BJP British Journal of Pharmacology

DOI:10.1111/bph.12519
www.brjpharmacol.org

Themed Section: Cannabinoids 2013

RESEARCH PAPER

Sex-dependent long-term effects of adolescent exposure to THC and/or MDMA on neuroinflammation and serotoninergic and cannabinoid systems in rats

Ana Belen Lopez-Rodriguez[1,2], Alvaro Llorente-Berzal[1], Luis M Garcia-Segura[2] and Maria-Paz Viveros[1]

[1]*Department of Animal Physiology (Animal Physiology II), Faculty of Biology, Complutense University of Madrid – Instituto de Investigación Sanitaria del Hospital Clínico San Carlos (IdISSC), Madrid, Spain, and* [2]*Instituto Cajal, Consejo Superior de Investigaciones Cientificas (CSIC), Madrid, Spain*

Correspondence
Maria-Paz Viveros, Department of Animal Physiology (Animal Physiology II), Faculty of Biology, Complutense University of Madrid, Calle Jose Antonio Novais, 2, Complutense University of Madrid, 28040 Madrid, Spain. E-mail: pazviver@bio.ucm.es

Keywords
adolescence; THC; MDMA; astrocytes; reactive microglia; CB_1 receptor; serotonin transporter; sex differences

Received
31 May 2013
Revised
7 November 2013
Accepted
13 November 2013

BACKGROUND AND PURPOSE
Many young people consume ecstasy as a recreational drug and often in combination with cannabis. In this study, we aimed to mimic human consumption patterns and investigated, in male and female animals, the long-term effects of Δ^9-tetrahydrocannabinol (THC) and 3,4-methylenedioxymethamphetamine (MDMA) on diverse neuroinflammation and neurotoxic markers.

EXPERIMENTAL APPROACH
Male and female Wistar rats were chronically treated with increasing doses of THC and/or MDMA during adolescence. The effects of THC and/or MDMA on glial reactivity and on serotoninergic and cannabinoid systems were assessed by immunohistochemistry in the hippocampus and parietal cortex.

KEY RESULTS
THC increased the area staining for glial fibrilar acidic protein in both sexes. In males, both drugs, either separately or in combination, increased the proportion of reactive microglia cells [ionized calcium binding adaptor molecule 1 (Iba-1)]. In contrast, in females, each drug, administered alone, decreased of this proportion, whereas the combination of both drugs resulted in a 'normalization' to control values. In males, MDMA reduced the number of SERT positive fibres, THC induced the opposite effect and the group receiving both drugs did not significantly differ from the controls. In females, MDMA reduced the number of SERT positive fibres and the combination of both drugs counteracted this effect. THC also reduced immunostaining for CB_1 receptors in females and this effect was aggravated by the combination with MDMA.

CONCLUSIONS AND IMPLICATIONS
Adolescent exposure of rats to THC and/or MDMA induced long-term, sex-dependent neurochemical and glial alterations, and revealed interactions between the two drugs.

LINKED ARTICLES
This article is part of a themed section on Cannabinoids 2013. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2014.171.issue-6

Abbreviations

BDNF, brain-derived neurotrophic factor; GFAP, glial fibrilar acidic protein; Iba-1, ionized calcium binding adaptor molecule 1; MDMA, 3,4-methylenedioxymethamphetamine; pnd, post-natal day; SERT, serotonin transporter protein; THC, Δ^9-tetrahydrocannabinol; Vh, vehicle

Introduction

Polydrug use among young people is a very frequent phenomenon and has increased in the last few years. In particular, cannabis is the most widely taken, illegal, co-drug in 3,4-methylenedioxymethamphetamine (MDMA) users, especially among younger adults (Parrott *et al.*, 2007; Schulz, 2011). Thus, 98% of the ecstasy users had also taken cannabis in a sample of East Coast college students (Wish *et al.*, 2006). Motivation for polydrug use might be influenced by psychophysiological aspects, for example, the transient relief exerted by one of the drugs of some undesired effects caused by the other substance. Specifically, acute cannabis consumption has been described by MDMA (ecstasy) abusers as a symptomatic relief against the feeling of anhedonia and depression, which follows ecstasy's high (see Schulz, 2011; Parrott *et al.*, 2007).

Cannabis is the most commonly used illicit drug among young people in Europe (EMCDDA, 2012). It is obtained from extracts of the hemp *Cannabis sativa* and its main psychotropic substance is Δ^9-tetrahydrocannabinol (THC) (Bossong and Niesink, 2010; Klein *et al.*, 2011). THC binds to cannabinoid CB_1 receptors (receptor nomenclature follows Alexander *et al.*, 2013) that are highly expressed in the brain and are involved in multiple functions, including neural development (Keimpema *et al.*, 2011), inflammation (Massi *et al.*, 2008; Wolf *et al.*, 2008), and anxiety and stress responses (Viveros *et al.*, 2005; 2007; 2011) among many other aspects of homeostasis (Marco *et al.*, 2012). Many studies demonstrate that cannabis produces severe behavioural and neurophysiological impairments and that these deficits are more evident when this substance is taken during critical developmental periods like adolescence (Viveros *et al.*, 2012). In fact, previous reports have shown that chronic adolescent administration of CB_1 receptor agonists induces alterations of the emotional behaviour, the cognitive function as well as psychotic-like symptomatology in adult rats (Biscaia *et al.*, 2003; Schneider and Koch, 2003; 2007; O'Shea *et al.*, 2004; 2006; Llorente-Berzal *et al.*, 2011; 2013a; Mateos *et al.*, 2011; Zamberletti *et al.*, 2012). The administration of CB_1 receptor agonists during adolescence also induces long-term neurochemical changes in the brain (Rubino *et al.*, 2008; Llorente-Berzal *et al.*, 2013a) and sex-dependent changes in expression and functionality of hippocampal CB_1 receptors (Mateos *et al.*, 2011; López-Gallardo *et al.*, 2012).

Regarding the involvement of the endocannabinoid system in the control of neuroinflammation, glial cells, astrocytes and microglia cells all express cannabinoid receptors (Stella, 2010) that contribute to modulate the inflammatory response. Marchalant *et al.* (2007) showed that WIN55212-2, a synthetic cannabinoid agonist, produced a decrease in the number of activated microglia after treatment with LPS, suggesting that the cannabinoid system may play a role in the control of microglia reactivity in response to an insult.

MDMA (or ecstasy) is a psychostimulant drug usually consumed by young adults attending a range of different nightlife venues (Parrott, 2004; EMCDDA, 2012). MDMA is an analogue of methamphetamine, which induces a rapid release of serotonin and inhibition of its re-uptake, affecting also other neurotransmitters such as dopamine and noradrenaline (Baumann *et al.*, 2007), and increases the metabolic activity and the production of free radical and oxidative stress (Parrott, 2004). The most consistent effect of MDMA exposure in rats is the serotoninergic deficit in various regions of the forebrain, including striatum, hippocampus and cortex (Battaglia *et al.*, 1991; Piper, 2007). Other indicators of MDMA-related insults are markers of neurotoxicity, such as cell death rate (Schmued, 2003), glial fibrilar acidic protein (GFAP) levels (Johnson *et al.*, 2002; Frau *et al.*, 2013) and neuroinflammation via activation of microglial cells (Monks *et al.*, 2004; Connor *et al.*, 2005). Although it is known that MDMA consumption during adolescence induces several long-lasting behavioural impairments on mood and cognitive function (Piper, 2007; Llorente-Berzal *et al.*, 2013a), there is scarce information about long-lasting neurotoxicity induced by adolescent MDMA exposure.

A critical factor to be considered regarding drug use and abuse is that sex differences affect many psychobiological aspects, including addiction (Carroll *et al.*, 2004; Viveros *et al.*, 2006, 2009; 2011). For example, clinical studies have shown that women are more susceptible to the effects of drugs of abuse (Carroll *et al.*, 2004). In particular, the majority of research in humans suggests that women are more likely to be affected by cannabinoids than men (Craft *et al.*, 2013). We have extensively studied sexual dimorphisms after chronic cannabinoid treatment during adolescence, which has allowed us to show an important number of sex differences affecting behavioural, endocrine and neuronal parameters (for a review, see Viveros *et al.*, 2011; 2012), as well as sex-dependent effects on glial cells (GFAP positive cells) (López-Gallardo *et al.*, 2012). Although there have been only a few studies that have focused on sexual dimorphisms in responses to MDMA in adult animals, a number of sex differences have been reported. Thus, female animals are more sensitive than males to the locomotor effects of MDMA (Palenicek *et al.*, 2005; Walker *et al.*, 2007), and MDMA induced lower levels of acoustic startle response in the prepulse inhibition test in female rats than in males (Bubeníková *et al.*, 2005). Moreover, we have recently found that males were more sensitive than females to the rewarding effects of an adolescent treatment with MDMA, as assessed by the conditioned place preference (Llorente-Berzal *et al.*, 2013b). Sexual differences in both cannabinoid and MDMA-induced effects can be attributed to both pharmacodynamic and pharmacokinetic factors (Fonsart *et al.*, 2009; Craft *et al.*, 2013).

