Neuroimaging revealed long-lasting glucose metabolism changes to morphine withdrawal in rats pretreated with the cannabinoid agonist CP-55,940 during periadolescence

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Running title: Neuroimaging morphine withdrawal after CP-55,940

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ABSTRACT

This study evaluates the long-term effects of a six and 14-week morphine withdrawal in rats pretreated with a cannabinoid agonist (CP-55,940, CP) during periadolescence. Wistar rats (33 males; 32 females) were treated with CP or its vehicle (VH) from postnatal day (PND) 28-38. At PND100, rats performed morphine self-administration (MSA, 15d/12h/session). Eight groups were defined according to pretreatment (CP), treatment (morphine), and sex. Three [^{18}F]FDG-PET brain images were acquired: after MSA, and after six and 14 weeks of withdrawal. PET data were analyzed with SPM12. Endocannabinoid (EC) markers were evaluated in frozen brain tissue at endpoint. Females showed a higher mean number of self-injections than males. A main Sex effect on global brain metabolism was found. FDG uptake in males was discrete, whereas females showed greater brain metabolism changes mainly in areas of the limbic system after morphine treatment. Moreover, the morphine-induced metabolic pattern in females was exacerbated when CP was previously present. In addition, the CP-Saline male group showed reduced CB1R, MAGL expression, and NAPE/FAAH ratio compared to the control group, and morphine was able to reverse CB1R and MAGL expression almost to control levels. In conclusion, females showed greater and longer-lasting metabolic changes after morphine withdrawal than males, indicating a higher vulnerability and a different sensitivity to morphine in subjects pre-exposed to CP. In contrast, males primarily showed changes in EC markers. Together, our results suggest that CP pre-exposure contributes to the modulation of brain metabolism and EC systems in a sex-dependent manner.

Key words: cannabis, FDG-PET, opiate withdrawal, endocannabinoid system

INTRODUCTION

Cannabis is the most widely consumed illicit drug worldwide. It is a worrying fact that the age of initiation of cannabis use has decreased considerably in recent years—the United Nations Office on Drugs and Crime (UNODC) reported in the latest World Drug Report (2021) a dramatic increase in cannabis exposure at the age of 10. Early cannabis exposure can influence neurocognitive functioning affecting mood, anxiety, stress, memory, and learning processes (Crane et al., 2013), and has been associated with psychosis (Higuera-Matas et al., 2015; Sideli et al., 2020) and reinforcing effects of other drugs (Volkow et al., 2007). The neurobiological effects of cannabis use have been intensely investigated in clinical and preclinical studies in recent decades (Gunasekera et al., 2021; Kesner and Lovinger, 2021; Volkow et al., 2016). The findings of these studies support the hypothesis that cannabis use facilitates subsequent drug abuse. In line with these findings, we showed that chronic treatment with cannabinoids during periadolescence enhances cocaine self-administration in adult female but not male Wistar rats (Higuera-Matas et al., 2008). Furthermore, morphine self-administration increased in male Wistar rats who were exposed to cannabinoids during periadolescence, but only when the demands required by the reinforcement schedule were low, which could be related to the lower functionality of μ-opioid receptors in the Nucleus Accumbens (NAcc) observed in these animals (Biscaia et al., 2008).

Few studies have evaluated the lasting brain consequences of drug abstinence/withdrawal and brain recovery. Almost all of these studies were focused on abstinence periods of 25-28 days or less, as evidence suggests that cannabis metabolites are no longer detectable in urine and its effects on cognitive performance do not persist beyond this period (Schulte et al., 2014). Functional magnetic resonance imaging (fMRI) studies have shown decreased activation in the right lateral orbitofrontal and dorsolateral prefrontal cortices along with increased activation in the cerebellum and parietal lobe (Bolla et al., 2005), altered orbitofrontal activity, and dorsal striatal connectivity (Zimmermann et al., 2018) in dependent adult marijuana users. In addition, increased activation in the dorsolateral, ventral, prefrontal, and posterior cortices has been observed in adolescent cannabis users (Blest-Hopley et al., 2019).

In vivo functional neuroimaging could elucidate the neural mechanisms underlying drug abuse and withdrawal. In this sense, positron emission tomography (PET) studies represent an invaluable tool to investigate changes in brain function as a result of pharmacological manipulations. The PET tracer [^{18}F]-fluoro-2-deoxy-D-glucose (FDG) is a widely used glucose analog for imaging tissue glucose uptake and can be used as a surrogate marker of brain activity, mainly by glial cells (astrocytes and microglia cells) and neurons in physiological and pathological conditions (Jha and Morrison, 2018; Siracusa et

al., 2019; Zimmer et al., 2022). As microglial cells represent less than 10% of total cells in the brain, the remaining cells, are more likely to drive changes in the FDG-PET signal. Although the acute and chronic effects of drug abuse have been extensively studied using magnetic resonance imaging techniques, few studies have evaluated drug effects using FDG-PET (Gunasekera et al., 2021; Volkow et al., 2003). A recent study evaluated the chronic effect of Delta-9-tetrahydrocannabinol (THC) on glucose uptake in the rat brain, showing that stimulation of CB1 receptors (CB1R) by THC affects glucose uptake mainly in limbic structures, such as the hypothalamus and cerebellar cortex, increasing and decreasing their metabolism, respectively (Miederer et al., 2017). Morphine also decreases brain metabolism in limbic and forebrain regions, mainly in areas associated with motivation and emotion (Cohen et al., 1991; Park et al., 2017). In addition, FDG-PET imaging in combination with immunohistological studies revealed that the corpus callosum, retrosplenial cortex, and ventral pallidum were involved in opioid dependence (Chen et al., 2018). Clinical PET studies have shown that THC increased dopamine release in the mesolimbic reward system in cannabis users, patients with psychotic disorder and first-degree relatives, demonstrating differential sensitivity to THC in individuals at risk for psychosis (Kuepper et al., 2013). In this regard, differences in brain glucose metabolism patterns have also been reported in two inbred rat models with different vulnerability to morphine self-administration (Soto-Montenegro et al., 2022).

