that the number of GFAP-positive cells were increased in the VM groups compared with the VV groups (p < 0.05 for adolescents, p < 0.001 for adults Fig. 4), as well as in the DM groups compared with the DV groups (p < 0.05 for adolescents, p < 0.001 for adults Fig. 4). Moreover, Newman–Keuls post-hoc test revealed that the increase in GFAP-positive cells elicited by MDMA (VM and DM groups) was more marked in adult mice than in adolescent mice (p < 0.01, for all comparisons, Fig. 4).

3.5 Immunoreactivity for IBA-1 in the SNc

Treatment with MDMA increased the numbers of IBA-1-positive cells in the SNc of adolescent and adult mice, and this effect was not influenced by exposure to DEX. Three-way ANOVA revealed a significant effect of treatment with MDMA ($F_{1,45}$ = 48.57, p <0.01).

In both adolescent and adult mice, Newman–Keuls post-hoc test revealed that the number of IBA-1-positive cells was increased in the VM groups compared with the VV groups (p < 0.01 for both adolescents and adults, Fig. 5), as well as in the DM groups compared with the DV groups (p < 0.05 for adolescents, p < 0.01 for adults, Fig. 5).

3.6 Immunoreactivity for IBA-1 in the striatum

Treatment with MDMA increased the numbers of IBA-1-positive cells in the striatum of adolescent and adult mice, and this effect was not influenced by exposure to DEX. Three-way ANOVA revealed significant effects of treatment with MDMA ($F_{1,46}$ = 95.54, p <0.01) and age ($F_{1,46}$ = 22.39, p <0.01).

In both adolescent and adult mice, Newman–Keuls post-hoc test revealed that the number of IBA-1-positive cells was increased in the VM groups compared with the VV groups ($p < 0.01$ for both adolescents and adults, Fig. 6), as well as in the DM groups compared with the DV groups ($p < 0.001$ for both adolescents and adults, Fig. 6).

3.7 Immunoreactivity for IBA-1+IL-1β in SNc

Treatment with MDMA increased the expression of IL-1β in IBA-1-positive cells in the SNc of adolescent and adult mice, and this effect was not influenced by exposure to DEX. Three-way ANOVA revealed a significant effect of treatment with MDMA ($F_{1,41} = 31.43$, $p < 0.01$).

In adolescent and adult mice, Newman–Keuls post-hoc test revealed that an increased colocalization of IBA-1+IL-1β occurred in the VM and DM groups compared with the VV and DV groups, respectively ($p < 0.05$, Table 2).

	VV	**VM**	**DV**	**DM**
Adolescent mice	0.139±0.006	0.169±0.013*	0.135±0.007	0.185±0.018#
Adult mice	0.103±0.023	0.176±0.008*	0.140±0.002	0.172±0.006#

Table 2. Immunoreactivity for IBA-1+IL-1β in the SNc. * $p < 0.05$ compared with mice born from vehicle-treated dams and treated with vehicle (VV); # $p < 0.05$ compared with mice born from DEX-treated dams and treated with vehicle (DV).

3.8 Immunoreactivity for IBA-1+IL-1β in striatum

Treatment with MDMA increased the expression of IL-1β in IBA-1-positive cells in the striatum of adolescent and adult mice, and this effect was not influenced by exposure to DEX. Three-way ANOVA revealed a significant effect of treatment with MDMA ($F_{1,36} = 16.64$, $p < 0.01$) and age ($F_{1,36} = 100.46$, $p < 0.01$).

In adolescent and adult mice, Newman–Keuls post-hoc test revealed that the colocalization of IBA-1+IL-1β in the VM and DM groups was increased compared with the VV and DV groups, respectively ($p < 0.01$ for VM vs. VV group in adult mice; $p < 0.05$ for the other comparisons, table 3).

	VV	**VM**	**DV**	**DM**
Adolescent mice	0.107±0.010	0.179±0.034*	0.124±0.031	0.219±0.038#

Adult mice	0.303±0.025	0.420±0.015**	0.337±0.016	0.385±0.005$^{\#}$

Table 3. Immunoreactivity for IBA-1+IL-1β in the striatum. * $p < 0.05$ and ** $p < 0.01$ compared with mice born from vehicle-treated dams and treated with vehicle (VV); # $p < 0.05$ compared with mice born from DEX-treated dams and treated with vehicle (DV).