In a recent study, we have reported long-term endocrine, behavioural and molecular effects of an adolescent treatment with THC and/or MDMA by using a protocol that mimics

the pattern of drug consumption in young humans (Llorente-Berzal *et al.*, 2013a). In brief, we used an MDMA administration schedule based on Meyer *et al.* (2008), who developed and characterized an animal model that mimics human MDMA weekend consumption, whereas THC was daily administered in an increasing dose regime (Rubino *et al.*, 2008). The specific chronic and escalating THC administration schedule was chosen because (i) a substantial proportion of cannabis users develop stable use patterns characterized by continuous use of cannabis (Ramaekers *et al.*, 2011) and (ii) chronic cannabinoid administration usually induces tolerance to many of the effects of THC (Childers, 2006). In the present study, we carried out an immunohistochemical analysis in the hippocampus and parietal cortex of animals employed in our previous experiments (Llorente-Berzal *et al.*, 2013a). In particular, we focused on glial (astrocytes and microglia) cells and on the serotonin transporter (SERT) and CB_1 receptors. In view of the numerous sexual dimorphisms found in our previous study (Llorente-Berzal *et al.*, 2013a), we also analysed here males and females. The present results demonstrate that chronic treatment with THC and/or MDMA during adolescence induced sex-dependent long-term effects on astrocyte and reactive microglia markers, as well as on CB_1 receptor expression and serotoninergic neurotoxicity. As we discuss below, some of these effects might be related to behavioural outcomes reported previously and, as in our previous study, we also reveal here functional interactions between both drugs.

Methods

Animals

All animal care and experimental procedures complied with the Spanish Royal Decree 1201/2005, 21 October 2005 (BOE No. 252) about the protection of experimental animals, in close agreement with the European Communities Council Directive of 24 November 1986 (86/609/EEC), and were approved by the local Animal Ethics Committee. All efforts were made to minimize animal suffering and distress. Studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). The animals used were the offspring of Wistar albino rats purchased from Harlan Laboratories (Milan, Italy), which were mated (one male × two females) at least 2 weeks after their arrival. After 10 days, females were single-housed and control of birth was strictly controlled. On the day of birth, postnatal day (pnd) 0, litters were culled and sex-balanced to eight pups per dam (four males and four females). Pups were left undisturbed until pnd 22 when they were separately housed in pairs of siblings of the same sex per cage. A total of 128 animals coming from 16 litters were used for the present experiment. All animals were maintained at constant conditions of temperature (22 ± 2°C) and humidity (50 ± 2%) in a reverse 12 h dark/light cycle (lights on at 20.00), with free access to food (commercial diet for rodents A04/A03; Safe, Augy, France) and water until they were killed at pnd 89–92.

Pharmacological treatments

THC (Dronabinol) was purchased from THC Pharm GmbH (Frankfurt, Germany) and dispersed in ethanol, cremophor (Sigma-Aldrich, Madrid, Spain) and saline (1:1:18) as in previous experiments (Llorente *et al.*, 2007; Llorente-Berzal *et al.*, 2011). MDMA hydrochloride was purchased from Lipomed (Arlesheim, Switzerland) and solutions were daily prepared in saline (0.9% NaCl). Drug treatments were restricted to the adolescent period, from pnd 28 to pnd 45 (Spear, 2000) where animals received i.p. injections of increasing doses of THC (2.5 mg·kg^{-1} from pnd 28 to 34; 5 mg·kg^{-1} from pnd 35 to 40; 10 mg·kg^{-1} from pnd 41 to 45) or vehicle, according to a slightly modified protocol from Rubino *et al.* (2008), the assignment to each group was randomised and under blind code during all the experiment. Additionally, every 5 days, from pnd 30, animals received two daily injections of MDMA (10 mg·kg^{-1}, s.c., calculated as the salt) or saline (Sal), with an inter-dose interval of 4 h, following a modified protocol from Meyer *et al.* (2008). Both drugs were administered at a volume of 2 mL·kg^{-1} (see Figure 1). Animals in the present study were exposed to exactly the same timeline, pharmacological treatments and behavioural tests as those described in Llorente-Berzal *et al.* (2013a), where the methodological details and results obtained in the first part of this study are explained.

Tissue fixation and immunohistochemistry

For histological analysis, at adulthood (pnd 89–92), a total of 48 animals (6 animals per experimental group) were anaesthetized with sodium pentobarbital (100 mg·kg^{-1}, i.p.; Vetoquinol, San Fernando de Henares, Spain) and perfused intracardially, first with saline (0.9% NaCl) and then with fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer). Brains were removed and immersed overnight at 4°C in the same fixative solution and then rinsed with phosphate buffer. Coronal sections, 50 µm thick, were obtained using a Vibratome (VT 1000 S; Leica Microsystems, Wetzlar, Germany) and each batch of sections was used for an independent immunohistochemical marker. Sections for all animals were processed for all the studied antigens.

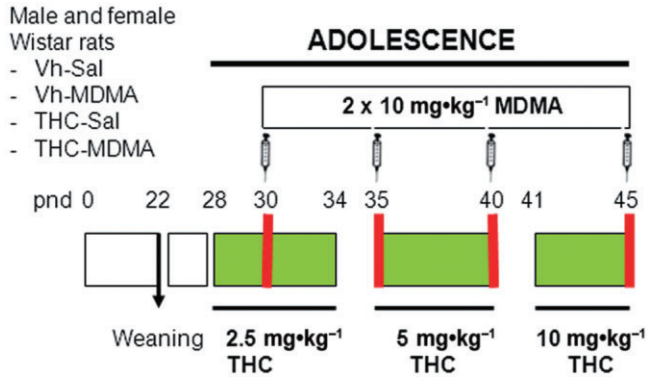

Figure 1

Scheme of the pharmacological treatment. Animals were exposed to increasing doses of THC (2.5, 5 and 10 mg·kg^{-1} or vehicle, i.p.) from pnd 28 to 45, and to MDMA (10 mg·kg^{-1} or saline, s.c.) twice a day every 5 days from pnd 30 to 45 with an inter-dose interval of 4 h.

Immunohistochemistry was carried out on free-floating sections. All washes and incubations were performed in 0.1 M phosphate buffer (pH 7.4), containing 0.3% BSA and 0.3% Triton X-100 (Sigma-Aldrich, St Louis, MO, USA). The endogenous peroxidase activity was quenched in a solution of 3% hydrogen peroxide in 30% methanol. Sections were incubated overnight at 4°C with either a rabbit polyclonal antibody against GFAP, a marker of astrocyte reactivity (diluted 1:1000; Dako, Glostrup, Denmark), a rabbit polyclonal antibody against the ionized calcium binding adaptor molecule 1(Iba-1), a marker of microglia (diluted 1:2000; Wako Pure Chemical Industries, Osaka, Japan), a rabbit polyclonal antibody against the transporter protein SERT (diluted 1:500; Calbiochem, Darmstadt, Germany) and a rabbit polyclonal antibody against CB_1 receptors (diluted 1:500; Thermo Scientific, Rockford, IL, USA). For these last two antibodies, a previous antigen retrieval step was needed (10 mM sodium citrate buffer, pH 8.7, 85°C). After primary antibodies, sections were incubated for 2 h with biotinylated goat anti-rabbit IgG (diluted 1:300; Pierce, Rockford, IL, USA). After several washes, sections were incubated for 90 min with avidin–biotin–peroxidase complex (diluted 1:250; ImmunoPure ABC peroxidase staining kit; Pierce) and the reaction product was revealed with 2 $\mu g \cdot mL^{-1}$ 3,3′-diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA) and 0.01% hydrogen peroxide in 0.1 M phosphate buffer. Finally, sections were dehydrated, mounted on gelatinized slides, coverslipped and examined with a Leitz Laborlux microscope (Leica Microsystems). Incubation of the tissue without the primary antibody was used as a control for immunohistochemistry. The hippocampus and parietal cortex were selected for immunohistochemical assessments for the following reasons. The implication of the hippocampus in cognition and memory is well known, and heavy MDMA users show an impaired cognitive function, including memory deficits (Quednow *et al.*, 2006; 2007). Moreover, recent data have shown oxidative damage in the hippocampus of mice acutely treated with MDMA (Ros-Simó *et al.*, 2013). CB_1 receptors are highly expressed in the hippocampus (Herkenham *et al.*, 1990) and are crucially involved in cognitive function and memory (Sullivan, 2000; Moreira and Lutz, 2008). Chronic exposure to THC in adolescent rats has been shown to induce long-term alterations of hippocampal CB_1 receptors, brain-derived neurotrophic factor (BDNF) and GFAP (López-Gallardo *et al.*, 2012), as well as memory deficits (Schneider and Koch, 2003; Rubino *et al.*, 2009a,b; Mateos *et al.*, 2011). Regarding the measurement of SERT density surface in parietal cortex, we selected this area for technical reasons. In the hippocampus or other areas such as the amygdala, the disposition of SERT fibres is diffuse, whereas in the cortex, it is very similar to a net where we can relatively easily distinguish vertical and horizontal fibres and quantify the surface density.

Morphometric analysis

All observations were made without knowledge of the treatments and samples were coded. We examined every section obtained from 2.5 to –2 mm bregma (Paxinos and Watson, 1998) and sections from 4 to 6 animals in each experimental group were analysed. GFAP presence was determined in the hilus area of the hippocampus by the calculation of the percentage of area immunostained for GFAP in high-quality microphotographs taken under the 10× objective. Six counting frames per animal were analysed by measuring densitometry with the software ImageJ 1.46o (NIH, Bethesda, MD, USA). The images were all changed to binary code and the positive staining was used to set the threshold, then, by using always the same measure area, the optical density was automatically determined and the percentage of stained area was calculated over the total area of the microphotograph.

The morphology of Iba-1 immunoreactive microglia was assessed with the 40× objective. Cells were classified, as described in (Diz-Chaves *et al.*, 2012), in five morphological types: type I, cells with few cellular processes (two or less); type II, cells showing three to five short branches; type III, cells with numerous (>5) and longer cell processes and a small cell body; type IV, cells with large somas and retracted and thicker processes; and type V, cells with amoeboid cell body, numerous short processes and intense Iba-1 immunostaining. Iba1-immunoreactive cells type III, type IV and type V were classified as reactive microglia (Diz-Chaves *et al.*, 2012). For each animal, we analysed a total of 100 cells within four different slices in the hilus area of the hippocampus and classified them into one of the five groups to finally sum them and determine the percentage of each group over the total.