However, few studies have evaluated the consequences of long-term drug withdrawal on brain metabolism while controlling for sex (Santoro et al., 2017). Only one study evaluated the underlying metabolic effects of spontaneous opioid withdrawal (Santoro et al., 2017), showing that replacement with methadone or buprenorphine abolished impaired glucose metabolism in regions associated with reward and opioid dependence in a sex-dependent manner. To our knowledge, this is the first study to evaluate long-term effects of a six and 14-week morphine withdrawal on brain metabolism in male and female rats pretreated with a cannabinoid agonist during periadolescence using *in vivo* FDG-PET imaging. Notably, a period of 14 weeks in rats is approximately equivalent to 10 human years (Sengupta, 2013), thus representing the first *in vivo* report on brain metabolism after morphine withdrawal over an equivalent period of 10 years in humans. In addition, we also measured changes in several biomarkers of the endocannabinoid system (ECS) as outcomes.

MATERIAL & METHODS

A schematic representation of the study design is shown in figure 1.A.

Animals

Male (N=33) and female (N=32) Wistar rats were maintained at constant temperature (24±0.5ºC) under a 12-hour light/dark cycle, with free access to food and water. Sample size was based on previous data of our group in opiate self-administration studies showing differences between groups (Soto-Montenegro et al., 2022; Ucha et al., 2019). Experimental procedures were conducted in conformity with Directive 2010/63/EU of the European Parliament and approved by the Ethics Committee for Animal Experimentation of Hospital Gregorio Marañón (ES280790000087) and the National University for Distance Learning (UNED) (ES280790000185).

Drug treatment

Cannabinoid agonist CP-55,940 (CP) (0.4mg/kg) or its vehicle (VH) (ethanol: Cremophor: saline solution [1:1:18] [Cremophor, Fluka BioChemiKa]) was administered intraperitoneally daily during periadolescence, from post-natal day (PND) 28 to PND 38 (Higuera-Matas et al., 2011).

Morphine sulphate (1 mg/kg dissolved in saline solution) or its saline was self-administered intravenously during 15 days at adulthood (PND100-115) (Garcia-Lecumberri et al., 2011; Soto-Montenegro et al., 2022).

Four groups per sex were established, according to the treatments: VH-Saline (male=10; female=7); VH-Morphine (male=7; female=9); CP-Saline (male=8; female=9); and CP-Morphine (male=8; female=7).

Experimental procedures

Morphine/saline self-administration study

The self-administration procedure was performed as described elsewhere (Garcia-Lecumberri et al., 2011; Soto-Montenegro et al., 2022) (Figure 1A). Briefly, on PND75, animals were submitted to an autoshaping fixed-ratio 1 (FR1) schedule of food reinforcement over 30 minutes for five days, and were allowed to recover their free-feeding weight for 10 days. On PND 90, a catheter was placed in the right jugular vein to allow saline/morphine self-administration. On PND100, rats started the self-administration study, which consisted of 12-hour day sessions during dark cycle with morphine or saline under a FR1 schedule of reinforcement for 15 consecutive days. This procedure was performed in an operant conditioning chamber, and lever-pressing data were recorded daily, as previously described by our group (Soto-Montenegro et al., 2022). A limit of 50 infusions per session was set in order to avoid overdosing.

Imaging studies

Animals were scanned at three different time points: MSA (PND115), and after six and 14 weeks of morphine withdrawal.

PET: Animals were scanned using a dedicated PET/CT scan for small animals (ARGUS PET/CT, SEDECAL, Madrid) under isoflurane anesthesia (3% induction, 1.5% maintenance in 100% O_2). FDG (~1.5 mCi) was injected intravenously and after an uptake time of 45 minutes, animals were imaged for 60 minutes. Tomographic images were reconstructed with a 3D-OSEM (Ordered Subsets Expectation Maximization) algorithm. Decay and deadtime corrections were applied.

Computed tomography (CT): Studies were acquired using the above-mentioned PET/CT scanner with 360 projections at 40 kV and 340 mA. Images were reconstructed using a Feldkamp algorithm (Abella et al., 2012). These images were used for the spatial normalization of PET images.

MRI (Magnetic Resonance Imaging): MR image from one random animal of each sex were obtained at PND 115 with a 7-Tesla Biospec 70/20 scanner (Bruker, Ettingen, Germany) under isoflurane anesthesia. A coronal T2-weighted spin echo sequence was acquired with previously described parameters (Casquero-Veiga et al., 2021; Romero-Miguel et al., 2021). These images were used as an anatomic template to localize the statistical parametric mapping (SPM) results.

PET data pre-processing and intensity normalization were performed as previously described (Gasull-Camos et al., 2017). PET data were analysed by using SPM12 (http://www.fil.ion.ucl.ac.uk/spm/software/spm12/). Groups were compared using ANOVA tests, setting a threshold of $p<0.05$ uncorrected (voxel-level significance), but only results corrected at cluster-level by False Discovery Rate (FDR) were considered significant in order to control type I errors. A 100-voxel clustering (spatial-extent) threshold was also applied. In addition, a region of interest (ROI) analysis of the whole brain was performed to rule out global differences in brain metabolism.