3.9 Immunoreactivity for IBA-1+IL-10 in SNc

Treatment with MDMA did not significantly modify the expression of IL-10 in IBA-1-positive cells in the SNc of adolescent and adult mice. Three-way ANOVA revealed a significant effect of age ($F_{1,42} = 16.46$, $p < 0.01$) and exposure to DEX ($F_{1,36} = 5.03$, $p < 0.05$), but no significant effect of treatment with MDMA. Adult mice in all the experimental groups displayed a more pronounced expression of IL-10 in IBA-1-positive cells in the SNc, compared with the respective groups of adolescent mice (Table 4).

	VV	**VM**	**DV**	**DM**
Adolescent mice	0.104±0.021	0.077±0.024	0.103±0.024	0.117±0.028
Adult mice	0.134±0.013	0.162±0.014	0.176±0.014	0.198±0.013

Table 4. Immunoreactivity for IBA-1+IL-10 in SNc. .

3.10 Immunoreactivity for IBA-1+IL-10 in striatum

Treatment with MDMA did not significantly modify the expression of IL-10 in IBA-1-positive cells in the striatum of adolescent and adult mice. Three-way ANOVA revealed no significant effects of treatment with MDMA, exposure to DEX or age.

	VV	**VM**	**DV**	**DM**
Adolescent mice	0.488±0.074	0.467±0.051	0.379±0.057	0.456±0.040
Adult mice	0.496±0.019	0.359±0.074	0.515±0.055	0.436±0.077

Table 5. Immunoreactivity for IBA-1+IL-10 in striatum.

4. Discussion

Several lines of clinical evidence indicate that *in utero* exposure to glucocorticoids may cause a series of neurological and behavioral abnormalities that are manifested later in life (Barker et al. 2002; Seckl and Holmes, 2007; Alexander et al. 2012, 2016; Drozdowicz and Bostwick, 2014). Interestingly, studies in experimental rodents have demonstrated that *in utero* exposure to DEX may induce IUGR, a condition associated with detrimental consequences in humans. Consistent with this, rodents exposed to DEX *in utero* display abnormalities in neurons and glial cells in several regions of the brain (McArthur et al. 2007; Shende et al. 2015; Spulber et al. 2015; Caetano et al. 2017; Tsiarli et al. 2017; Conti et al. 2017). These findings indicate that prenatal exposure to DEX may induce persistent alterations in brain function, and led us to hypothesize that it could also exacerbate the detrimental effects that can be elicited in the brain by substances with neurotoxic/neuroinflammatory properties that are experienced later in life. The results of the present study support this view, by demonstrating that C57BL6/J male mice exposed to DEX *in utero* displayed an exacerbation of dopamine neuron degeneration and astrogliosis that was induced in the SNc and striatum by the amphetamine-related drug MDMA administered at adolescence and/or adulthood.

In line with earlier findings, we observed that MDMA administration decreased the total number of TH-positive neurons in the SNc of both adolescent and adult mice (Frau et al. 2016b; Costa et al. 2019b). Moreover, we found that in adolescent mice exposed to DEX *in utero*, treatment with MDMA caused a more marked decrease in the total number of TH-positive nigral neurons, compared with adolescent mice born from vehicle-treated dams. These findings indicate that prenatal exposure to DEX may render the SNc of adolescent mice more vulnerable to the dopaminergic damage induced by MDMA, and substantiate our hypothesis that prenatal exposure to DEX is a factor that may worsen the central toxicity of psychoactive drugs consumed later in life. Moreover, our results are particularly interesting given that MDMA is mostly consumed by adolescents (Strote et al. 2002; Barrett et al. 2006; Costa et al. 2020b), and that adolescence is a critical neurodevelopmental period in which the brain may be heavily affected by exposure to psychoactive drugs (Casey et al. 2000). Indeed, our results could suggest that the potentially detrimental effects that MDMA may exert on brain remodeling are more severe in the adolescents who were exposed to DEX *in utero*; it remains to be determined whether this hypothesis may extend also to other amphetamine-related drugs consumed at adolescence and/or to the exposure to other glucocorticoids during the prenatal life. A Global Survey released from the United Nations Office on Drugs and Crime in 2003 (United Nations publication, Sales No. E.03.XI.15) revealed that during the 1990s, the rates of amphetamine-related drugs abuse increased more than those for any other drugs. Amphetamine-related drugs are the second most abused drugs after cannabis among high school students in all regions, except in South-East Asia, where rate of use of amphetamines are among the highest in the world and may exceed cannabis. Moreover, the prenatal administration of corticosteroids is most beneficial for improving neonatal outcomes