SERT expression was assessed by analysing the surface density of SERT positive (+) fibres within the parietal cortex area on high-resolution microphotographs taken with the 40× objective. The surface density of SERT positive fibres was estimated according to the method of Weibel (1979) using a morphometric grid defining an area of 195 × 146 μm and presenting vertical and horizontal lines separated by a distance of 30 μm. The grid was superimposed over the microphotograph and we counted the number of vertical and horizontal intersections of the lines of the grid with the SERT positive fibres, and then the total number of intersections was summed.

Quantification of CB_1 receptor immunostaining was carried out on high-resolution microphotographs taken with the 10× objective and under the same conditions of light and contrast, by measuring optical density using the software ImageJ 1.46o (NIH), because this antibody presents a punctuate and diffuse staining. We analysed five slices per animal and focused on CA1 and CA3 areas of Ammon's horn and the hilus of the dentate gyrus of the hippocampus.

Data analysis

Data were analysed using a three-way ANOVA, with factors being sex (males vs. females), cannabinoid treatment (Vehicle vs. THC) and intermittent MDMA administration (Sal vs. MDMA). Normality and homocedasticity were assessed with Kolmogorov–Smirnov and Levene's tests respectively. When necessary, data were transformed to achieve a normal distribution. *Post hoc* comparisons were performed using the Bonferroni test with a level of significance, $P < 0.05$. In the case of CB_1 receptor data, additional Student's *t*-tests were performed between control males and females, as detailed in the Results section. Statistical analyses were carried out with the SPSS 19.0 software package (SPSS, Inc., Chicago, IL, USA).

Results

GFAP

The analysis of the percentage of area immunoreactive for GFAP by a three-way ANOVA rendered a significant effect of THC treatment [$F_{(1,35)}$ = 40.85; P < 0.001] and a significant interaction between THC and MDMA [$F_{(1,35)}$ = 17.32; P < 0.001].

As Figure 2 shows, THC induced a significant increase in the proportion of GFAP-positive cells in both sexes, and the same trend was observed for MDMA. No additive effects were found when both drugs were administered in combination. Rather, in females, MDMA tended to counteract the effect of THC.

Iba-1

Three-way ANOVA of the percentage of microglia cells with reactive phenotype rendered significant effects of THC [$F_{(1,34)}$ = 16.31; P < 0.001] and MDMA [$F_{(1,34)}$ = 4.79; P < 0.05], significant interactions between sex and THC [$F_{(1,34)}$ = 10.74; P < 0.01], and the two pharmacological treatments (THC × MDMA) [$F_{(1,34)}$ = 5.76; P < 0.05] and a significant triple interaction [$F_{(1,34)}$ = 34.72; P < 0.001]. *Post hoc* comparisons (Figure 3) revealed a significant difference between male and female control groups, with females exhibiting higher levels of reactive microglial cells. In males, both drugs, either separately or in combination, induced a significant increase of the percentage of reactive microglia cells. In contrast, in females, each drug, when administered alone, produced a significant decrease of this parameter, whereas the combination of both drugs resulted in 'normalization' to control values (see Figure 3).

SERT

Three-way ANOVA of the number of SERT positive fibres rendered significant effects of sex [$F_{(1,35)}$ = 26.16, P < 0.001], THC

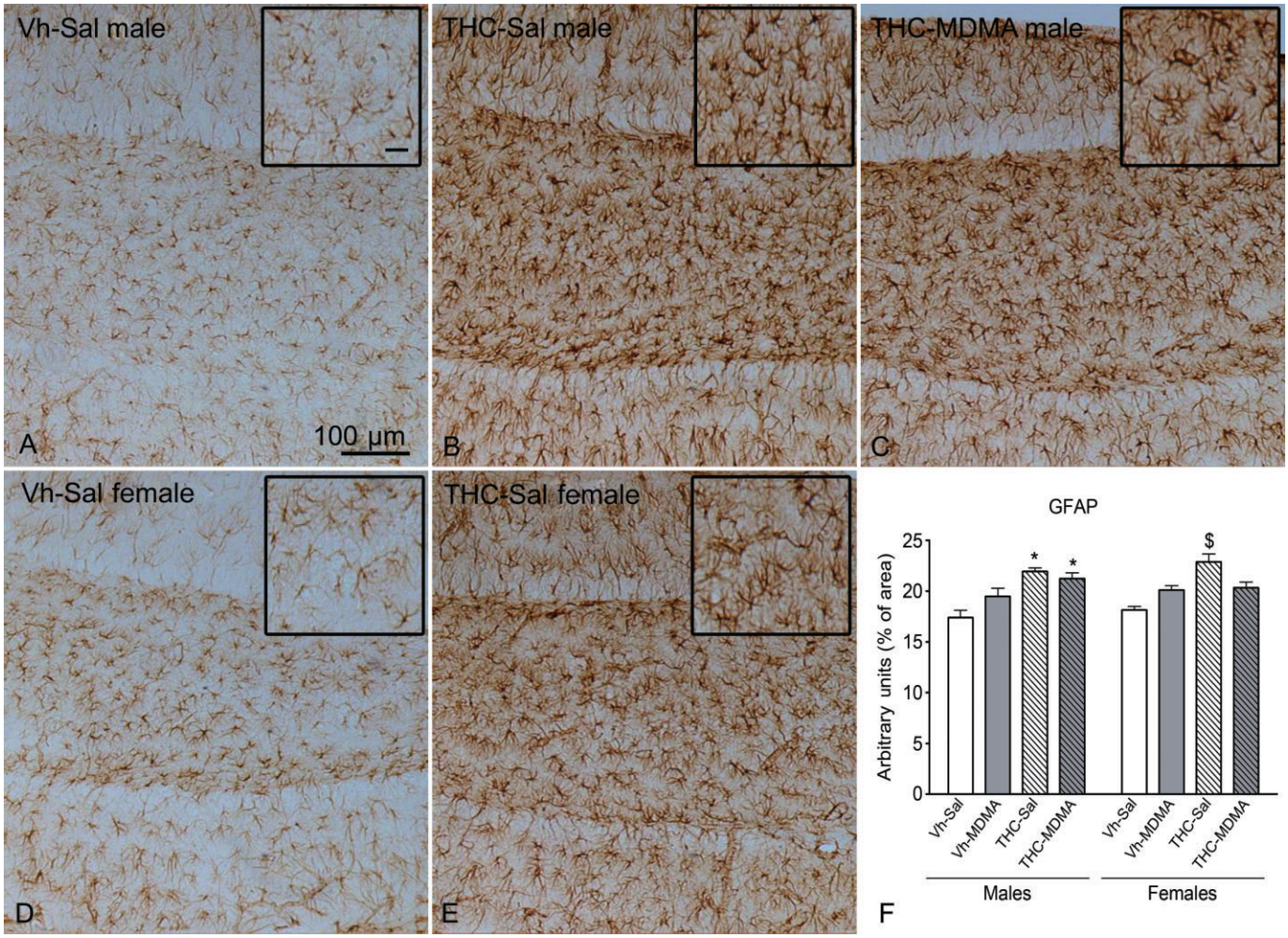

Figure 2

Percentage of area occupied by GFAP positive cells in the hilus of adult male and female rats. Animals were exposed to increasing doses of THC, 2.5, 5 and 10 mg·kg^{-1} or vehicle (Vh), from pnd 28 to 45, and to MDMA 10 mg·kg^{-1} or saline (Sal), twice a day every 5 days from pnd 30 to 45. Representative images of GFAP immunoreactivity in the hilus. (A) Vh-Sal male; (B) THC-Sal male; (C) THC-MDMA male; and (D) Vh-Sal female; (E) THC-Sal female. Scale bar of the insets: 100 µm. (F) Histogram representing the mean + SEM (n = 4–6) of the percentage of area occupied by GFAP positive cells. Bonferroni's *post hoc* test, P < 0.05, significantly different, * from, male Vh-Sal; $, from female Vh-Sal.

