Early-life stress exacerbates the effects of WIN55,212-2 and modulates the cannabinoid receptor type 1 expression

Ana Martín-Sánchez¹, Alba García-Baos², Adriana Castro-Zavala², Laia Alegre-Zurano², Olga Valverde^{2*}

1. Neurobiology of Behaviour Research Group (GReNeC-NeuroBio), Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona, Spain; Neuroscience Research Program, IMIM-Hospital del Mar Research Institute, Barcelona, Spain.

2. Neurobiology of Behaviour Research Group (GReNeC-NeuroBio), Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona, Spain.

*Correspondence should be addressed to:

Olga Valverde

Department of Experimental and Health Sciences (DCEXS)

Universitat Pompeu Fabra

Carrer Doctor Aiguader 88, Barcelona 08003 Spain

Tel: +34 93 316 08 67

E-mail: olga.valverde@upf.edu

HIGHLIGHTS

- Maternal separation modifies emotional and spatial memories.
- Maternal separation promotes a delay in tolerance to cannabinoids effects.
- MSEW increases Purkinje's cells and CB₁-expressing neurons.
- MSEW increases Iba1-positive cells in BLA and cerebellum.
- CB₁ receptors are reduced in BLA and CA1 after WIN55,212-2 withdrawal in MSEW mice

ABSTRACT

Early-life stress induces an abnormal brain development and increases the risk of psychiatric diseases, including depression, anxiety and substance use disorders. We have developed a reliable model for maternal neglect, named maternal separation with early weaning (MSEW) in CD1 mice. In the present study, we evaluated the long-term effects on anxiety-like behaviours, nociception as well as the Iba1-positive microglial cells in this model in comparison to standard nest (SN) mice. Moreover, we investigated whether MSEW alters the cannabinoid agonist WIN55,212-2 effects regarding reward, spatial and emotional memories, tolerance to different cannabinoid responses, and physical dependence. Adult male offspring of MSEW group showed impaired responses on spatial

and emotional memories after a repeated WIN55,212-2 treatment. These behavioural impairments were associated with an increase in basolateral amygdala and hippocampal CB₁-expressing fibres and higher number of CB₁-containing cells in cerebellum. Additionally, MSEW promotes a higher number of Iba1-positive microglial cells in basolateral amygdala and cerebellum. As for the cannabinoid-induced effects, rearing conditions did not influence the rewarding effects of WIN55,212-2 in the conditioned place preference paradigm. However, MSEW mice showed a delay in the development of tolerance to the cannabinoid effects. Moreover, CB₁-positive fibres were reduced in limbic areas in MSEW mice after cannabinoid withdrawal precipitated with the CB₁ antagonist SR141617A. These findings support that early-life stress promotes behavioural and molecular changes in the sensitivity to cannabinoids, which are mediated by alterations in CB₁ signalling in limbic areas and it induces an increased Iba1-microglial marker which could interfere in emotional memories formation.

Keywords: Maternal separation, WIN55,212-2, cannabinoid receptor type 1, physical dependence, memory, Iba1.

ABBREVIATIONS

BLA	Basolateral amygdala
CA1	Cornu Ammonis layer 1
CB ₁	Cannabinoid receptor type 1
CPP	Conditioning place preference
CUD	Cannabis use disorder
EPM	Elevated plus maze
gl	Glomerular layer
Iba1	Ionized calcium-binding adapter molecule 1
ml	Molecular layer
MP	Maximum possible effect
MSEW	Maternal separated with early weaning
PC	Purkinje's cell
PBS	Phosphate saline solution
PD	Postpartum day
PostC	Post-conditioning
PreC	Pre-conditioning
SN	Standard nest
TBS	Tris buffered saline
Δ ⁹ -THC	Δ ⁹ -tetrahydrocannabinol
WIN55,212-2	<i>R</i> -(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone mesylate

1. INTRODUCTION

Parental care, particularly maternal behaviour, has a deep impact on the normal neurological development of the offspring, influencing some phenotypical features of newborns (Mehta et al., 2009; Pan et al., 2014; Rutter et al., 2012). In fact, some studies in humans revealed that disruption of mother-infant interactions during the early postnatal period, such as maternal neglect, affects in a negative way the individuals later in life (Rutter et al., 2012). Among others, child maltreatment is linked to a number of outcomes, including alterations in the neuroendocrine regulation in response to stress (Li et al., 2015; Meaney, 2001), memory disabilities and aggression (Demeusy et al., 2018), and it becomes a risk factor for the later onset of psychiatric disorders, such as depression, anxiety and substance use disorders (Danese, 2019; Kim et al., 2019; Moran et al., 2004; Norman et al., 2012).