among patients who give birth preterm, and is used worldwide according to the WHO recommendations on interventions (2015). Based on these considerations, the possible detrimental interactions between DEX and MDMA may represent a serious risk for health that should be taken into account. Besides, elucidation of this issue may be relevant to further clarify the impact that behavioral stress and altered function of glucocorticoid signaling during pregnancy may have on the neurotoxic and neuroinflammatory effects of MDMA experienced later in life. Indeed, a previous evidence obtained in rats has demonstrated that stressful events during gestation may aggravate the detrimental effects of MDMA in the brain, since adolescent offspring born from dams subjected to restrainer-stress displayed increased metabolic rate of MDMA and more marked MDMA-induced motor alterations, compared with adolescent rats born from non-stressed dams (Morley-Fletcher et al. 2004). Activation of glucocorticoid signaling critically regulates the behavioral and neurochemical effects of stress; accordingly, it may be speculated that alterations in glucocorticoid signaling may be a key mechanism that may explain the exacerbation of the noxious effects of MDMA in the offspring from dams subjected to stress during gestation. On this basis, further studies are warranted to thoroughly characterize the possible interactions between exposure to glucocorticoids, such as DEX, during gestation and modifications in the effects of MDMA, and other psychoactive agents, experienced later in life.

Interestingly, *in utero* exposure to DEX did not significantly affect the dopaminergic damage induced by MDMA in the SNc of adult mice, as well as in the striatum of adolescent and adult mice. These findings indicate that the influence of prenatal exposure to DEX on the nigrostriatal damage induced by MDMA in later life is region- and age-specific. The inability of prenatal DEX to amplify the striatal dopaminergic damage induced by MDMA could be explained by considering that in the present study, and consistent with earlier investigations by us and others, the dopaminergic damage elicited by MDMA appeared to be more pronounced in the SNc rather than in the striatum (Costa et al. 2013; Frau et al. 2016b; Moratalla et al. 2017; Noël et al. 2018), possibly reflecting region-specific mechanisms of toxicity. The higher vulnerability of adolescents may be substantiated by the results of a previous study by our group that evaluated the effects of cage crowding on the dopaminergic damage induced by MDMA in the SNc, which demonstrated that crowding exacerbated the loss of nigral neurons induced by MDMA in adolescent but not in adult mice (Frau et al. 2016a). Hence, it could be possible that MDMA induces dopaminergic toxicity in the SNc of adolescent mice by means of mechanisms that are selectively activated at adolescence, but not adulthood. Oxidative stress is a possible mechanism that may explain the potentiation of the dopaminergic damage induced by MDMA in the SNc of adolescent mice exposed to DEX prenatally. In fact, MDMA administration is known to increase the generation of reactive oxygen and nitrogen species (Gorska et al. 2014, 2018; Schiavone et al. 2019). Developmental exposure to DEX has been shown to induce a long lasting increased susceptibility to oxidative stress in both *in vivo* (Ahlbom et al.

2000) and *in vitro* models (Raciti et al. 2016, Bose et al. 2010, 2015), which may explain the increased susceptibility to MDMA observed in the present study.