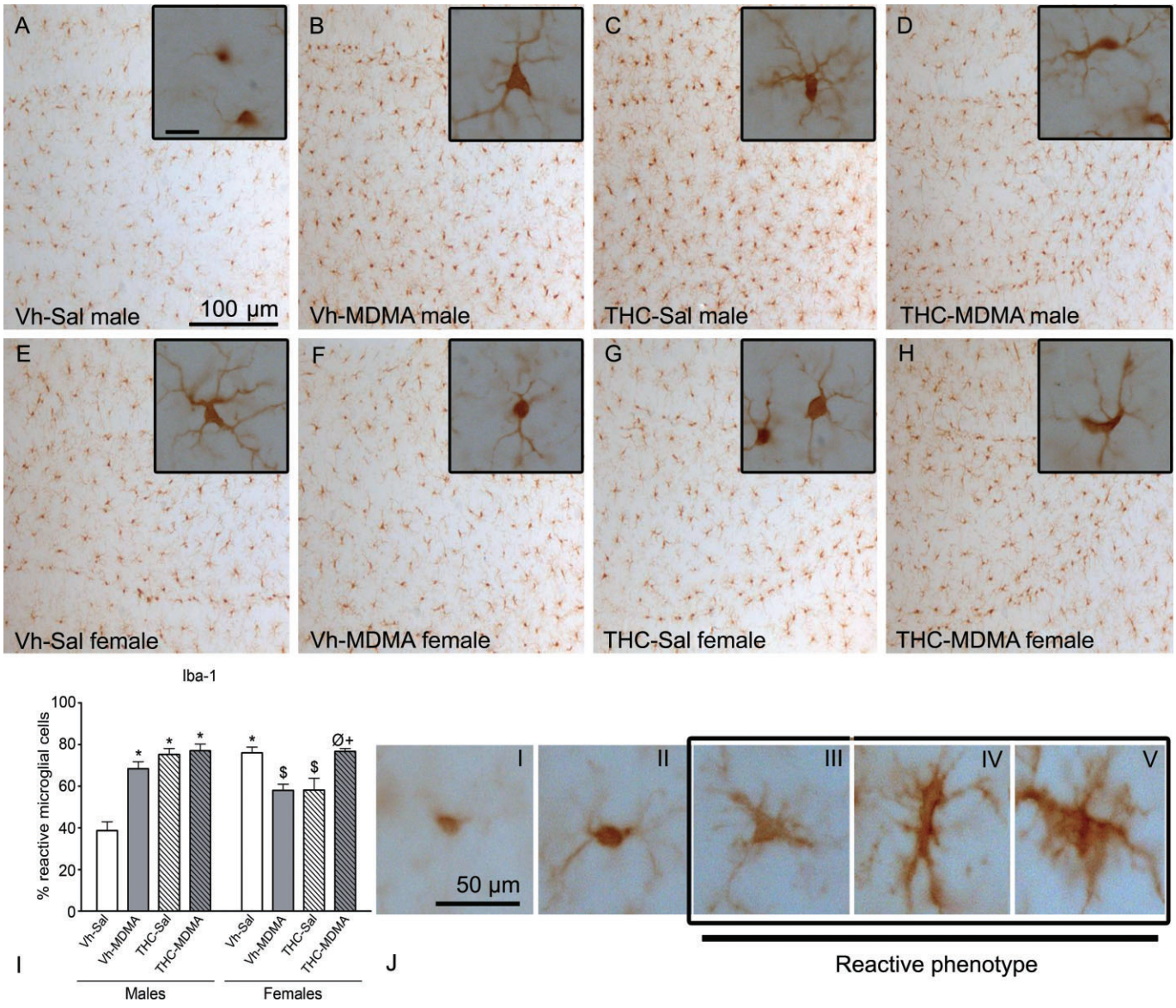

Figure 3

Percentage of reactive microglial cells in the hilus of adult male and female rats. Animals were exposed to increasing doses of THC, 2.5, 5 and 10 mg·kg^{-1} or vehicle (Vh), from pnd 28 to 45, and to MDMA, 10 mg·kg^{-1} or saline (Sal), twice a day every 5 days from pnd 30 to 45. Representative images of Iba-1 immunoreactivity in the hilus. (A) Vh-Sal male; (B) Vh-MDMA male; (C) THC-Sal male; (D) THC-MDMA male; (E) Vh-Sal female; (F) Vh-MDMA female; (G) THC-Sal female; and (H) THC-MDMA female. Scale bar of the insets: 50 µm. (I) Histogram representing the mean + SEM (n = 4–6) of the percentage of reactive microglial cells. Bonferroni's *post hoc* test, $P < 0.05$, significantly different, * from male Vh-Sal; $ from female Vh-Sal; Ø from female Vh-MDMA; + from female THC-Sal. (J) Microglia cells stained with Iba-1 and classified according to morphological aspects (Diz-Chaves *et al.*, 2012). Highlighted, reactive phenotype (from type III to type V).

[$F_{(1,35)}$ = 100.93; $P < 0.001$] and MDMA [$F_{(1,35)}$ = 102.61; $P < 0.001$]. It also revealed that the double sex × THC interaction [$F_{(1,35)}$ = 4.84; $P < 0.05$] and the triple interaction [$F_{(1,35)}$ = 4.61; $P < 0.05$] were significant. *Post hoc* comparisons showed a significant difference between control male and female animals. In addition, in males, MDMA induced the expected significant reduction of SERT positice fibres, THC induced the opposite effect, that is, a significant increase in this parameter, and the group receiving both drugs did not significantly differ from the control group. In females, MDMA reduced the number of SERT positive fibres and, in the absence of any effect of THC *per se*, the combination of both drugs counteracted the effect of MDMA (Figure 4).

CB_1 receptors

Optical densitometry of CB_1 receptor expression has been studied in three different areas of the hippocampal formation: CA1, CA3 and hilus. The three-way ANOVA revealed, for each hippocampal area studied, a significant effect of the THC treatment [CA1: $F_{(1,34)} = 5.34$, $P < 0.05$; CA3: $F_{(1,34)} = 11.54$,

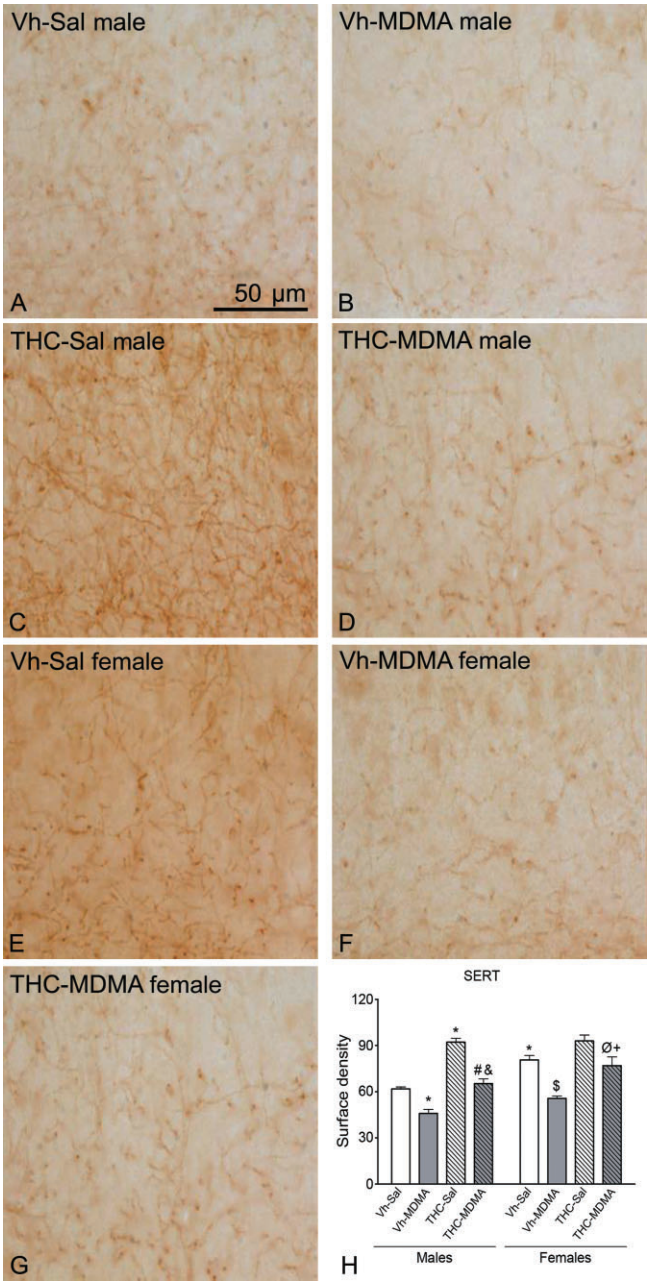

Figure 4

Surface density of SERT positive fibres in the parietal cortex of adult male and female rats, assessed using a morphometric grid according to the method of Weibel (1979). Animals were exposed to increasing doses of THC, 2.5, 5 and 10 mg·kg^{-1} or vehicle (Vh), from pnd 28 to 45, and to MDMA, 10 mg·kg^{-1} or saline (Sal), twice a day every 5 days from pnd 30 to 45. Representative images of SERT immunoreactivity in the parietal cortex. (A) Vh-Sal male; (B) Vh-MDMA male; (C) THC-Sal male; (D) THC-MDMA male; (E) Vh-Sal female; (F) Vh-MDMA female; and (G) THC-MDMA female. (H) Histogram representing the mean + SEM (n = 4–6) of the surface density of SERT positive fibres. Bonferroni's *post hoc* test, $P < 0.05$, significantly different * from male Vh-Sal; # from male Vh-MDMA; & from male THC-Sal; $ from female Vh-Sal; Ø from female Vh-MDMA; + from females THC-Sal.

$P < 0.01$; hilus: $F_{(1,34)} = 10.69$, $P < 0.01$] and significant interactions between sex and THC [CA1: $F_{(1,34)} = 11.34$, $P < 0.01$; CA3: $F_{(1,34)} = 7.22$, $P < 0.05$; hilus: $F_{(1,34)} = 4.94$, $P < 0.05$]. Furthermore, in CA3, it also rendered a significant effect of the MDMA treatment [$F_{(1,34)} = 7.70$; $P < 0.01$]. *Post hoc* comparisons did not reveal any difference among male animals, whereas among females, the group exposed to both drugs showed the lowest CB_1 expression in the three subareas analysed (Figure 5). A visual inspection of the histograms showed at least a trend towards a higher expression of CB_1 receptors in control females than in control males. In order to clarify this point, we carried out the corresponding comparisons using Student's *t*-test, which rendered the following results: CA1 area, vehicle (Vh)-Sal male versus Vh-Sal female, t = 0.052; CA3 area, Vh-Sal male versus Vh-Sal female, t = 0.044; hilus area, Vh-Sal male versus Vh-Sal female, t = 0.079.

Discussion and conclusions

In spite of the frequent combined use and abuse of ecstasy and cannabis among adolescents, little is known about the long-term consequences of this pattern of drug consumption. To the best of our knowledge, only two experimental studies have investigated the long-term effects of exposure to these drugs, administered in combination, to adolescents and both reports have focused on physiological and behavioural parameters (Shen *et al.*, 2011; Llorente-Berzal *et al.*, 2013a). We provide here the first evidence of sex-dependent, persistent consequences of THC and/or MDMA administration on neuroinflammation and serotoninergic and cannabinoid systems.