Biochemical determinations

Since the cannabinoid signaling system could be altered by the use of CP-55,940, cannabinoid receptors (CB1 and CB2) and critical enzymes involved in endocannabinoid metabolism, such as N-acyl phosphatidylethanolamine-specific phospholipase D (NAPE), diacylglycerol lipase (DAGL), fatty acid amidohydrolase (FAAH), and monoacylglycerol lipase (MAGL), were studied. For this purpose, frozen tissue samples from the NAcc and/or striatum (6-9 animals per group) were used to study these endocannabinoid components by Western blot techniques. We also determined the NAPE/FAAH and DAGL/MAGL ratios as indirect index of anandamide (AEA) and 2-arachidonoylglycerol (2-AG) levels respectively

(Notarstefano et al., 2020). These determinations were performed in cytosolic extracts. Protein levels were measured using the Bradford method.

Proteins were loaded into electrophoresis gel, and then blotted onto a membrane with a semi-dry transfer system. Blots were blocked with 5% BSA (Sigma, Spain) 1 hour at RT and probed overnight at 4°C with rabbit anti-CB1 (ab23703, 1:1000 BSA 0.5%; Abcam, UK), rabbit anti-CB2 (101550, 1:1000 BSA 0.5%; Cayman, Estonia), goat anti-DAGL (ab81984, 1:1000 BSA 0.5%; Abcam, UK), rabbit anti-FAAH (101600, 1:1000 BSA 0.5%; Cayman, Estonia), rabbit anti-MAGL (100035, 1:1000 BSA 0.5%; Cayman, Estonia), rabbit anti-NAPE (10306, 1:1000 BSA 0.5%; Cayman, Estonia) and mouse anti-β-actin (A5441, 1:10000 TBSt; Sigma, Spain). Respective horseradish peroxidase-linked secondary antibodies were employed to detect each protein. The binding was detected by an Odyssey Fc System (LI-COR®, Germany). Housekeeping β-actin was used as a loading control and all western blots were performed at least three times in separate assays.

Statistical analysis

Results were analyzed by means of two- and three-way analyses of variance (ANOVA) followed by LSD post-hoc tests. Normality and homoscedasticity of the data were tested using Shapiro-Wilk's and Levene's tests, respectively. Non-normal or heteroscedastic variables were analyzed by Kruskal-Wallis' test, followed by Dunn's post-hoc. A p-value <0.05 was considered statistically significant. Data are represented as mean ± S.E.M.

RESULTS

The results of MSA, ROIs and biochemical determinations analyzed by three-way ANOVA and separated by sex (two-way ANOVA) are shown in Table 1 and Table 2, respectively. The estimated size effect of these analyses are shown in Supplementary Table 1 and Supplementary Table 2. Finally, SPM results are shown in Table 3 (females) and Table 4 (males).

Morphine/saline self-administration study

Three-way ANOVA showed a significant interaction between Sex and Morphine ($p<0.01$). In addition, we conducted an analysis for each sex separately, showing a main effect of Morphine ($p<0.001$) in females but not males (Table 1). Thus, post-hoc tests showed that morphine-treated females self-administered a higher mean number of injections than controls (VH-Saline), especially when pre-exposed to CP (Figure 1B). Besides, both morphine and CP-morphine females self-administered a higher mean number of injections than males (Figure 1B).

Global brain analysis

At the end of the MSA study, three-way ANOVA showed a significant main effect of Sex in global brain metabolism ($p<0.05$). Significant interactions between Sex and Morphine ($p<0.05$) and Sex, CP, and Morphine ($p<0.05$) were also found (Table 1). CP-Morphine females showed increased global metabolism compared to CP-Morphine males. The analysis for each sex separately showed a significant interaction between CP and Morphine ($p<0.01$) in males (Table 2). Thus, CP pre-exposure in males induced increased metabolism compared to the control group (VH-saline) that was reduced by morphine. In addition, in females, morphine induced increased metabolism in CP-treated animals compared to the control group (Figure 2A).

After six-week withdrawal, three-way ANOVA showed a significant main effect of Sex in global brain metabolism ($p<0.001$) together with a significant interaction between Sex and Morphine ($p<0.001$) (Table 1). Thus, VH-females showed increased brain metabolism compared to males. The analysis for each sex separately showed a significant main effect of Morphine in females ($p<0.05$) (Table 2). CP-Morphine females showed a higher brain metabolism compared to the control group, while males showed the opposite effect (Figure 2A).

After fourteen-week withdrawal, ANOVA showed a significant main effect of Sex in global brain metabolism ($p<0.001$) (Table 1). Thus, VH-females showed increased brain metabolism compared to males (Figure 2A).

SPM study

Figures 3 and 4 show the brain metabolic changes measured via PET at adulthood in females and males, respectively.

At the end of the MSA study (Figure 3A and Figure 4A), females pre-exposed to CP showed no statistically significant brain changes, whereas CP-males showed increased metabolism in the subiculum, visual and somatosensory cortex, and caudate-putamen (CPu). Morphine increased glucose uptake in the brainstem, Locus Coeruleus (Lc), and hippocampus in VH- and CP-females, and in the thalamus, periaqueductal gray (PAG), ventral tegmental area (VTA), habenula, and insular cortex in CP-females. In contrast, morphine induced increased metabolism in the cerebellum and the somatosensory and motor cortices in VH-males, whereas no statistically significant changes were found in CP-males.