In order to further characterize the interplay between *in utero* exposure to DEX and noxious effects of MDMA on the nigrostriatal tract we also evaluated glia activation. MDMA increased the number of GFAP-positive cells in the striatum but not SNc in both adolescent and adult mice, in agreement with our earlier report (Frau et al. 2016b). Prenatal exposure to DEX amplified the effects of MDMA on astroglia activation in the SNc of adolescent and adult mice, leading to significant astrogliosis in this region. These findings may suggest that induction of astrogliosis, although apparently not being a crucial player in the neurotoxic effects of MDMA in the SNc, may be one of the mechanisms by means of which prenatal exposure to DEX exacerbates the dopaminergic nigral damage induced by MDMA. While this hypothesis may be consistent with our findings obtained in the adolescent mice, adult mice did not display any exacerbation in MDMA-induced nigral dopaminergic damage by prenatal exposure to DEX. Nevertheless, it has to be remarked that adolescent mice are more susceptible than adult mice to the neurotoxic effects of MDMA, which may explain these apparently discrepant findings. In this regard, it is necessary to make some considerations on the interplay between activation of glial cells and nigrostriatal neurotoxicity induced by MDMA. Previous studies have demonstrated that astroglia activation may sustain neurodegeneration in the dopaminergic nigrostriatal system (Ciesielska et al. 2009; Gil-Martínez et al. 2018), and this mechanism has also been proposed to participate in the neurotoxic dopaminergic damage induced by MDMA (O'Callaghan et al. 2014). Support to a possible role of astrogliosis in MDMA-induced dopaminergic damage may also come from the finding of the present study that adult mice, which displayed marked astrogliosis in the striatum, also exhibited a significant loss of dopaminergic fibers in the same region, an effect that was not observed in adolescent mice, which displayed a weak striatal astrogliosis. As suggested above, it may be possible that the mechanisms that mediate MDMA-induce dopaminergic damage vary with the specific brain region and age. Accordingly, we may speculate that in mice exposed to DEX *in utero* and treated with MDMA at adolescence the induction of astrogliosis is a crucial mechanism that mediates the neuronal loss in the SNc, whereas this mechanism is not critically involved in the degeneration of dopaminergic nigral neurons in mice exposed to DEX *in utero* and treated with MDMA at adulthood. Additionally, we may hypothesize that in adolescent mice other, yet undefined, mechanisms of toxicity may interact with astrogliosis to cause degeneration of dopaminergic nigral neurons, and that such a noxious interaction does not occur, or does only in part, in the SNc of adult mice treated with MDMA, thus not resulting in overt neurodegeneration.

In addition to astrogliosis, we evaluated the presence of microgliosis and the microglial phenotype induced by MDMA in the nigrostriatal tract, and whether prenatal exposure to DEX could influence this effect. In line with previous results obtained with CD11b (Frau et al. 2016b), another marker of microglia activation, MDMA given alone increased the

number of IBA-1-positive cells in both the SNc and striatum of adolescent and adult mice. The increase in the immunoreactivity for IBA-1 induced by MDMA in the SNc and striatum was not significantly affected by *in utero* exposure to DEX. Similarly, prenatal exposure to DEX did not significantly modify the microglia phenotype induced by MDMA at either adolescence or adulthood. Taken together, these findings suggest that activation of microglia is not a major mechanism by means of which prenatal DEX facilitates dopaminergic nigrostriatal degeneration following administration of MDMA later in life. Nevertheless, it has to be considered that the present study was performed in male mice only, and that gender may be a factor that could potentially affect the interactions between prenatal exposure to DEX and detrimental effects of MDMA experienced later in life. Thus, prenatal exposure to DEX in rats has been reported to induce a gender-specific remodeling of microglial cell processes in the prefrontal cortex, with a hyper-ramification and increased length in males and decreased numbers and length of microglia processes in females (Caetano et al. 2017). Similarly, studies in rodents have demonstrated that the toxic effects of MDMA in the brain may differ between males and female. Thus, a previous study by our group characterized a selective neuroinflammatory and neurotoxic effect of MDMA in adult and middle-aged male Rhes knock-down (KO) mice, compared with female Rhes KO mice (Costa et al., 2019). After MDMA, we observed in adult and middle-aged male Rhes KO mice astrogliosis in SNc and microgliosis in striatum, SNc, motor cortex, ventral tegmental area and nucleus accumbens. Moreover, MDMA administration also affected TH immunoreactivity in both striatum and SNc of male but not female Rhes KO mice (Costa et al. 2019). Moreover, a study by other performed in rats has demonstrated that males are more susceptible than females to the hyperthermic and hyperlocomotor effect of MDMA administration (Koenig et al. 2005). All these data considered, additional studies are warranted to determine whether male and female mice born from DEX-treated dams display differences in the degeneration of dopamine neurons and fibers in the nigrostriatal system and in glia activation that are elicited by MDMA administered later in life, and whether gender-specific mechanisms exist that may underlie the exacerbation of the central effects of MDMA in mice exposed to DEX *in utero*.