MDMA (ecstasy) has neurotoxic and neuroinflammatory properties that are more evident and potent in microglia than in astroglia (Frau *et al.*, 2013). In agreement with this observation, our results show that MDMA did not induce a significant effect on GFAP immunoreactivity in the hippocampus, whereas it did exert clear effects on microglial reactivity. The above-mentioned study, where the effects of MDMA on glial reactivity were analysed in nucleus accumbens, striatum, substantia nigra and cortex, showed that MDMA exerted its major effect in the striatum (Frau *et al.*, 2013). However, the hippocampus has not been extensively studied yet. The present results show that, in this brain region, THC significantly increased the percentage of GFAP immunoreactive area in both sexes. Although a trend in the same direction was observed for MDMA, no additive effects were found when both drugs were administered in combination. Rather, in females, MDMA counteracted the effect of THC, supporting other studies which report functional interactions between THC and MDMA in the control of astrocytic activation (Touriño *et al.*, 2010). The mechanisms underlying the interaction of MDMA and THC on astrocytes are unknown. THC may have direct effect on astrocytes, which express CB receptors, or exert indirect effects acting on neurons. In turn, MDMA may alter the release of neurotransmitters that affect astrocyte function, such as serotonin (Quesseveur *et al.*, 2013). Indeed, sex differences in the levels of serotonin (Ortiz *et al.*, 1988; Mitsushima *et al.*, 2006) may contribute to the different effects of MDMA on male and female astrocytes.

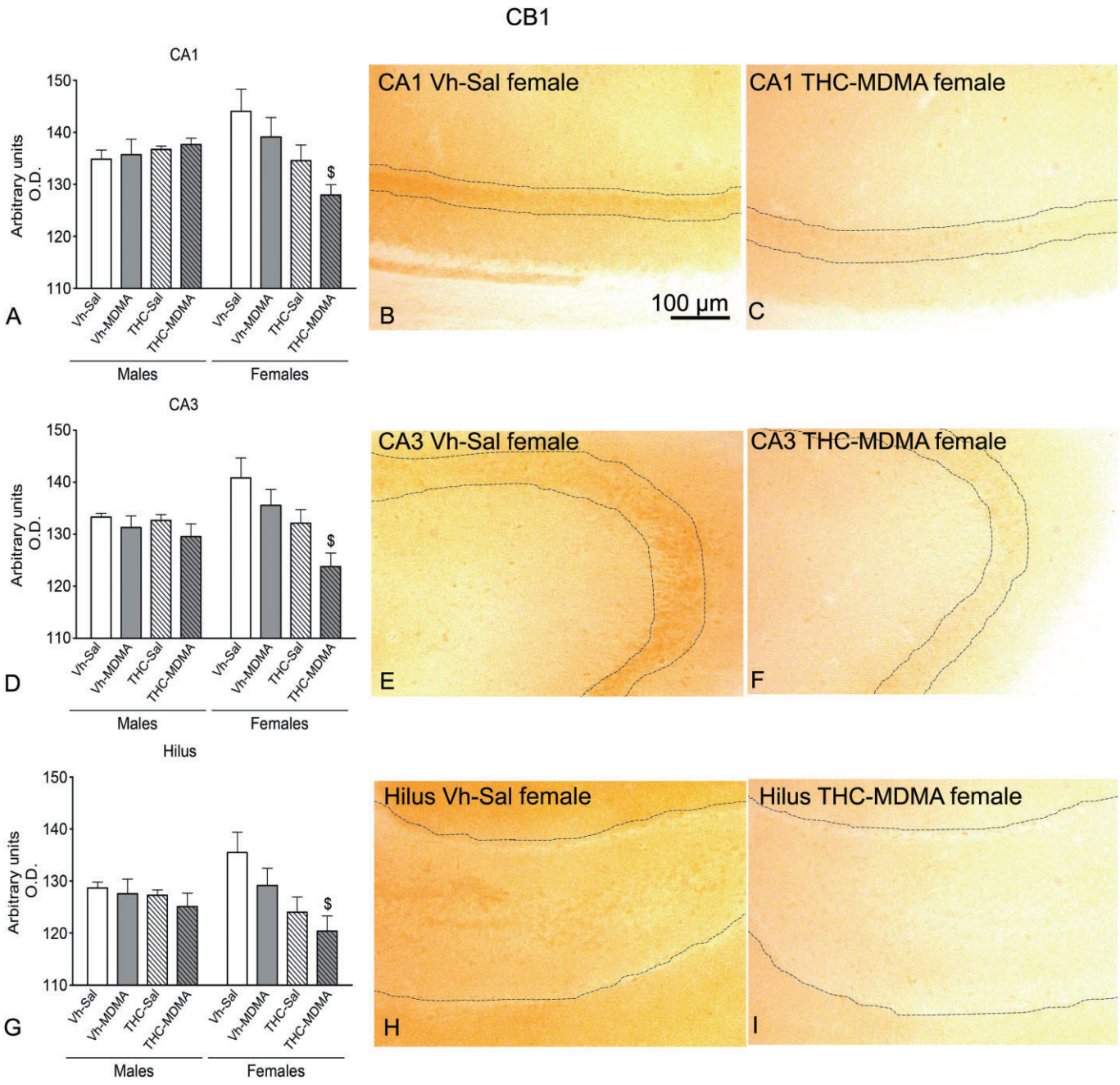

Figure 5

Optical densitometry of CB_1 receptor expression in three hippocampal areas, CA1, CA3 and hilus, of adult male and female rats. Animals were exposed to increasing doses of THC [2.5, 5 and 10 mg·kg^{-1} or vehicle (Vh)] from pnd 28 to 45, and to MDMA [10 mg·kg^{-1} or saline (Sal)] twice a day every 5 days from pnd 30 to 45. Representative images of CB_1 receptor immunoreactivity in CA1, CA3 and hilus. (B) CA1 Vh-Sal female; (C) CA1 THC-MDMA female; (E) CA3 Vh-Sal female; (F), CA3 THC-MDMA female; (H) hilus Vh-Sal female; and (I) hilus THC-MDMA female. Histograms representing the mean + SEM (n = 4–6) of optic densitometry of CB_1 receptor expression of CA1 (A), CA3 (D) and hilus (G) areas of the hippocampus. Bonferroni's *post hoc* test, $ $P < 0.05$, significantly different from female Vh-Sal.

Thus, it is likely that the interaction of THC and MDMA on astrocytes is mediated by modifications in neuroglia crosstalk.

The present results also show that, in males, both drugs, either separately or in combination, induced a significant increase of the percentage of reactive microglia cells (Iba-1 positive). Previous studies have shown that an acute treatment with MDMA in male Dark Agouti rats induced an increase in the levels of IL-1β (Orio *et al.*, 2004), which is a key mediator of microglia responses in health and disease (Giulian *et al.*, 1988; Sheng *et al.*, 1996; Griffin *et al.*, 1998). With respect to THC, *in vitro* studies with mouse cells have

demonstrated that this compound modulates the bioactivity of IL-1β (Shivers *et al*., 1994), controlling the activation of microglia. Our present findings, showing long-term effects of MDMA and THC treatments on the percentage of reactive microglia, suggests that prolonged administration of these drugs may result in persistent modifications of microglia reactivity.

The results that we found in females regarding the effects of the drugs on microglia reactivity were just the opposite of the ones observed in males. Thus, in females, each drug, when administered alone, produced a significant decrease of the percentage of reactive microgial cells, whereas the combination of both drugs resulted in a 'normalization' of this parameter, which reached control values. It is important to highlight that the basal state of microglia cells is completely different in male and female control animals, with females presenting higher levels of cells with reactive phenotype. These findings are in agreement with previous studies, indicating that males show more microglia cells at early post-natal development (pnd 4), whereas female rats show a higher number of reactive microglia cells during adolescence and in adulthood (pnd 30–60). This sex difference is extended to the levels of chemokines and the activity of microglia cells, which may account for their different role and contribution in the response to several insults (Schwarz *et al*., 2012). In view of these data, it is likely that depending on the 'starting' activational state of microglia, THC and MDMA produce different effects. Previous studies have shown that WIN55212-2, a synthetic cannabinoid agonist, administered daily for 21 days to young male Sprague-Dawley rats, decreased the number of activated microglia when it was administered together with the pro-inflammatory molecule LPS; however, it did not decrease the microglia reactivity when LPS was not present (Marchalant *et al*., 2007). This observation indicates that cannabinoid agonists may reduce the microglia reactivity when there is a previous activated state. Furthermore, Marchalant *et al*. (2007) also found that the control of microglia reactivity mediated by this cannabinoid agonist occurred in the dentate gyrus and CA1 of the hippocampal formation but not in CA3 or the entorhinal cortex, which further indicates an site-dependent effect. The fact that in the present study THC only reduced the percentage of microglia cells in females might be attributed to their high percentage of reactive microglia in basal conditions when compared with their male counterparts. Regarding the effects of MDMA on microglia cells in males and females, Connor *et al*. (2005) showed that MDMA administered acutely to male Sprague-Dawley rats increased the levels of the anti-inflammatory IL-10 and decreased the pro-inflammatory TNF-α when it was co-administered with LPS *in vivo* and *in vitro*(Connor *et al*., 2005); and other authors found that MDMA exerts pro-inflammatory effects when injected in basal conditions (Touriño *et al*., 2010). These findings suggest that MDMA also needs a previous activated state of microglia cells to reduce the microglia reactive phenotype. In line with this reasoning, and similar to what we described earlier for THC, our results indicate that MDMA reduced the percentage of microglia cells with a reactive phenotype only in females, which is the sex that showed more activated microglia cells in basal conditions. The fact that THC and MDMA when administered separately significantly decreased microglia activation with respect to the control group, what could be considered an 'anti-inflammatory' effect, should not be viewed as a beneficial effect since what the drugs are doing is changing the baseline (physiological) situation. Also within females, the combination of both drugs resulted in a return of the microglia activation to control levels. To explain these results, we propose the following explanation. According with our experimental protocol (see Figure 1), the animals exposed to THC + MDMA had already received two injections of THC before starting with the MDMA treatment. Thus, it is likely that when MDMA began to be administered, THC had already reduced the originally increased basal reactive microglia of females. In this situation, MDMA, which appears to be in need of a previous activated state to reduce microglia activation (Connor *et al*., 2005), would have increased microglia activation (as it did in males), which would result in a return to control values. It is known that CB_2 receptors are present in microglia cells and that depending on their activational state, there are changes in the expression of these cannabinoid receptors. In fact, CB_2 receptors are highly expressed by activated microglia (Ashton and Glass, 2007). Although an immunohistochemical analysis of these receptors have not been included in this study, it is plausible that the treatments that have induced an increase of reactive microglia have also caused an increase of CB_2 receptor expression.