By far, cannabis is the most widely used illicit drug worldwide (The United Nations publications, 2019). Although it is known that childhood abuse is an important predictor of substance use disorder (Fergusson et al., 2008a), few studies have examined the underpinning mechanisms that link early life adversities and cannabis use disorders (CUD). Fergusson et al. (2008b) observed that childhood sexual and physical abuse correlate with cannabis use during adolescence, which could initiate the use of other drugs, such as alcohol. Moreover, Abajobir et al., (2017) found that not only physical but also emotional abuse and neglect, among other early-life adversities, intimately predict cannabis abuse and dependence later in adulthood.

The endocannabinoid system mainly consists of the two canonical cannabinoid receptors type 1 and 2 (CB₁ and CB₂, respectively), their endogenous ligands (N-arachidonylethanolamine and 2-arachidonoylglycerol), and the enzymes responsible for their synthesis and degradation. Due to its widespread expression in the central nervous system, the endocannabinoid system participates in many physiological functions, such as homeostasis, learning, memory and stress (Lutz et al., 2015; Marsicano et al., 2002). Moreover, a substantial body of findings strongly suggests that the endocannabinoid system also contributes to modulate the synaptic transmission of the mesocorticolimbic dopaminergic system and the abuse of different drugs, such as cannabinoids (Maldonado et al., 2006; Morena and Campolongo, 2014). Despite these advances, there is no well-established animal model for cannabinoids consumption. Although the operant self-

administration paradigm is a very useful tool to assess reinforcement effect induced by different drugs of abuse in animal models (Halladay et al., 2020; Hay et al., 2018; Luján et al., 2018; Towers et al., 2019), the effects of cannabinoids is not easily replicated using this paradigm. Early studies have shown that drug-naïve and cocaine-experienced squirrel monkeys self-administered Δ^9 -tetrahydrocannabinol (Δ^9 -THC), the psychoactive component of cannabis (Justinova et al., 2003; Tanda et al., 2000). In rodents, some studies have achieved the self-administration of cannabinoids, using intravenous Δ^9 -THC (Spencer et al., 2018), vaporized Δ^9 -THC (Freels et al., 2020), or intravenous *R*-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate (WIN55,212-2), a synthetic cannabinoid receptor agonist (Kirschmann et al., 2017; Mendizábal et al., 2006). The low reliability of the cannabinoids self-administration could be due to the low rewarding effects of Δ^9 -THC observed in the conditioning place preference paradigm (Kubilius et al., 2018). Indeed, there are contradictory results, because i.p. Δ^9 -THC administration could promote either conditioning place preference (CPP) or even aversion in a dose-dependent manner (Kubilius et al., 2018; Manwell et al., 2014; Murray and Bevins, 2010). By contrast, more consistent results have been reported using WIN55,212-2, since it induced CPP (Castañé et al., 2004; Manzanedo et al., 2010) probably due to its higher affinity for CB₁ and CB₂ receptors than Δ^9 -THC (Thomas et al., 1998). Nonetheless, one of the most reproducible and objective paradigms to assess the cannabinoid effects are models that reveal tolerance and physical dependence symptoms (Castañé et al., 2004; Valverde et al., 2000). In this context, up to now there are no studies shedding light on the influence of the early-life stress in consumption, rewarding effects and physical dependence to cannabinoids in animal models.