5. Conclusions

In conclusion, the present study demonstrates that prenatal exposure to glucocorticoids, such as DEX, exacerbates dopaminergic damage and astrogliosis induced by MDMA administered later in life and may be a risk factor that increases neurotoxic and neuroinflammatory effects induced by amphetamine-related drugs. While these results are an important warning on the increasing use of corticosteroids during pregnancy and on its possible influence on the effects of amphetamine-like drugs consumed later in life, they paved the way for further studies (currently running in our laboratory) aimed at understanding the mechanisms of this negative interaction.

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Figure legends

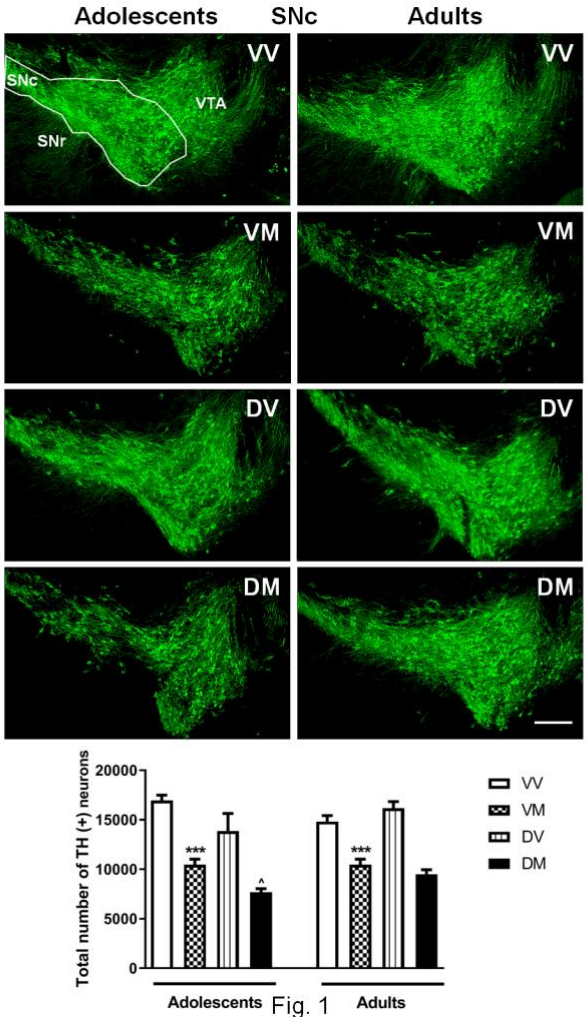

Fig. 1

Fig. 1. Effect of the administration of 3,4-methylenedioxymethamphetamine (MDMA) in adolescent and adult male mice born from vehicle- or dexamethasone (DEX)-treated dams on the immunoreactivity for tyrosine hydroxylase (TH) in the substantia nigra pars compacta (SNc). Representative sections of the SNc acquired at 10× magnification of adolescent (left panels) and adult (right panels) mice immunostained for TH and histogram of the total number of TH-immunoreactive neurons in the SNc, evaluated with the stereological analysis. *** $p < 0.001$, compared with mice born from vehicle-treated dams and treated with vehicle (VV); ^ $p < 0.05$ compared with mice born from vehicle-treated dams and treated with MDMA (VM). Scale bar: 50 μm. DM, mice born from DEX-treated dams and treated with MDMA; SNr, substantia nigra pars reticulata, VTA, ventral tegmental area. Quantification was performed in the SNc only.