Many studies have demonstrated that MDMA produces a reduction of serotonin levels, tryptophan hydroxylase and SERT (Baumann *et al*., 2007; Piper, 2007; Biezonski and Meyer, 2011). In agreement with these data, the present results show that adolescent exposure to MDMA induced a long-term significant reduction of SERT positive fibres in both sexes. This reduction has been classically considered a neurotoxic effect of MDMA on serotoninergic axons. However, nowadays, there is some evidence that points to a down-regulation of SERT gene expression induced by MDMA (for review, see Biezonski and Meyer, 2011). Therefore, the observed reduction in the surface density of SERT positive fibres may be due to serotoninergic axonal depletion and/or to decreased SERT gene expression. Our findings also indicate that THC induced a significant increase of SERT positive fibres in males with no changes in females. It is known that the endocannabinoid system plays a crucial role in neurodevelopmental processes during adolescence (Viveros *et al*., 2012) and modulates the activity of the serotoninergic system by inhibiting serotonin release via CB_1 receptor activation (Haj-Dahmane and Shen, 2011). It may be that chronic THC treatment and the resulting reduction of serotonin levels lead to a reorganization of serotoninergic fibres, which, in turn, could be reflected in an increase of SERT expression in the parietal cortex. In fact, previous data indicate that changes of the post-natal expression patterns of SERT may be the result of a reorganization of the serotoninergic innervation (Hansson *et al*., 1998). In contrast with the present findings, Shen *et al*. (2011) did not find any significant alteration of SERT expression in the parietal cortex of adult animals treated chronically with THC ($5\ mg{\cdot}kg^{-1}{\cdot}day^{-1}$) in the adolescent period. This apparent discrepancy might be attributed to the very different kinds of treatment, since in our case, we administered increasing doses of THC (2.5, 5 and $10\ mg{\cdot}kg^{-1}$) (see Figure 1), which likely prevented the development of

tolerance to the THC effect. In our hands, a normalization of the number of SERT positive fibres to control values was found in the animals exposed to both drugs.

There are several reports suggesting that acute administration of THC prevents some of the neurotoxic effects of MDMA (Morley *et al*., 2004; Parrott *et al*., 2007; Touriño *et al*., 2007; 2010). This idea comes from the assumption that THC and MDMA induce, when administered acutely, in the short-term, opposite pharmacological effects on certain parameters such as locomotion, thermal response and anxiety (Touriño *et al*., 2010). Most of the research about this topic have studied the acute effects and/or short-term outcomes of THC + MDMA combination and in adult animals, whereas only a few studies have used chronic treatments during the adolescent period, which is the temporal window when these drugs are usually consumed by humans (EMCDDA, 2012), and analysed the effects in the long term, after a relatively long washout period (between 44 and 47 days after the end of the pharmacological treatment). In these latter conditions, which mimic more adequately the usual human consumption pattern, the outcomes are far more complex. We have previously found that adolescent co-administration of THC and MDMA induced a stronger deterioration of working memory in females and attentional capabilities in both sexes compared with the effect of each drug when administered alone (Llorente-Berzal *et al*., 2013a). Other authors have reported that THC may somehow counteract the anxiogenic-like effect of MDMA (Morley *et al*., 2004; Shen *et al*., 2011), whereas we did not find any interaction of MDMA and THC in the plus maze (a test that is used to evaluate anxiety-related behaviour) (Llorente-Berzal *et al*., 2013a). Thus, in our case, the normalization of SERT expression induced by the combination of THC and MDMA does not appear to be reflected in 'normalization' at the behavioural level.

The present results show that SERT expression was higher in control females than in control males. The serotoninergic system presents sexual dimorphisms. Ortiz *et al*. (1988) reported higher plasmatic levels of serotonin in women than in men, and Mitsushima *et al*. (2006) found that male rats have higher levels of serotonin than females in the basolateral amygdala, possibly due to a higher synthesis rate in males in several brain regions, including the parietal cortex (Nishizawa *et al*., 1997). Our results show that control females show a higher density of SERT positive fibres in the parietal cortex, supporting previous studies in humans (Staley *et al*., 2001).

Regarding the results of CB_1 receptor expression, we did not observe significant effects of the pharmacological treatments in males, whereas in females, a nice 'staircase' effect appeared, with the group exposed to both drugs showing the lowest CB_1 expression. This same profile was found for the discrimination index in the novel object test for male and female animals, that is, no effects in males and a decrease in females with the highest decrease found in females treated with both THC and MDMA (Llorente-Berzal *et al*., 2013a). This test is usually employed to assess hippocampal-related long-term memory reconsolidation, and the discrimination index provides a measurement of the animals' ability to discriminate a novel object from a familiar one, which is known as object recognition memory (ORM) (Antunes and Biala, 2012). As it has been previously shown that endocannabinoid system is crucial for ORM (Clarke *et al*., 2008; Rubino and Parolaro, 2011), it is conceivable that the marked reduction of hippocampal CB_1 receptors in the female animals exposed to both drugs, THC and MDMA, is related to their impaired memory function (Llorente-Berzal *et al*., 2013a).

Previous studies with similar protocols have demonstrated long-term changes in CB_1 receptor density after chronic treatment with a CB_1 receptor agonist during adolescence, showing a decrease in CB_1 receptor immunoreactivity in males (Rubino *et al*., 2008), and similar results were obtained with a treatment with CP55940 in adolescent rats (López-Gallardo *et al*., 2012). The discrepancy between these data and the ones found in the present study could be due to the differences in the CB_1 receptor agonists, rats strain and/or previous manipulation, that is, the set of behavioural tests that the animals used in this case had performed (Llorente-Berzal *et al*., 2013a). We have already reported a basal sexual dimorphism in CB_1 receptor levels in the hippocampus, with females exhibiting lower levels of CB_1 receptors than males (Mateos *et al*., 2011; López-Gallardo *et al*., 2012; Llorente-Berzal *et al*., 2013c); however, the results of the present study shows the opposite trend. CB_1 receptor expression can be altered by disturbances during neurodevelopmental periods (Suárez *et al*., 2009; López-Gallardo *et al*., 2012). In the present experiments, control animals were submitted to chronic injections of vehicle and saline, rectal temperature measurements, and holeboard and elevated plus maze behavioural tests during adolescence (Llorente-Berzal *et al*., 2013a), which is a determinant neurodevelopmental period of the endocannabinoid system (Viveros *et al*., 2011). This could be a plausible explanation for the apparent discrepancy between the present results and the previous studies.

In summary, the results presented here showed that THC and/or MDMA administration during adolescence induced long-term alterations in neuroinflammation-related parameters and changes in serotoninergic and cannabinoid systems. The majority of the studies aimed at clarifying the effects of these two drugs do not represent the actual pattern of human consumption. For this reason, animal models such as the one used in here, which mimics more adequately the human pattern of drug use and abuse could provide more valuable evidence for the long-lasting effects of drugs. These results also point out that sex is a crucial factor. There is evidence for the existence of sex differences in the metabolism of several neurotransmitters such as dopamine, serotonin or GABA (Andreano and Cahill, 2009), as well as various neuropeptidergic systems (Bielsky *et al*., 2005; Kauffman, 2010). Therefore, it is plausible that compounds that interact with these systems, such as cannabinoids or psychostimulants, induce different responses in males and females.

Acknowledgements

This work was supported by Plan Nacional sobre Drogas Orden SAS/1250/2009, GRUPOS UCM-BSCH 951579, Red de Trastornos Adictivos RD06/0001/1013 and RD12/0028/0021 and Ministerio de Economía y Competitividad, Spain (Grant No. BFU2011-30217-C03-01).

Conflict of interest

None.

References

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Catterall WA, Spedding M, Peters JA, Harmar AJ and CGTP Collaborators (2013). The Concise Guide to PHARMACOLOGY 2013/14: Overview. Br J Pharmacol 170: 1449–1867.

Andreano JM, Cahill L (2009). Sex influences on the neurobiology of learning and memory. Learn Mem 16: 248–266.

Antunes M, Biala G (2012). The novel object recognition memory: neurobiology, test procedure, and its modifications. Cogn Process 13: 93–110.

Ashton JC, Glass M (2007). The cannabinoid CB2 receptor as a target for inflammation-dependent neurodegeneration. Curr Neuropharmacol 5: 73–80.

Battaglia G, Sharkey J, Kuhar MJ, de Souza EB (1991). Neuroanatomic specificity and time course of alterations in rat brain serotonergic pathways induced by MDMA (3,4-methylenedioxymethamphetamine): assessment using quantitative autoradiography. Synapse 8: 249–260.