After six-week withdrawal (Figure 3B and Figure 4B), CP pre-exposed animals showed no brain changes. VH-morphine females showed increased metabolism in the

brainstem, Lc, PAG, somatosensorial cortex, hippocampus, subiculum, thalamus, and habenula, and reduced metabolism in the cerebellum and retrosplenial cortex. Males showed no statistically significant changes. Brain differences in brainstem, Lc, PAG, and thalamus were maintained in CP-Morphine females, while in CP-Morphine males no statistical significant brain changes were maintained.

After 14-week withdrawal (Figure 3C and Figure 4C), CP pre-exposed females showed reduced metabolism in the retrosplenial, ectorhinal, somatosensory, and piriform cortices, whereas males showed no brain changes. VH-morphine females showed decreased FDG uptake in the amygdalo-piriform area and entorhinal cortex, whereas males showed no statistically significant effects. Brain changes in the amygdala and the ectorhinal and entorhinal cortices were maintained in CP-Morphine females, along with reduced metabolism in the retrosplenial cortex, while no significant clusters were found in CP-Morphine males.

Biochemical determinations

Cannabinoid receptors and endocannabinoid enzymes were determined *post-mortem* in Striatum and NAcc (Figure 2B).

CB1R (Figure 2.B.1)**:** The Kruskal-Wallis test showed significant differences between groups in CB1R expression in the NAcc ($p<0.001$) (Table 1). Thus, CP-Saline males showed reduced CB1R expression compared to CP-Saline females. The analyses separated by sex showed that CP-Saline males showed reduced CB1R expression compared to the control group (VH-Saline) that was reversed by morphine. In addition, we found an interaction between CP and Morphine ($p<0.01$) in males (Table 2). Thus, morphine reduced CB1R expression in VH-males, while increased it in CP-males. In VH-females, we only found that morphine increased CB1R expression in the striatum in CP-animals.

CB2R (Figure 2.B.1)***:*** In the NAcc, three-way ANOVA showed a significant main effect of Sex in the expression of CB2R in the NAcc ($p<0.05$), and a significant interaction between Sex, CP, and Morphine ($p<0.05$) (Table 1). CP-males showed lower concentrations of CB2R compared to CP-females. In the striatum, the Kruskal-Wallis test showed significant differences between groups in the expression of CB2R ($p<0.01$) (Table 1).

In females, the analysis by sex revealed a significant main effect of CP ($p<0.001$) in the striatum, and an interaction between CP and Morphine in both the striatum ($p<0.01$) and NAcc ($p<0.05$) (Table 2). Morphine increased CB2R expression in VH-females compared to controls in the NAcc, while in the striatum, decreased this expression in CP-females compared to controls (VH-Saline) and CP-Saline females (Figure 2.B.1).

NAPE (Figure 2.B.2)***:*** The Kruskal-Wallis test showed significant differences in NAPE expression ($p<0.01$) between groups (Table 1). CP-Saline males showed lower NAPE expression than CP-Saline females. The analysis by sex showed a significant main effect of CP ($p<0.001$) and Morphine ($p<0.05$) in males (Table 2). CP decreased the expression of NAPE in both saline and Morphine-treated groups compared to controls (VH-Saline) in males (Figure 2.B.2).

DAGL (Figure 2.B.2)***:*** Three-way ANOVA showed a significant main effect of Sex in DAGL expression ($p<0.05$), and a significant interaction between Sex and Morphine ($p<0.05$), and between Sex, CP, and Morphine ($p<0.05$) (Table 1). VH-Morphine females showed lower DAGL expression than males. In addition, Morphine decreased DAGL in VH-females compared to controls (VH-Saline).

FAAH (Figure 2.B.2)***:*** Three-way ANOVA showed a significant main effect of Sex in FAAH expression ($p<0.01$) and a significant interaction between Sex, CP, and Morphine ($p<0.05$) (Table 1). Thus, VH-Morphine males showed decreased FAAH compared to VH-Morphine females. In addition, CP-Saline males showed lower FAAH expression than CP-Saline females, as well as compared to their control group (VH-Saline).

MAGL (Figure 2.B.2)***:*** Three-way ANOVA showed a significant main effect of Sex ($p<0.001$), CP ($p<0.001$), and Morphine ($p<0.05$), and a significant interaction between Sex and CP ($p<0.05$) (Table 1). Thus, CP-Saline males showed reduced DAGL expression compared to CP-Saline females. The analysis by sex showed a CP effect in males ($p<0.001$) (Table 2). Thus, CP reduced MAGL expression in males while morphine reversed it.

NAPE/FAAH (Figure 2.B.3)***:*** The Kruskal-Wallis test showed significant differences between groups ($p<0.01$) (Table 1). The analysis by sex showed a main effect of CP ($p<0.001$) and Morphine ($p<0.05$) in males (Table 2). Morphine increased the NAPE/FAAH ratio in VH-males while this ratio was reduced CP-males.

DAGL/MAGL (Figure 2.B.3)***:*** The Kruskal-Wallis test showed significant differences between groups ($p<0.01$) (Table 1). Thus, VH-Morphine females showed a reduced DAGL/MAGL ratio compared to VH-Morphine males. The analysis by sex showed a main effect of Morphine in females ($p<0.01$) (Table 2).