We have previously established a mice model of maternal separation with early weaning (MSEW) (Castro-Zavala et al., 2020; Gracia-Rubio et al., 2016; Portero-Tresserra et al., 2018). This model somehow replicates the pathophysiology of patients that suffer early-neglect or emotional abandonment during childhood, and its effects on both emotional and substance use disorders. The MSEW mice show anxiety-like and despair-like behaviours, as well as anhedonia-like state and deficits in emotional memory that persist until the adulthood (Gracia-Rubio et al., 2016). Studies in maternal deprivation rodent models have demonstrated that early-life stress has a deep impact in different types of learning, such as olfactory memory (Czarnabay et al., 2019) and social

fear-related memories (Zoicas and Neumann, 2016), both stimuli converging within the associative nuclei of the basolateral amygdaloid complex (BLA) (Pardo-Bellver et al., 2012). Our model of maternal separation reflects the increase of markers associated to neuroinflammation and alterations in the tryptophan-kynurenine pathways (Gracia-Rubio et al., 2016) related to an abnormal serotonin availability in the brain, which is crucial for the monoaminergic transmission (Clark et al., 2009). Additionally, MSEW mice show an increased consumption of cocaine (Castro-Zavala et al., 2020) and alcohol (Portero-Tresserra et al., 2018) during adulthood, as also occurs in humans with an early-life adverse experiences (Teicher et al., 2016). Indeed, MSEW mice show an imbalance in the glutamatergic, serotonergic and endocannabinoid systems signalling (Castro-Zavala et al., 2020; Gracia-Rubio et al., 2016; Portero-Tresserra et al., 2018), which interact with the mesocorticolimbic dopaminergic system (Tan et al., 2014) and can influence the drug-seeking behaviour.

Changes in the endocannabinoid system in response to stress induced by early-life adversity, especially CB₁ receptor expression, have been previously documented in animal models. A 24h-single episode of maternal deprivation on PD9 promotes brain region-dependent changes in the endocannabinoid system during adolescence (Marco et al., 2015) in Wistar rats. Whereas a decrease in the hippocampal formation expression of CB₁ was observed in male rats on PD13 in a sex-dependent manner (Suárez et al., 2009), changes in diacylglycerol lipase (biosynthesis of 2-arachidonoylglycerol) and monoacylglycerol lipase (degradation) enzymes were observed in this maternal deprivation model (Suárez et al., 2010). However, Zamberletti et al., (2012) did not find differences in CB₁ density and functionality in both female and male adult rats after maternal deprivation. Recently, Papilloud et al., (2018) observed that peripubertal stress induced by fear induction, increases the CB₁ gene expression in nucleus accumbens shell. Other studies found alterations in the CB₁ receptor expression in limbic brain regions in maternally separated animals (Alteba et al., 2016; Dow-Edwards et al., 2016; Hill et al., 2019; Romano-López et al., 2012). Thus, the CB₁ receptor expression seems to be modulated by early stress and could be a crucial factor for a differential vulnerability to cannabinoids during adulthood.

To that end, the present study aims to assess how MSEW may modulate cannabinoids effects, neuroinflammation process and cannabinoid physical dependence after a repeated treatment. We focused on whether there are differences in the emotional

and spatial memories between the MSEW group and the SN control group after a WIN55,212-2 repeated treatment, and if it could produce changes in CB₁ expression in hippocampus, BLA and cerebellum. Then, we assessed whether MSEW might alter WIN55,212-2-induced reward, tolerance to different behavioural effects, and withdrawal syndrome precipitated by CB₁ antagonist SR141716A in comparison to Standard Nest (SN) control group. Finally, we studied the anatomical consequences of physical withdrawal syndrome precipitation in MSEW mice after SR141617 administration in the CB₁ receptor expression.

2. MATERIAL AND METHODS

2.1. Animals and rearing conditions

CD1 male and female mice purchased from Charles River (France) were used as breeders. On arrival, breeding pairs were formed and housed in Plexiglas cages (369 x 156 x 132 mm). After ten days, male mice were removed, and pregnant females were housed individually. Mice were randomly assigned to one of the two different experimental groups, SN and MSEW animals. Maternal separation protocol has been previously conducted in our laboratory (Castro-Zavala et al., 2020; Gracia-Rubio et al., 2016; Portero-Tresserra et al., 2018), adapted from George et al., (2010).