Baumann MH, Wang X, Rothman RB (2007). 3,4-Methylenedioxymethamphetamine (MDMA) neurotoxicity in rats: a reappraisal of past and present findings. Psychopharmacology (Berl) 189: 407–424.

Bielsky IF, Hu SB, Young LJ (2005). Sexual dimorphism in the vasopressin system: lack of an altered behavioral phenotype in female V1a receptor knockout mice. Behav Brain Res 164: 132–136.

Biezonski DK, Meyer JS (2011). The nature of 3,4-methylenedioxymethamphetamine (MDMA)-induced serotonergic dysfunction: evidence for and against the neurodegeneration hypothesis. Curr Neuropharmacol 9: 84–90.

Biscaia M, Marin S, Fernandez B, Marco EM, Rubio M, Guaza C *et al.* (2003). Chronic treatment with CP 55,940 during the peri-adolescent period differentially affects the behavioural responses of male and female rats in adulthood. Psychopharmacology (Berl) 170: 301–308.

Bossong M, Niesink R (2010). Adolescent brain maturation, the endogenous cannabinoid system and the neurobiology of cannabis-induced schizophrenia. Prog Neurobiol 92: 370–385.

Bubeníková V, Votava M, Horácek J, Pálenícek T (2005). Relation of sex and estrous phase to deficits in prepulse inhibition of the startle response induced by ecstasy (MDMA). Behav Pharmacol 16: 127–130.

Carroll ME, Lynch WJ, Roth ME, Morgan AD, Cosgrove KP (2004). Sex and estrogen influence drug abuse. Trends Pharmacol Sci 25: 273–279.

Childers SR (2006). Activation of G-proteins in brain by endogenous and exogenous cannabinoids. AAPS J 8: E112–E117.

Clarke JR, Rossato JI, Monteiro S, Bevilaqua LR, Izquierdo I, Cammarota M (2008). Posttraining activation of CB1 cannabinoid receptors in the CA1 region of the dorsal hippocampus impairs object recognition long-term memory. Neurobiol Learn Mem 90: 374–381.

Connor TJ, Harkin A, Kelly JP (2005). Methylenedioxymethamphetamine suppresses production of the proinflammatory cytokine tumor necrosis factor-alpha independent of a beta-adrenoceptor-mediated increase in interleukin-10. J Pharmacol Exp Ther 312: 134–143.

Craft RM, Marusich JA, Wiley JL (2013). Sex differences in cannabinoid pharmacology: a reflection of differences in the endocannabinoid system? Life Sci 92: 476–481.

Diz-Chaves Y, Pernía O, Carrero P, Garcia-Segura LM (2012). Prenatal stress causes alterations in the morphology of microglia and the inflammatory response of the hippocampus of adult female mice. J Neuroinflammation 9: 71.

EMCDDA (2012). Annual Report 2012: The State of the Drug Problem in Europe. European Monitoring Centre for Drugs and Drug Addiction: Luxembourg.

Fonsart J, Menet MC, Debray M, Hirt D, Noble F, Scherrmann JM *et al.* (2009). Sprague-Dawley rats display sex-linked differences in the pharmacokinetics of 3,4-methylenedioxymethamphetamine (MDMA) and its metabolite 3,4-methylenedioxyamphetamine (MDA). Toxicol Appl Pharmacol 241: 339–347.

Frau L, Simola N, Plumitallo A, Morelli M (2013). Microglial and astroglial activation by 3,4-methylenedioxymethamphetamine (MDMA) in mice depends on S(+) enantiomer and is associated with an increase in body temperature and motility. J Neurochem 124: 69–78.

Giulian D, Woodward J, Young DG, Krebs JF, Lachman LB (1988). Interleukin-1 injected into mammalian brain stimulates astrogliosis and neovascularization. J Neurosci 8: 2485–2490.

Griffin WS, Sheng JG, Royston MC, Gentleman SM, McKenzie JE, Graham DI *et al.* (1998). Glial-neuronal interactions in Alzheimer's disease: the potential role of a 'cytokine cycle' in disease progression. Brain Pathol 8: 65–72.

Haj-Dahmane S, Shen RY (2011). Modulation of the serotonin system by endocannabinoid signaling. Neuropharmacology 61: 414–420.

Hansson SR, Cabrera-Vera TM, Hoffman BJ (1998). Infraorbital nerve transection alters serotonin transporter expression in sensory pathways in early postnatal rat development. Brain Res Dev Brain Res 111: 305–314.

Herkenham M, Lynn AB, Little MD, Johnson MR, Melvin LS, de Costa BR *et al.* (1990). Cannabinoid receptor localization in brain. Proc Natl Acad Sci U S A 87: 1932–1936.

Johnson EA, Shvedova AA, Kisin E, O'Callaghan JP, Kommineni C, Miller DB (2002). d-MDMA during vitamin E deficiency: effects on dopaminergic neurotoxicity and hepatotoxicity. Brain Res 933: 150–163.

Kauffman AS (2010). Gonadal and nongonadal regulation of sex differences in hypothalamic Kiss1 neurones. J Neuroendocrinol 22: 682–691.

Keimpema E, Mackie K, Harkany T (2011). Molecular model of cannabis sensitivity in developing neuronal circuits. Trends Pharmacol Sci 32: 551–561.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). Animal research: reporting *in vivo* experiments: the ARRIVE guidelines. Br J Pharmacol 160: 1577–1579.

Klein C, Karanges E, Spiro A, Wong A, Spencer J, Huynh T *et al.* (2011). Cannabidiol potentiates Δ^9-tetrahydrocannabinol (THC) behavioural effects and alters THC pharmacokinetics during acute and chronic treatment in adolescent rats. Psychopharmacology (Berl) 218: 443–457.

Llorente R, Arranz L, Marco EM, Moreno E, Puerto M, Guaza C *et al*. (2007). Early maternal deprivation and neonatal single administration with a cannabinoid agonist induce long-term sex-dependent psychoimmunoendocrine effects in adolescent rats. Psychoneuroendocrinology 32: 636–650.

Llorente-Berzal A, Fuentes S, Gagliano H, Lopez-Gallardo M, Armario A, Viveros MP *et al*. (2011). Sex-dependent effects of maternal deprivation and adolescent cannabinoid treatment on adult rat behaviour. Addict Biol 16: 624–637.

Llorente-Berzal A, Puighermanal E, Burokas A, Ozaita A, Maldonado R, Marco E *et al*. (2013a). Sex dependent behavioural and molecular effects of THC and MDMA in an animal model of adolescent drug consumption. PLoS ONE 8: e78386.

Llorente-Berzal A, Manzanedo C, Daza-Losada M, Valero M, López-Gallardo M, Aguilar MA *et al*. (2013b). Sex-dependent effects of early maternal deprivation on MDMA-induced conditioned place preference in adolescent rats: possible neurochemical correlates. Toxicology 311: 78–86.

Llorente-Berzal A, Assis MA, Rubino T, Zamberletti E, Marco EM, Parolaro D *et al*. (2013c). Sex-dependent changes in brain CB1R expression and functionality and immune CB2R expression as a consequence of maternal deprivation and adolescent cocaine exposure. Pharmacol Res 74: 23–33.

López-Gallardo M, López-Rodríguez AB, Llorente-Berzal Á, Rotllant D, Mackie K, Armario A *et al*. (2012). Maternal deprivation and adolescent cannabinoid exposure impact hippocampal astrocytes, CB1 receptors and brain-derived neurotrophic factor in a sexually dimorphic fashion. Neuroscience 204: 90–103.

McGrath J, Drummond G, McLachlan E, Kilkenny C, Wainwright C (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

Marchalant Y, Rosi S, Wenk GL (2007). Anti-inflammatory property of the cannabinoid agonist WIN-55212-2 in a rodent model of chronic brain inflammation. Neuroscience 144: 1516–1522.

Marco EM, Romero-Zerbo SY, Viveros MP, Bermudez-Silva FJ (2012). The role of the endocannabinoid system in eating disorders: pharmacological implications. Behav Pharmacol 23: 526–536.

Massi P, Valenti M, Bolognini D, Parolaro D (2008). Expression and function of the endocannabinoid system in glial cells. Curr Pharm Des 14: 2289–2298.

Mateos B, Borcel E, Loriga R, Luesu W, Bini V, Llorente R *et al*. (2011). Adolescent exposure to nicotine and/or the cannabinoid agonist CP 55,940 induces gender-dependent long-lasting memory impairments and changes in brain nicotinic and CB(1) cannabinoid receptors. J Psychopharmacol 25: 1676–1690.

Meyer JS, Piper BJ, Vancollie VE (2008). Development and characterization of a novel animal model of intermittent MDMA ('Ecstasy') exposure during adolescence. Ann N Y Acad Sci 1139: 151–163.

Mitsushima D, Yamada K, Takase K, Funabashi T, Kimura F (2006). Sex differences in the basolateral amygdala: the extracellular levels of serotonin and dopamine, and their responses to restraint stress in rats. Eur J Neurosci 24: 3245–3254.

Monks TJ, Jones DC, Bai F, Lau SS (2004). The role of metabolism in 3,4-(+)-methylenedioxyamphetamine and 3,4-(+)-methylenedioxymethamphetamine (ecstasy) toxicity. Ther Drug Monit 26: 132–136.

Moreira FA, Lutz B (2008). The endocannabinoid system: emotion, learning and addiction. Addict Biol 13: 196–212.

Morley KC, Li KM, Hunt GE, Mallet PE, McGregor IS (2004). Cannabinoids prevent the acute hyperthermia and partially protect against the 5-HT depleting effects of MDMA ('ecstasy') in rats. Neuropharmacology 46: 954–965.