DISCUSSION

Morphine self-administration study

In the present study, we found periadolescent exposure to the cannabinoid CP-55,940 altered the reinforcing properties of morphine in female but not in male rats, with a significant

increase in the mean number of morphine injections. These results are consistent with studies finding increased morphine self-administration in females exposed perinatally to THC, with no change in males exposed to THC (Vela et al., 1998), and no alteration in heroin self-administration in males exposed prenatally to THC under the same reinforcement schedule used in our study (Spano et al., 2007). However, other studies have shown increased morphine self-administration in males exposed to CP-55,940 during adolescence (PND35-PND45) with no change in females (Biscaia et al., 2008), and increased heroin self-administration in males (PND28-PND49) exposed to THC (Ellgren et al., 2007) under the same schedule of reinforcement. In this respect, the differences between the studies could be related to the varying vulnerability of different rat strains to drugs, as previously reported (Soto-Montenegro et al., 2022). Notably, morphine by itself did not increase self-administration in VH-males, but it did when these animals were pretreated with CP-55,940 during adolescence, similar to what has been previously published by our group (Biscaia et al., 2008). The lack of this effect in VH-males could be related to the low number of subjects in this group compared to the other groups.

Much is known about sex differences in opioid self-administration, including reinforcing properties (Biscaia et al., 2008; Bobzean et al., 2014; Cicero et al., 2003). Our results are consistent with greater opioid self-administration in females, as previously reported by our group (Ambrosio et al., 1999), supporting the notion that females find opiates more reinforcing than males, work harder to get the drug, and are more vulnerable to the effects of CP-55,940 compared to THC (Cicero et al., 2003).

Long-term effects of cannabis on glucose metabolism

Most neuroimaging studies focus on the acute effects of cannabinoids, and few studies have examined the long-term effects of its withdrawal using *in vivo* imaging techniques. Most PET studies have focused on abstinence ranges from 12-72 hours (Block et al., 2002; Block et al., 2000) to 25-28 days (Bolla et al., 2005; Pope et al., 2001), making it difficult to determine whether many of the published results are due to persistent drug impairment or withdrawal symptoms. In these studies, the prefrontal cortex (PFC), hippocampus, and cerebellum were the brain regions most affected by cannabis use, and their dysfunction has been associated with poor cognitive abilities, mainly in executive function, memory, and decision-making (Bolla et al., 2005).

In our study, males showed increased glucose metabolism in limbic system areas such as CPu, hippocampal-subiculum area, and visual cortex at adulthood, whereas females showed non-significant metabolic reductions in cortical areas until the end of the study. In contrast, the metabolic changes found in males in limbic system areas were not maintained

throughout the study, suggesting that males may recover a normal metabolic pattern from the detrimental effects of CP during periadolescence. ROI analysis appears to support this finding, as males showed constant global brain metabolic levels along the study.

Long-term effects of morphine on glucose metabolism

PET results showed that morphine was associated with increased metabolism in the cortex and cerebellum in males, whereas these changes were subcortical in females, mainly in the hippocampus and brainstem. These metabolic differences between sexes could explain the greater rates of compulsive drug-seeking in females. Midbrain dopaminergic cells have been shown to be involved in incentive salience and reward (Koob and Volkow, 2016), and this process may reinforce the learned association with repeated morphine exposure, which could create strong reward seeking and explain the increased metabolism observed in the hippocampus.

Multiple neural circuits are involved in the withdrawal process. Activation of the hypothalamic-pituitary-adrenal axis underlying stress and anxiety-like responses, together with the extended amygdala, are important components in the production of negative emotional states leading to negative reinforcement (Koob and Volkow, 2016). Here, we demonstrated that morphine induces long-lasting changes in brain glucose metabolism in females in areas related to the limbic system, such as the amygdala and piriform and entorhinal cortices. The metabolic reductions found in the amygdalo-piriform area are supported by functional decreases in the extended amygdala neurocircuitry after withdrawal (Koob and Volkow, 2016), and are associated with functional decreases in the dopaminergic component of the reward system and dysregulation of afferent projections from the PFC to the amygdala, among others (Koob and Volkow, 2016). Nearly significant reductions were also found in the amygdalo-piriform area in males. The piriform cortex is part of the olfactory cortex, which is involved in memory processing and encoding (Vismer et al., 2015) and connects with several limbic areas such as the amygdala, hippocampus, and rhinal cortex. Our results are consistent with an fMRI study in an animal model of precipitate morphine withdrawal, in which positive changes in BOLD contrast were found within the dentate gyrus, visual, auditory, insular, cingulate, and piriform cortices, supporting the importance of these structures in withdrawal, and their involvement in impairments in attentional performance following prolonged opioid abstinence (Prosser et al., 2009).

In addition, we showed sex-dependent differences in the mesolimbic reward circuitry. Specifically, females showed increased glucose metabolism in the hippocampus after six and 14 weeks of morphine withdrawal. This area is involved in the formation of drug-context memories and drug-cue associations (Kokane and Perrotti, 2020; Kutlu and Gould, 2016).

Furthermore, long-term structural and functional changes in this area are associated with chronic drug use (Nestler, 2016). It should be noted that most of what is known about morphine and opioid withdrawal comes from studies conducted only in males. However, the transition to addiction is faster in women, who also have more difficulty maintaining abstinence (Kokane and Perrotti, 2020). Among the possible factors that could explain these differences are ovarian hormones, specifically estrogen and estradiol, which have been involved in the sensitization of dopaminergic neurons by increasing their responsiveness to opioids (Becker et al., 2005). In addition, these hormones have been associated with a redistribution of δ-μ opioid receptors in hippocampal circuits (Ryan et al., 2018). These characteristics could explain the facilitation of opioid-associative learning processes and the increased susceptibility of females to opioid addiction. In our study, the estrous cycle was not controlled in female rats, as most human neuroimaging studies do not consider the hormonal conditions of women. In line with this fact, there has recently been growing interest in performing human neuroimaging studies based on the menstrual cycle, as brain structure, chemistry, and function might be affected (Dubol et al., 2021). This fact may explain some of the sex differences observed in our animals.