Briefly, pregnant females were daily observed at 9 and 17h for parturition. For each litter, the date of delivery was assigned as a postpartum day (PD) 0. In the MSEW group, offspring were separated from their mothers for 4 h per day on PD2-5 (09:30–13:30h) and 8 h per day on PD6-16 (09:30–17:30 h). We neither culled the pups nor adjusted the number of female and male mice, because MSEW protocol does not affect body weight, mortality or morbidity (George et al., 2010; Gracia-Rubio et al., 2016). For separation, mothers were moved to another cage, while the offspring were maintained in their home cages with a heating blanket (32–34 °C) for thermoregulation. Pups from MSEW group were early weaned at PD17, and to facilitate their access to food and avoid a possible dehydration, wet regular chow and hydrogel (Bio-Services, Uden, The Netherlands) were provided in their home cages until PD21. In the SN group, offspring remained undisturbed with their mothers for 21 days, when they were weaned (PD21). After weaning, offspring were housed in groups of 4 to 5 animals of the same sex. In accordance with previous results in our laboratory (Gracia-Rubio et al., 2016), we observed a similar average number of pups delivered by a CD1 dam (n=12; 52% male) among groups and

each litter was randomly assigned to MSEW or SN group before the parturition. Moreover, we randomly distributed the pups of each litter between vehicle and WIN55,212-2 treatment in order to avoid a litter size effect. We assigned a maximum of 3 male pups per litter to vehicle or WIN55,212-2 treatment in all the experiments. Female littermates were dismissed for the experiment and were used for other experiments.

All animals were housed in a room maintained at 21 ± 1 °C, humidity $55 \pm 10\%$ and a 12:12h light:dark cycle, with lights on at 08:00h. Water and food were available ad libitum. Cages were cleaned weekly except during postpartum days, when dams were left undisturbed until PD10. All procedures were conducted in accordance with national (BOE-2013-1337) and EU (Directive 2010-63EU) guidelines regulating animal research and were approved by the local ethics committee (CEEA-PRBB).

2.2. *Drugs*

All drugs were administered by i.p. route. For conditioned place preference (CPP) procedure the dose of non-selective cannabinoid receptor agonist R(+)-WIN55,212-2 mesylate salt (Sigma Chemical Co., Madrid Spain) was administered at 0.5 mg/kg (Manzanedo et al., 2010), whereas for physical dependence induction it was administered at 1mg/kg in a volume of 0.1ml/10g of body weight, following Hutcheson et al., (1998). The selective CB₁ cannabinoid receptor antagonist SR141716A (*Rimonabant*; Sigma-Aldrich, Darmstadt, Germany) was administered at 10mg/kg in a volume of 0.2ml/10g of body weight, as previously described (Hutcheson et al., 1998; Valverde et al., 2000). Both drugs were respectively dissolved in the vehicle solution consisting of ethanol/cremophor EL (*Kelliphor*; Sigma-Aldrich, Darmstadt, Germany)/distilled water (1:1:18).

2.3. *Behavioural procedures*

2.3.1. *Elevated plus maze*

Elevated plus maze (EPM) was performed using a black maze elevated 300 mm above the ground (Gracia-Rubio et al., 2016; Portero-Tresserra et al., 2018) in PD 56 SN (n=14) and MSEW (n=16) mice. Each mouse was placed in the centre of the maze, and was allowed to freely explore for 5 min. The software SMART 2.5 (Panlab s.l.u., Barcelona, Spain) automatically reported the time spent in the open arms, entries in open arms and the total entries.

2.3.2. *Tail-immersion test*

The nociceptive response was evaluated in two different sets of animals at both SN and MSEW groups at PD57 and 84 (MSEW at PD57 n=20, MSEW at PD84 n=7, SN at PD57 n=16 and SN at PD84 n=6), using the tail immersion test (Valverde et al., 2000). The water used for immersing the tail was maintained at a constant temperature of $50 \pm 0.5^{\circ}\text{C}$ in a warm bath (Clifton, Nickel-Electro LTD, England, UK). The time (in seconds) to withdraw the tail from the bath was evaluated with a cut-off latency of 15s to prevent tissue damage.

2.3.3. *Hot-plate test*

The test was based on that described by Simonin et al., (1998) and it was conducted four hours later the tail-immersion test. We evaluated this test at PD57 and PD84 in two different sets of mice (MSEW at PD57 n=20, MSEW at PD84 n=7, SN at PD57 n=16 and SN at PD84 n=6), in two different sets of mice. A glass cube was used to keep the mice on the heated surface of the plate (254 x 254 x 19 mm, Columbus Instruments, Ohio USA), which was heated up to $50 \pm 0.5^{\circ}\text{C}$ of temperature. Two thermal nociceptive responses were evaluated: latency to paw licking and latency to jump. The time (s) was evaluated with a cut-off latency of 240s to prevent tissue damage, as previously reported (Alegre-Zurano et al., 2020; Simonin et al., 1998) .