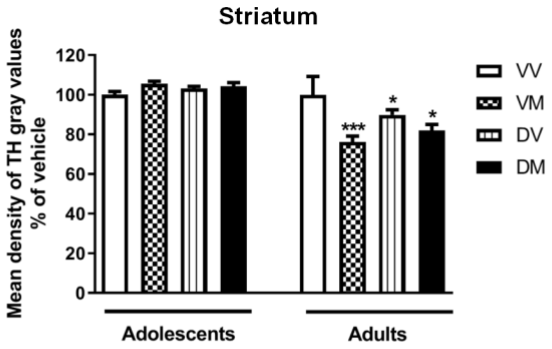

Fig. 2

Fig. 2. Effect of the administration of 3,4-methylenedioxymethamphetamine (MDMA) in adolescent and adult male mice born from vehicle- or dexamethasone (DEX)-treated dams on the immunoreactivity for tyrosine hydroxylase (TH) in the striatum. Representative histogram of the mean density of TH-immunoreactive fibers in the striatum. The values are expressed as a percentage of the respective group of mice (either adults or adolescents) born from vehicle-treated dams and treated with vehicle (VV).* $p < 0.05$ and *** $p < 0.001$, compared with the respective group of VV mice. DV, mice born from DEX-treated dams and treated with vehicle; VM, mice born from vehicle-treated dams and treated with MDMA; DM, mice born from DEX-treated dams and treated with MDMA.

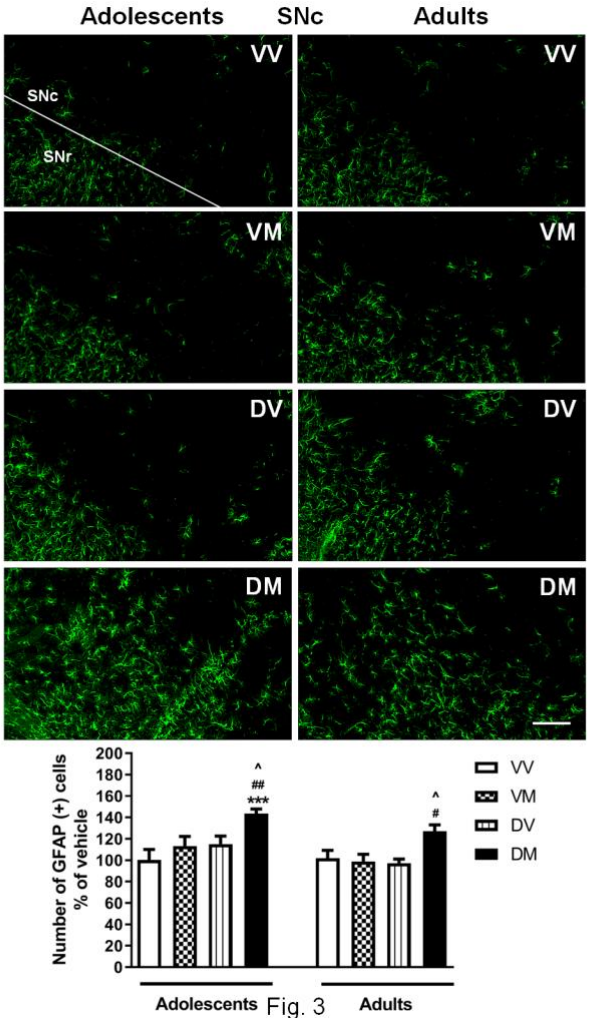

Fig. 3

Fig. 3. Effect of the administration of 3,4-methylenedioxymethamphetamine (MDMA) in adolescent and adult male mice born from vehicle- or dexamethasone (DEX)-treated dams on the immunoreactivity for glial fibrillary acidic protein (GFAP) in the substantia nigra pars compacta (SNc). Representative sections of the SNc acquired at 10× magnification of adolescent (left panels) and adult (right panels) mice immunostained for GFAP and histogram of the number of GFAP-positive cells in the SNc. *** $p < 0.001$ compared with the respective group of mice born from vehicle-treated dams and treated with vehicle (VV); # $p < 0.05$ and ## $p < 0.01$ compared with the respective group of mice born from DEX-treated dams and treated with vehicle (DV); ^ $p < 0.05$ compared with the respective group mice born from vehicle-treated dams and treated with MDMA (VM). Scale bar: 50 μm. DM, mice born from DEX-treated dams and treated with MDMA; SNr, substantia nigra pars reticulata. Quantification was performed in the SNc only.