Long-term effects of CP and morphine on glucose metabolism

The functional brain circuitry associated with morphine withdrawal in subjects pre-exposed to a cannabinoid agonist has not been studied in depth, and no long-term *in vivo* neuroimaging study using FDG-PET as proposed in this study has been previously published. Here, we show that the morphine-induced metabolic pattern in females was exacerbated when CP was present mainly at the end of the MSA, but also during withdrawal, suggesting that CP may cause permanent brain metabolic changes in females.

Morphine induced enhanced metabolism in females in the brainstem, hippocampus, thalamus, PAG, VTA, habenula, insular, and somatosensorial cortices, all of which are related to the reward system. Of importance, the endocannabinoid and opioid systems share the distribution of CB1R and μ-opioid receptors in many brain areas, which may provide interactions between the two systems in reward and withdrawal (Wiese and Wilson-Poe, 2018). Indeed, a bidirectional relationship between CB1R and μ-opioid receptors has been previously reported among the rewarding properties of drug abuse (Wills and Parker, 2016). This could explain the enhanced glucose metabolism observed at the end of the MSA in females but not in males where no brain metabolic differences were found. In fact, ROI analysis showed a greater pattern of metabolic changes in females, which could be related to the higher mean number of morphine injections compared to males.

Two brain structures of interest in opioid addiction and drug withdrawal are the PAG, which is related to chronic exposure to drugs of abuse, modulating anxiety, fear, and nociception (Vazquez-Leon et al., 2021), and the habenula, related to negative reinforcement, such as lack of expected reward (Curtis et al., 2017; McIlwrath et al., 2020), and drug-associated impulsive behavior (Zapata and Lupica, 2021). Here, we show an increase in glucose metabolism in the PAG and habenula after MSA that recovered throughout withdrawal to normal values. The PAG sends and receives projections between the VTA, the extended amygdala, the medial PFC, and the hypothalamus, among others. In addition, the habenula has significant concentrations of μ-opioid, CB1 (Zapata and Lupica, 2021), and nicotinic receptors (Neugebauer et al., 2013). Specifically, the PAG sends glutamatergic and GABAergic inputs to dopamine- and GABA-containing cells in the VTA, and this VTA-PAG circuitry plays an important role in the early phases of drug addiction (George et al., 2019). Thus, the VTA-PAG circuitry, together with the basolateral amygdala and the NAcc, form a circuit in response to a prolonged stressful situation (Horovitz et al., 2017), as in the case of chronic opioid use. As the stressful situation disappears, there is a gradual normalization of glucose metabolism in these areas.

Furthermore, the metabolic changes observed in females were maintained after a six-week morphine withdrawal, whereas they almost disappeared after 14 weeks, suggesting that recovery in females is slower compared to males. However, this metabolic recovery was not complete, as some metabolic reductions appeared in the restrosplenial, entorhinal, and ectorhinal cortices, which are brain areas associated with cognitive deficits in memory as has been shown in cannabis- and opioid-dependent adolescents (Vo et al., 2014).

Long-term effects of CP and morphine on endocannabinoid system

The endocannabinoid system plays a key role in several aspects of neurodevelopment, including neuronal migration, glia formation, brain cell proliferation, axonal migration and connectivity, and synaptogenesis (Martinez-Pena et al., 2021). Thus, prenatal or adolescence cannabinoid exposure has been associated with a large number of behavioral alterations (Campolongo et al., 2011; Pinky et al., 2019), as well as abnormalities in the maturation of different neurotransmitter systems including the opioidergic system (Martinez-Pena et al., 2021) and progressive changes in the cannabinoid receptor density (Biegon and Kerman, 2001; Mato et al., 2003) among others. In this regard, we have found a reduction in CB1/2R and EC enzymes (NAPE, MAGL, FAAH) in the NAcc in males exposed to CP-55,940 during periadolescence, but not in females. Cannabinoid exposure is known to cause downregulation of CB1R, especially in cortical areas (D'Souza et al., 2016; Sim-Selley, 2003). However, the time course of CB1R changes after abstinence/withdrawal remains controversial. Thus, two days of monitored cannabis abstinence resulted in

normalization of CB1R number and function in cortical areas, as measured by *in vivo* PET imaging using the reversible [^{11}C]OMAR ligand (D'Souza et al., 2016), and this result was maintained after 28 days of abstinence. In male mice, a 15-day THC administration produced a down-regulation of CB1R in the striatum/globus pallidus and hippocampus that returned to control levels after two weeks of withdrawal (Sim-Selley et al., 2006).

Our findings contrast with previous literature, as our male animals exposed to CP-55,940 showed reductions in CB1R levels in the NAcc after 14 weeks of withdrawal. Several factors could explain these differences, such as the different cannabinoid agonist used, the dose and magnitude of exposure, the time of evaluation, and the brain region studied. Furthermore, an interaction between cannabis and testosterone has been reported, which could also account for these differences in NAPE/FAAH and DAGL/MAGL indices in males (Hsiao and Clavijo, 2018). These findings of reduced endocannabinoid signaling in males were unexpected, as these animals showed a recovery of normal brain metabolism at the end of the study, whereas the opposite pattern was found in females.
It should be highlighted that tissue glucose uptake measured by PET can be used as a surrogate marker of glial and neuronal activity but does not provide information on the endocannabinoid system *per se*. Furthermore, the lower resolution of *in vivo* PET imaging may prevent masking and detection of significant differences in the NAcc that would be associated with reduced signaling of the endocannabinoid system.