2.3.4. *Conditioning place preference (CPP)*

For place conditioning, we employed four identical Plexiglas boxes with two equally sized compartments (307 mm length x 315 mm width x 345 mm height) separated by a grey central area (138 mm, length x 315 mm, width x 345 mm height). The compartments have different colour walls (black vs white) and distinct floor textures (smooth black compartment vs rough white compartment). Four infrared light beams in each compartment of the box and six in the central area allowed the recording of the position of the animal and its crossings from one compartment to the other. The equipment was controlled by one computer using MONPRE 2Z software (CIBERTEC, SA, Spain).

Place conditioning protocol was adapted from Manzanedo et al., (2010). This procedure consisted of three phases and took place during the light cycle following an

unbiased procedure in terms of initial spontaneous preference. For this experiment we used MSEW and SN mice vehicle- (MSEW-Vehicle n=14 and SN-Vehicle n=14) and WIN55, 212-2 (MSEW- WIN55, 212-2 n=13 and SN- WIN55, 212-2 n=11) treated animals on PD 70 at the beginning of the experiment (Figure 2A). At the beginning of the experiment, all the animals were weighted to calculate the volume of the drug/vehicle to inject.

In brief, during pre-conditioning (PreC, day 1) mice were allowed access to both compartments of the apparatus for 900 s recording the time spent by the animal in each compartment. None of the mice showed a strong unconditioned aversion (less than 33% of the session time) or preference (more than 67%) for one of the compartments. In the second phase (conditioning), animals were conditioned with saline or WIN55, 212-2 through four pairings with the respective compartment (one pairing each day). Animals received an injection of the corresponding drug immediately prior to confinement in the drug-paired compartment for 1800s on days 2, 4, 6 and 8, and received vehicle injection before being confined to the vehicle-paired compartment for 1800s on days 3, 5, 7 and 9. Control animals received a vehicle injection during all days of conditioning phase. During the third phase, or post-conditioning (PostC), which took place on day 10, the guillotine doors separating the two compartments were removed and the time spent by the untreated mice in each compartment during an observation period of 900 s was recorded. CPP score was calculated as the difference between the time spent in the vehicle- or WIN55, 212-2-paired compartment during the PostC session and the time spent in the same compartment during the PreC session.

2.3.5. Left-right discrimination test

In a different cohort of animals, we administered a repeated treatment with WIN 55, 212-2 (1mg/kg, i.p; twice daily) and they were divided in two different sets: i) in the first set of animals, we evaluated the memory impairments due to the WIN 55, 212-2 administration; ii) in the second set of animals, we administered WIN 55, 212-2 or vehicle to SN and MSEW animals for the anatomical study (n=5/group) and they were sacrificed 24h after the last injection.

Animals were injected with WIN55,212-2 or its vehicle twice daily from PD70-80 (SN-Vehicle, MSEW-Vehicle, SN-WIN and MSEW-WIN, n=10/group; Figure 3A).

The left-right discrimination test was carried out on PD75 and PD77, using a T-maze apparatus, previously documented (Cantacorps et al., 2019; Filali et al., 2009). Briefly, this T-maze was filled with water (25 ± 1 °C) at a height of 120 mm. The animals were first assessed for side preferences. To do so, during the first two trials, two identical platforms ($110 \times 110 \times 110$ mm) were submerged on the end of both arms. Then, only the least chosen arm was selected to place the platform. Mice were placed in the stem of the T-maze and swam either to the left or the right until finding the submerged platform up to a maximum of 1 min. If the animals did not find the platform within this 1 min, it was gently guided to it, and this trial was counted as an error. After reaching the platform, the mice remained on it for 20 s and then placed back in the maze for up to a maximum of 20 trials, except for a 10-min resting period after each 10-trial block. A mouse was considered to have achieved criterion after 5 consecutive trials without errors. The reversal learning phase was then conducted 48h later, applying the same protocol except that the mice were trained to reach the escape platform on the opposite arm. Escape latencies and number of trials to reach the criterion were manually recorded.