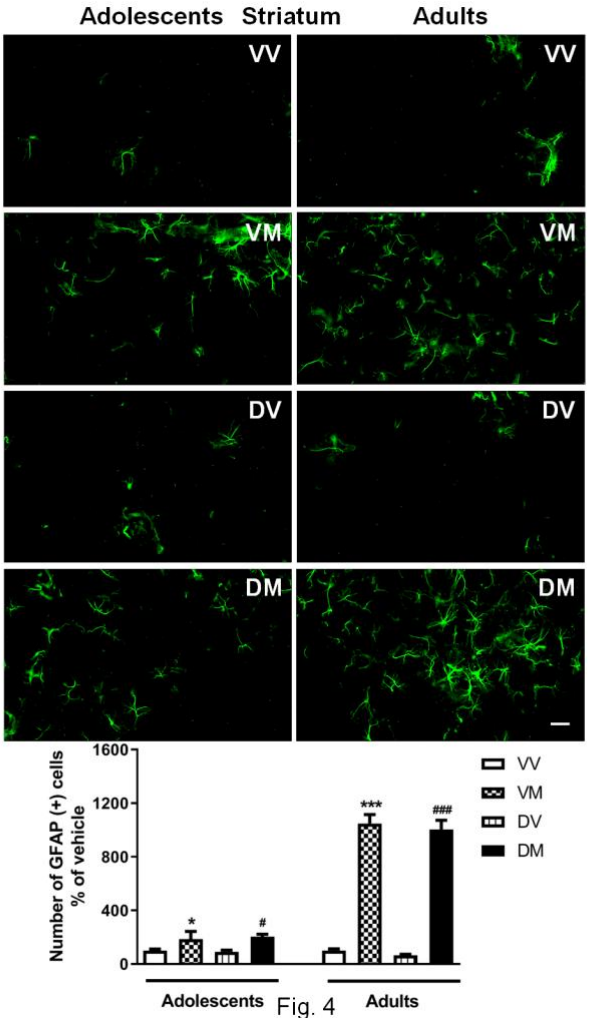

Fig. 4

Fig. 4. Effect of the administration of 3,4-methylenedioxymethamphetamine (MDMA) in adolescent and adult male mice born from vehicle- or dexamethasone (DEX)-treated dams on the immunoreactivity for glial fibrillary acidic protein (GFAP) in the striatum. Representative sections of the striatum acquired at 20× magnification of adolescent (left panels) and adult (right panels) mice immunostained for GFAP and histogram of the number of GFAP-positive cells in the striatum. * $p < 0.05$ and *** $p < 0.001$, compared with the respective group of mice born from vehicle-treated dams and treated with vehicle (VV); # $p < 0.05$ and ### $p < 0.001$ compared with the respective group of mice born from DEX-treated dams and treated with vehicle (DV). Scale bar: 50 μm. DM, mice born from DEX-treated dams and treated with MDMA; VM, mice born from vehicle-treated dams and treated with MDMA.