Data regarding CB2R and withdrawal is null, as until recently CB2R was considered a peripheral cannabinoid receptor involved in the regulation of the immune system (Cabral and Griffin-Thomas, 2009). However, in recent years it has also been detected in glia (Navarrete et al., 2018) and neurons (Zhang et al., 2014), mainly in brain areas related to the reward system, which supports its relevance in the field of addiction (Navarrete et al., 2021). CB2R activation is associated with anti-inflammatory and neuroprotective properties, due to its suppressive effect on reactive microglia (Bie et al., 2018), which has made CB2R as potential targets to treat psychiatric disorders such as schizophrenia and depression, and chronic neurodegenerative disorders (Cortez et al., 2020). In this sense, we found a reduction in CB2R expression in the NAcc in males exposed to CP-55,940 during periadolescence, similar to that found for CB1R expression. Due to the possible role of CB2R in exerting neuroprotection by blocking microglial activation (Bie et al., 2018), we elucidated whether this reduction of CB2R expression could have a negative effect on the progression of withdrawal syndrome associated with cannabis abuse.

Regarding biosynthetic and degradative enzymes in the NAcc, we found a reduction of NAPE, the main enzyme involved in AEA synthesis, together with a reduction of degradative enzymes (FAAH and MAGL) in males pre-exposed to CP. Moreover, the

NAPE/FAAH ratio was reduced, whereas the DAGL/MAGL ratio increased in CP-exposed males, indicating a reduction in AEA levels and an increase in 2-AG levels. Notably, these changes were reversed by morphine, suggesting an interaction between the endocannabinoid and opioid systems, as previously reported (Spanagel, 2020). In relation to these results, compensatory changes in the receptors might be expected. Along these lines, our data on cannabinoid receptors showed an interesting interaction between CP and morphine, with a reduction of the expression of CB1R in VH-animals and an increase in male CP-animals, whereas the opposite pattern was found for CB2R in females.

Limitations of the study

Some limitations need to be addressed in this study. First, the use of anesthesia has been shown to reduce brain neural activity, brain metabolism, and cerebral blood flow (Sicard et al., 2003). In our case, FDG uptake was carried out in unanesthetized animals, thus ruling out anesthesia effects on brain metabolism. Moreover, images were acquired under inhaled anesthesia, which hardly affects neurological activity (Shimoji et al., 2004). Second, the low number of subjects in the study, mainly due to surgical difficulties, may limit statistical analyses of the data and reduce the impact of the final results. However, we were able to observe statistically significant results when comparing the different groups of treatment. Finally, the estrous cycle was not controlled in female rats, which might have an impact on brain metabolism and ECS system. In human neuroimaging studies, the ovarian cycle in women is usually not controlled. Moreover, clinical trials controlling for the ovarian cycle in neuroimaging studies are extremely unusual, and the results are not consistent enough to support a large impact on FDG metabolism (Rettberg et al., 2014). Furthermore, although the estrus cycle has been reported to influence the ECS, other studies including ovariectomized (OVX) rats have shown differences in CB1R expression in the amygdala compared to normally cycling/non-OVX females, while the opposite pattern was found in the hippocampus and hypothalamus (Kim et al., 2022). In any case, an equal representation of all cycle phases would have been desirable to reduce the impact of female hormones in the ECS.

In conclusion, there are three main findings in this study. First, increased morphine self-administration was found in females but not males. Second, the metabolic pattern induced by morphine in females was exacerbated when CP was present, mainly at the end of morphine self-administration but also to a lesser extent during withdrawal, suggesting that CP may cause long-lasting metabolic changes in females. Third, pre-exposure to CP during periadolescence modulated the reward and endocannabinoid systems, inducing long-lasting metabolic changes after a 14-week morphine withdrawal. This was more notable in females than in males, which could explain sex differences in vulnerability to the use of drugs of

abuse such as opiates. Thus, pre-exposure to CP during adolescence contributes to the modulation of the reward and endocannabinoid systems, inducing long-lasting metabolic changes that remain at least 14 weeks after morphine withdrawal, mainly in females, which could explain sex differences in vulnerability to drugs of abuse such as opiates.

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FIGURES LEGEND

Figure 1. Design of the study and MSA study. A) Timeline of the experimental workflow including CP/VH pretreatment and saline/Morphine self-administration protocol. **B)** Mean number of morphine/saline injections during MSA protocol. Each column represents Mean ± SEM of 5-10 animals of the two- and three-way ANOVA results [**p<0.01 vs Saline group; $$$p<0.01 vs control group (VH-Saline); ###p<0.001 vs females].

Figure 2. ROI analysis and biochemical determinations A) Graphs indicate differences between Sex, CP and Morphine after acquisition and six and 14 weeks of withdrawal periods. Values are represented as Mean ± SEM of 7-9 animals per group. Two- and three-way ANOVA followed by LSD post-hoc test results [*p<0.05, **p<0.01 vs Saline group; $p<0.05 vs control group (VH-Saline); #p<0.05, ##p<0.01, ###p<0.001 vs females]. Graphs represent the **B.1)** concentration of CB1 and CB2 cannabinoid receptors in both Nucleus Accumbens (NAcc) and Striatum (St); **B.2)** expression of synthesis (NAPE and DAGL) and degradation (FAAH and MAGL) endocannabinoid enzymes and; **B.3)** indexes of NAPE/FAAH and DAGL/MAGL as indirect measures of Anandamide and 2-arachidonoylglycerol. Results are represented as Mean ± SEM of 7-10 animals per group. Two- and three-way ANOVA followed by LSD post-hoc test or Kruskall-Wallis followed by Dunn's post-hoc test [*p<0.05, **p<0.01 vs Saline group; $p<0.05, $$p<0.01, $$$p<0.001 vs control group (VH-Saline); #p<0.05, ##p<0.01, vs females].