2.3.6. *Passive avoidance test*

After the reversal learning test, animals underwent the passive avoidance test on PD 79-80, following Saavedra et al., (2013). The experiment was conducted in an apparatus divided into weakly and brightly lit chambers (2–5 and 160 lx respectively; dimensions, 190x190x270mm) (Panlab s.l.u., Barcelona, Spain). The dark compartment had a stainless-steel grid floor for shock delivery. On the acquisition day, each mouse was placed into the bright compartment. A sliding door between the compartments was opened after 30s, and the latency to enter into the dark compartment was recorded for up to 90s. When the animal entered into the dark compartment, the door closed and the mouse received a foot shock (0.5mA, 3s), and it was immediately removed from the apparatus. 24h later, mice were placed into the brightly lit compartment, and the procedure was repeated without the foot shock (retention trial). The latency to enter the dark compartment was evaluated with a cut-off of 300 s.

2.3.7. *Tolerance to WIN55, 212-2 effects*

In a different set of animals, we administered a repeated treatment with WIN 55, 212-2 (1mg/kg, i.p; twice daily; Figure 4A), and we evaluated the tolerance to different

behavioural responses induced by the cannabinoid (locomotor activity, rectal temperature and antinociceptive responses), the subsequent physical dependence precipitation (CB₁ antagonist SR141716, 10mg/kg i.p; n=6/group) and CB₁ receptor expression. For the tolerance experiment, we evaluated body weight changes, rectal temperature changes and nociception responses during cannabinoid treatment, comparing with the vehicle-treated group.

Body weight was recorded for each animal, using an electronic balance (Emerald JE120, Ohaus, with a sensitivity of 0.01 g), twice per day before every morning and evening injections.

Rectal temperature was measured in each individual using a thermocouple flexible rectal probe (Panlab s.l.u., Barcelona, Spain). The probe was placed 30mm into the rectum of the mice for 30s before the temperature was recorded. On days 1 and 2, measures were taken immediately prior to, and 20min after, each injection. On days 3, 4, 5 and 6 rectal temperature was recorded before and 20min after the morning injection only.

Nociceptive responses were evaluated using the tail immersion test, with a cut-off of 15s. This evaluation was performed just after rectal temperature measurements before (only on day 1 considered as pre-treatment latency value) and after 20min after injection, during mornings (day 1 to 6) and evenings (day 1 and 2), as post-treatment latency values. For animals that did not respond within the cut-off time of 15s, the value of the cut-off was considered as latency period for the animal. Results were expressed as % maximum possible effect (MPE) following Jacob et al., (2013): % MPE = [(Post-injection latency - Pre-treatment latency) x 100/ (Pre-treatment latency)].

After tail immersion test, animals were placed in locomotor activity boxes for 20min, as previously described (Esteve-Arenys et al., 2017). Animals were placed in plastic rectangular area (240 mm × 240 mm × 240 mm; LE8811 IR, Panlab s.l.u., Barcelona, Spain) with two crossed photocells in a low luminosity room.

2.3.8. Physical dependence induced by WIN55,212-2 and evaluation of withdrawal syndrome after systemic administration of SR141716A

The evaluation of withdrawal syndrome in the same set of animals that tolerance experiment (n=6/group) was conducted as previously reported (Castañé et al., (2004). On day six after receiving the WIN55,212-2 treatment (1mg/kg, i.p) for five days, mice received the first injection of WIN55,212-2 and 2h later mice were placed in a circular plastic cylinder for 15min of habituation. Then, SR141716A was injected (10mg/kg i.p) and the abstinence signs were evaluated during 45min: wet dog shakes, ptosis, body tremor, ataxia, front paw tremors, piloerection, hunched-back posture, mastication, penile licking, abdominal twist and sniffing. The number of wet dog shakes, front paw tremors and sniffing behaviour were counted. Ptosis, body tremor, ataxia, piloerection, hunched-back posture, mastication and genital licks were scored 1 for appearance and 0 for non-appearance within 5 min-period. A quantitative value was calculated in each animal for different checked signs by adding the scores obtained in each 5 min-period. A global score was calculated for each animal giving to each sign a relative weight (Valverde et al., 2000).