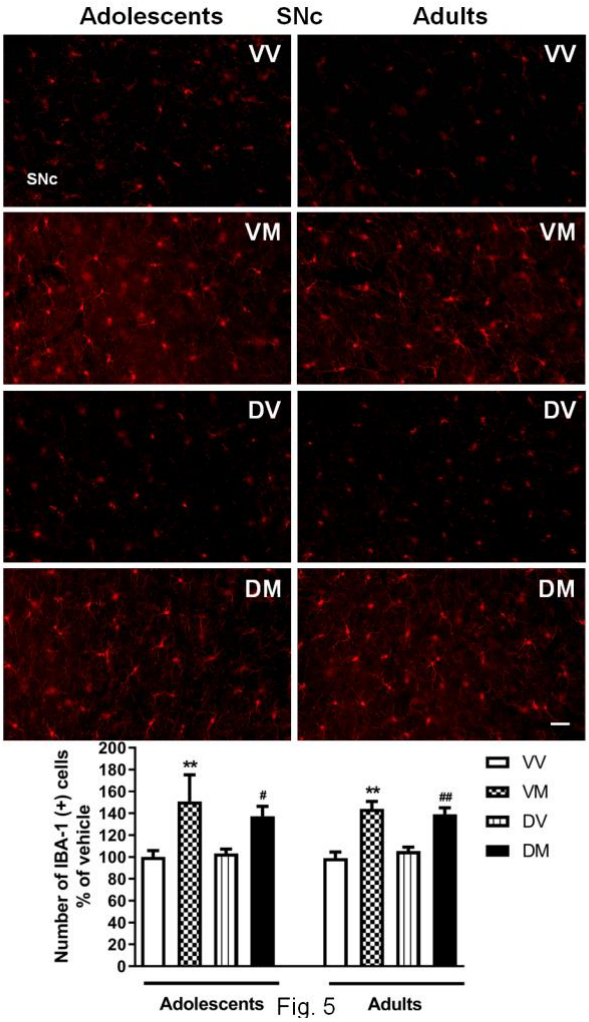

Fig. 5

Fig. 5. Effect of the administration of 3,4-methylenedioxymethamphetamine (MDMA) in adolescent and adult male mice born from vehicle- or dexamethasone (DEX)-treated dams on the immunoreactivity for ionized calcium-binding adapter molecule 1 (IBA-1) in the substantia nigra pars compacta (SNc). Representative sections of the SNc acquired at 20× magnification of adolescent (left panels) and adult (right panels) mice immunostained for IBA-1 and histogram of the number of IBA-1-positive cells in the SNc. ** $p < 0.01$ compared with the respective group of mice born from vehicle-treated dams and treated with vehicle (VV); # $p < 0.05$ and ## $p < 0.01$ compared with the respective mice born from DEX-treated dams and treated with vehicle (DV). Scale bar: 50 μm. DM, mice born from DEX-treated dams and treated with MDMA; VM, mice born from vehicle-treated dams and treated with MDMA.

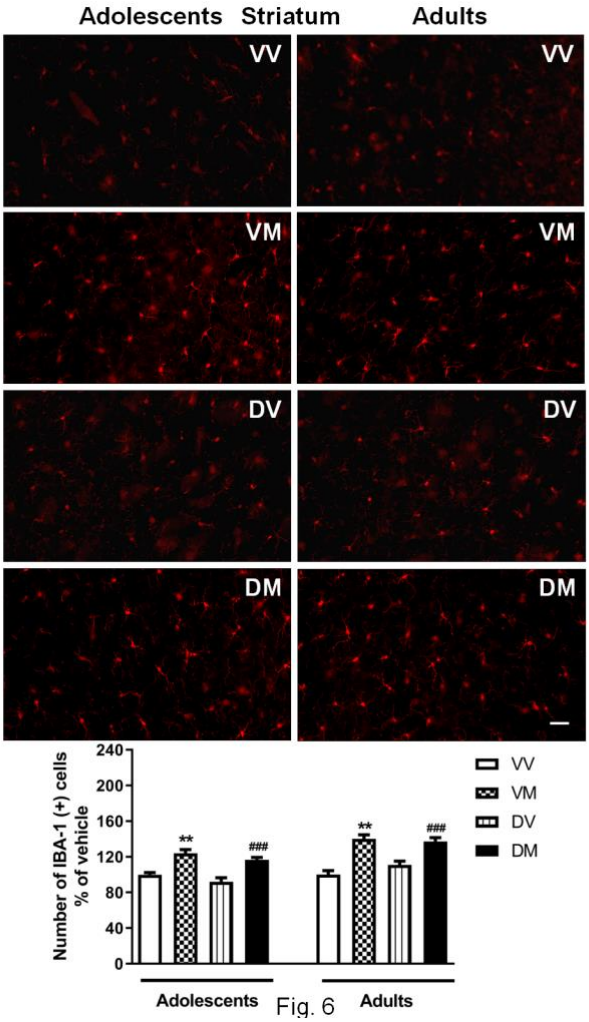

Fig. 6

Fig. 6. Effect of the administration of 3,4-methylenedioxymethamphetamine (MDMA) in adolescent and adult male mice born from vehicle- or dexamethasone (DEX)-treated dams on the immunoreactivity for ionized calcium-binding adapter molecule 1 (IBA-1) in the striatum. Representative sections of the striatum acquired at 20× magnification of adolescent (left panels) and adult (right panels) mice immunostained for IBA-1 and histogram of the number of IBA-1-positive cells in the striatum. ** $p < 0.01$ compared with the respective group of mice born from vehicle-treated dams and treated with vehicle (VV); ### $p < 0.001$ compared with the respective group of mice born from DEX-treated dams and treated with vehicle (DV). Scale bar: 50 μm. DM, mice born from DEX-treated dams and treated with MDMA; VM, mice born from vehicle-treated dams and treated with MDMA.

Acknowledgments and conflict of interest disclosure
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