Figure 3. Brain metabolic changes measured via PET at adulthood in females. SPM results on T-maps overlaid on a T2-MR template showing brain metabolic changes due to CP-pre-exposure in periadolescence (first row, CP), morphine self-administration in adulthood (middle row, M) and morphine self-administration in animals pre-exposure to CP during periadolescence (third row, CP + M), after Acquisition, and six and 14 weeks of morphine withdrawal. The color bars represent the T-values corresponding to higher (warm) and lower (cold) gray matter volume. Voxel-based p<0.05, k=100 voxels. Side: Right (R) and Left (L). Abb.: T: student t; K: cluster size; CP: CP-55,940; M: morphine; AA: amygdala; BS: brainstem; Cb: cerebellum; Col: colliculus; Ect: ectorhinal cortex; Ent: entorhinal cortex; Hb: habenula; Hipp: hippocampus; IC: insular cortex; Lc: Locus Coeruleus; PAG: periaqueductal gray; Pir: piriform cortex; RSA: retrosplenial cortex; Spt: septum; SSC: somatosensory cortex; S: subiculum; Th: thalamus; VTA: ventral tegmental area.

Figure 4. Brain metabolic changes measured via PET at adulthood in males. SPM results on T-maps overlaid on a T2-MR template showing brain metabolic changes due to CP-pre-exposure in periadolescence (first row, CP), morphine self-administration in adulthood (middle row, M) and morphine self-administration in adult animals pre-exposure to CP during periadolescence (third row, CP + M), after Acquisition, and six and 14 weeks of morphine withdrawal. The color bars represent the T-values corresponding to higher (warm) and lower (cold) gray matter volume. Voxel-based p<0.05 k=100 voxels. Side: Right (R) and Left (L). Abb.: T: student t; K: cluster size; CP: CP-55,940; M: morphine; AA: amygdala; BS: brainstem; CPu: caudate-putamen; Cb: cerebellum; ICol: inferior colliculus; Lc: Locus Coeruleus; MC: motor cortex; Pir: piriform cortex; SSC: somatosensory cortex; S: subiculum; VC: visual cortex.

TABLES LEGEND

Table 1. **Group differences in MSA, ROIs and biochemical determinations.** Table represents three-way ANOVA results followed by LSD post-hoc test or Kruskall-Wallis test followed by Dunn's post-hoc test [*p<0.05, **p<0.01, ***p<0.001]. Abb.: CB: cannabinoid receptor; CP: CP-55,940; DAGL: diacylglycerol lipase; FAAH: fatty acid amidohydrolase; KW: Kruskall Wallis; MAGL: monoacylglycerol lipase; M: morphine; NAcc: nucleus accumbens; NAPE: N-acyl phosphatidylethanolamine-specific phospholipase D; St: striatum.

Table 2. Sex differences in MSA, ROIs and biochemical determinations. Table represents two-way ANOVA results followed by LSD post-hoc test or Kruskall-Wallis test followed by Dunn's post-hoc test [*p<0.05, **p<0.01, ***p<0.001]. Abb.: CB: cannabinoid receptor; CP: CP-55,940; DAGL: diacylglycerol lipase; FAAH: fatty acid amidohydrolase; KW: Kruskall Wallis; MAGL: monoacylglycerol lipase; M: morphine; NAcc: nucleus accumbens; NAPE: N-acyl phosphatidylethanolamine-specific phospholipase D; St: striatum.

Table 3. Brain metabolic changes measured via PET at adulthood in females. Table shows the main clusters in each group after Acquisition, six and 14 weeks of morphine withdrawal. Abb.: T: student t; K: cluster size; unc: uncorrected; FDR: false discovery rate; CP: CP-55,940; M: morphine; AA: amygdala; BS: brainstem; Cb: cerebellum; Col: colliculus; Ect: ectorhinal cortex; Ent: entorhinal cortex; Hb: habenula; Hipp: hippocampus; IC: insular cortex; Lc: Locus Coeruleus; PAG: periaqueductal gray; Pir: piriform cortex; RSA: retrosplenial cortex; Spt: septum; SSC: somatosensory cortex; S: subiculum; Th: thalamus; VTA: ventral tegmental area.

Table 4. Brain metabolic changes measured via PET at adulthood in males. Table shows the main clusters in each group after Acquisition, six and 14 weeks of morphine withdrawal. Abb.: T: student t; K: cluster size; unc: uncorrected; FDR: false discovery rate; CP: CP-55,940; M: morphine; AA: amygdala; BS: brainstem; CPu: caudate-putamen; Cb: cerebellum; ICol: inferior colliculus; Lc: Locus Coeruleus; MC: motor cortex; Pir: piriform cortex; SSC: somatosensory cortex; S: subiculum; VC: visual cortex.

Supplementary Table 1. Estimated size effect of group differences in MSA, ROIs and biochemical determinations. Table shows partial Eta square (η2) of the three-way ANOVA comparisons. Values between 0.06 and 0.14 are considered as 'medium size effect' and are underlined. Values higher than 0.14 are considered 'high size effect' and are double underlined.

Supplementary Table 2. Estimated size effect in MSA, ROIs and biochemical determinations for each sex separately. Table shows partial Eta square (η2) of the two-way ANOVA comparisons for each sex separately. Values between 0.06 and 0.14 are considered as 'medium size effect' and are underlined. Values higher than 0.14 are considered 'high size effect' and are double underlined.