Nishizawa S, Benkelfat C, Young SN, Leyton M, Mzengeza S, de Montigny C *et al*. (1997). Differences between males and females in rates of serotonin synthesis in human brain. Proc Natl Acad Sci U S A 94: 5308–5313.

O'Shea M, Singh ME, McGregor IS, Mallet PE (2004). Chronic cannabinoid exposure produces lasting memory impairment and increased anxiety in adolescent but not adult rats. J Psychopharmacol 18: 502–508.

O'Shea M, McGregor IS, Mallet PE (2006). Repeated cannabinoid exposure during perinatal, adolescent or early adult ages produces similar long-lasting deficits in object recognition and reduced social interaction in rats. J Psychopharmacol 20: 611–621.

Orio L, O'Shea E, Sanchez V, Pradillo JM, Escobedo I, Camarero J *et al*. (2004). 3,4-Methylenedioxymethamphetamine increases interleukin-1beta levels and activates microglia in rat brain: studies on the relationship with acute hyperthermia and 5-HT depletion. J Neurochem 89: 1445–1453.

Ortiz J, Artigas F, Gelpí E (1988). Serotonergic status in human blood. Life Sci 43: 983–990.

Palenicek T, Votava M, Bubenikova V, Horacek J (2005). Increased sensitivity to the acute effects of MDMA ('ecstasy') in female rats. Physiol Behav 86: 546–553.

Parrott AC (2004). MDMA (3,4-methylenedioxymethamphetamine) or ecstasy: the neuropsychobiological implications of taking it at dances and raves. Neuropsychobiology 50: 329–335.

Parrott AC, Milani RM, Gouzoulis-Mayfrank E, Daumann J (2007). Cannabis and Ecstasy/MDMA (3,4-methylenedioxymethamphetamine): an analysis of their neuropsychobiological interactions in recreational users. J Neural Transm 114: 959–968.

Paxinos G, Watson C (1998). The Rat Brain in Stereotaxic Coordinates, 4th edn. Elsevier: New York.

Piper BJ (2007). A developmental comparison of the neurobehavioral effects of ecstasy (MDMA). Neurotoxicol Teratol 29: 288–300.

Quednow BB, Jessen F, Kuhn KU, Maier W, Daum I, Wagner M (2006). Memory deficits in abstinent MDMA (ecstasy) users: neuropsychological evidence of frontal dysfunction. J Psychopharmacol 20: 373–384.

Quednow BB, Kühn KU, Hoppe C, Westheide J, Maier W, Daum I *et al*. (2007). Elevated impulsivity and impaired decision-making cognition in heavy users of MDMA ('ecstasy'). Psychopharmacology (Berl) 189: 517–530.

Quesseveur G, Gardier AM, Guiard BP (2013). The monoaminergic tripartite synapse: a putative target for currently available antidepressant drugs. Curr Drug Targets 14: 1277–1294.

Ramaekers JG, Theunissen EL, de Brouwer M, Toennes SW, Moeller MR, Kauert G (2011). Tolerance and cross-tolerance to neurocognitive effects of THC and alcohol in heavy cannabis users. Psychopharmacology (Berl) 214: 391–401.

Ros-Simó C, Moscoso-Castro M, Ruiz-Medina J, Ros J, Valverde O (2013). Memory impairment and hippocampus specific protein oxidation induced by ethanol intake and 3, 4-methylenedioxymethamphetamine (MDMA) in mice. J Neurochem 125: 736–746.

Rubino T, Parolaro D (2011). Sexually dimorphic effects of cannabinoid compounds on emotion and cognition. Front Behav Neurosci 5: 64.

Rubino T, Realini N, Braida D, Alberio T, Capurro V, Viganò D *et al.* (2009a). The depressive phenotype induced in adult female rats by adolescent exposure to THC is associated with cognitive impairment and altered neuroplasticity in the prefrontal cortex. Neurotox Res 15: 291–302.

Rubino T, Realini N, Braida D, Guidi S, Capurro V, Viganò D *et al.* (2009b). Changes in hippocampal morphology and neuroplasticity induced by adolescent THC treatment are associated with cognitive impairment in adulthood. Hippocampus 19: 763–772.

Rubino T, Vigano D, Realini N, Guidali C, Braida D, Capurro V *et al.* (2008). Chronic delta 9-tetrahydrocannabinol during adolescence provokes sex-dependent changes in the emotional profile in adult rats: behavioral and biochemical correlates. Neuropsychopharmacology 33: 2760–2771.

Schmued LC (2003). Demonstration and localization of neuronal degeneration in the rat forebrain following a single exposure to MDMA. Brain Res 974: 127–133.

Schneider M, Koch M (2003). Chronic pubertal, but not adult chronic cannabinoid treatment impairs sensorimotor gating, recognition memory, and the performance in a progressive ratio task in adult rats. Neuropsychopharmacology 28: 1760–1769.

Schneider M, Koch M (2007). The effect of chronic peripubertal cannabinoid treatment on deficient object recognition memory in rats after neonatal mPFC lesion. Eur Neuropsychopharmacol 17: 180–186.

Schulz S (2011). MDMA & cannabis: a mini-review of cognitive, behavioral, and neurobiological effects of co-consumption. Curr Drug Abuse Rev 4: 81–86.

Schwarz JM, Sholar PW, Bilbo SD (2012). Sex differences in microglial colonization of the developing rat brain. J Neurochem 120: 948–963.

Shen EY, Ali SF, Meyer JS (2011). Chronic administration of THC prevents the behavioral effects of intermittent adolescent MDMA administration and attenuates MDMA-induced hyperthermia and neurotoxicity in rats. Neuropharmacology 61: 1183–1192.

Sheng JG, Ito K, Skinner RD, Mrak RE, Rovnaghi CR, Van Eldik LJ *et al.* (1996). In vivo and in vitro evidence supporting a role for the inflammatory cytokine interleukin-1 as a driving force in Alzheimer pathogenesis. Neurobiol Aging 17: 761–766.

Shivers SC, Newton C, Friedman H, Klein TW (1994). delta 9-Tetrahydrocannabinol (THC) modulates IL-1 bioactivity in human monocyte/macrophage cell lines. Life Sci 54: 1281–1289.

Spear L (2000). The adolescent brain and age-related behavioral manifestations. Neurosci Biobehav Rev 24: 417–463.

Staley JK, Krishnan-Sarin S, Zoghbi S, Tamagnan G, Fujita M, Seibyl JP *et al.* (2001). Sex differences in [123I]beta-CIT SPECT measures of dopamine and serotonin transporter availability in healthy smokers and nonsmokers. Synapse 41: 275–284.

Stella N (2010). Cannabinoid and cannabinoid-like receptors in microglia, astrocytes, and astrocytomas. Glia 58: 1017–1030.

Suárez J, Llorente R, Romero-Zerbo SY, Mateos B, Bermúdez-Silva FJ, de Fonseca FR *et al.* (2009). Early maternal deprivation induces gender-dependent changes on the expression of hippocampal CB(1) and CB(2) cannabinoid receptors of neonatal rats. Hippocampus 19: 623–632.

Sullivan JM (2000). Cellular and molecular mechanisms underlying learning and memory impairments produced by cannabinoids. Learn Mem 7: 132–139.

Touriño C, Maldonado R, Valverde O (2007). MDMA attenuates THC withdrawal syndrome in mice. Psychopharmacology (Berl) 193: 75–84.

Touriño C, Zimmer A, Valverde O (2010). THC prevents MDMA neurotoxicity in mice. PLoS ONE 5: e9143.

Viveros M, Marco E, File S (2005). Endocannabinoid system and stress and anxiety responses. Pharmacol Biochem Behav 81: 331–342.

Viveros M, Llorente R, López-Gallardo M, Suarez J, Bermúdez-Silva F, De la Fuente M *et al.* (2009). Sex-dependent alterations in response to maternal deprivation in rats. Psychoneuroendocrinology 34 (Suppl. 1): S217–S226.

Viveros MP, Marco EM, File SE (2006). Nicotine and cannabinoids: parallels, contrasts and interactions. Neurosci Biobehav Rev 30: 1161–1181.

Viveros MP, Marco EM, Llorente R, Lamota L (2007). The role of the hippocampus in mediating emotional responses to nicotine and cannabinoids: a possible neural substrate for functional interactions. Behav Pharmacol 18: 375–389.

Viveros MP, Marco EM, López-Gallardo M, Garcia-Segura LM, Wagner EJ (2011). Framework for sex differences in adolescent neurobiology: a focus on cannabinoids. Neurosci Biobehav Rev 35: 1740–1751.

Viveros MP, Llorente R, Suarez J, Llorente-Berzal A, López-Gallardo M, de Fonseca FR (2012). The endocannabinoid system in critical neurodevelopmental periods: sex differences and neuropsychiatric implications. J Psychopharmacol 26: 164–176.

Walker QD, Williams CN, Jotwani RP, Waller ST, Francis R, Kuhn CM (2007). Sex differences in the neurochemical and functional effects of MDMA in Sprague-Dawley rats. Psychopharmacology (Berl) 189: 435–445.

Weibel ER (1979). Stereological Methods. I. Practical Methods for Biological Morphometry. Academic Press: London.

Wish ED, Fitzelle DB, O'Grady KE, Hsu MH, Arria AM (2006). Evidence for significant polydrug use among ecstasy-using college students. J Am Coll Health 55: 99–104.

Wolf SA, Tauber S, Ullrich O (2008). CNS immune surveillance and neuroinflammation: endocannabinoids keep control. Curr Pharm Des 14: 2266–2278.

Zamberletti E, Prini P, Speziali S, Gabaglio M, Solinas M, Parolaro D *et al.* (2012). Gender-dependent behavioral and biochemical effects of adolescent delta-9-tetrahydrocannabinol in adult maternally deprived rats. Neuroscience 204: 245–257.