2.4.Perfusion, fixation and sectioning

One day after the evaluation of withdrawal syndrome or the last WIN55-212 injection (1mg/kg i.p.), animals were deeply anaesthetized using a Dolethal overdose (i.p. injection of 120 mg/kg of body weight of the pentobarbital-based solution). Then, animals were killed by transcardiac perfusion of phosphate saline solution 0.1M (PBS, pH 7.4) using a peristaltic pump (5.5 ml/min for 2 minutes) followed by 4% paraformaldehyde in 0.1M phosphate buffer pH 7.4 (same flux for 5 minutes). Brains were carefully removed from the skull and immediately post-fixed in the same fixative solution overnight at 4°C. Then, the brains were placed into 30% sucrose solution (in 0.1M PBS, pH 7.6, 4°C) until they sank. Finally, the brains were frozen, and we obtained 40-µm-thick coronal sections with a cryostat (Leica CM3050 S, Wetzlar, Germany). Free-floating sections were collected in five parallel sets.

2.5.Immunohistochemistry

Two out of five parallel series obtained from SN and MSEW groups (Vehicle- and WIN-treated animals) were employed for immunofluorescence for i) simultaneous of CB₁ receptor and nuclear immunostaining, ii) and for ionized calcium-binding adapter molecule 1 (Iba1), separately. In brief, sections were: (i) pre-incubated in 4% normal goat

serum in 0.05M TRIS buffered saline pH 7.6 (TBS) with 0.2% Triton X-100, at RT for 1 hour, for blocking unspecific labelling; (ii) incubated in primary antibodies, mouse anti-CB₁ IgG (1:500; Synaptic systems, Cat.#258011) or rabbit anti-Iba1 (1:1000; Wako Pure Chemicals, Oaza Ogohara, Japan, #AB_839504) diluted in TBS with 0.2% Triton X-100 with 4% normal goat serum (48h or 24h respectively, at 4°C); (iii) incubated with fluorescent-labelled secondary antibodies (90 min at RT) diluted in TBS with 0.2% Triton X-100, Alexa Fluor 555-conjugated Goat anti-mouse IgG (1:250; Thermofisher Cat. No. A21422) or Alexa Fluor 555-conjugated Goat anti-rabbit IgG (1:250; Abcam ab150078). After each step, sections were washed in TBS (except between steps i and ii). To reveal the cytoarchitecture, sections were counterstained prior to mounting by bathing them for 5min in Hoechst (a nuclear staining, 1:10000; Invitrogen, Cat.#33258) at room temperature. Sections were finally rinsed thoroughly in TB and mounted onto gelatinized slides and cover-slipped with fluorescence mounting medium (FluorSave Reagent, Merck Milipore, Cat#345789, Darmstadt, Germany). CB₁ immunofluorescence was analysed with a confocal microscope LEICA TCS SP5 upright (Wetzlar, Germany). Double scans were made to identify Alexa Fluor 555 (red, CB₁ or Iba1) and Hoechst (blue, nuclear) only for counterstaining.

2.6. Immunofluorescence analyses

CB₁ immunofluorescence was analysed with a Leica TCS SP5 upright confocal microscope system. Double scans were made to identify Hoechst and Alexa Fluor 555. Excitation wavelengths were 361 nm for Hoechst and 555 nm Alexa 546. Emission wavelengths were 497 and 565, respectively. Sections with 4µm Z distance of separation were taken from the regions of interest. To minimize the channel spillover, the images were sequentially acquired and saved as LIFF files. The stacks obtained were further processed with Fiji-ImageJ software. Photographs were taken in both hemispheres. Then, the third and fifth images of each stack were selected to be analysed. From the RGB colour images, they were binarized, using the same thresholding rule for all the animals: pixels whose grey level was lower than 75% of the mode of the histogram were selected as belonging to immunolabeled CB₁ puncta. The area fraction (%) that these pixels represent in all the frames of a given structure (both cerebral hemispheres) was registered for each mouse following Otero-Garcia et al., (2014), whereas CB₁-immunoreactive

cells located in cerebellum were manually counted. using ImageJ software (#SCR_003070, NIH, Bethesda, MD, USA) by an observer who was blind to treatment.

Photographs of Iba1-immunoreactive microglial cells were taken in both hemispheres using a microscope Leica DM6000B (Leica AG, Germany) with a digital camera (Leica DFC300 FX). To analyse the number of microglial cells using Iba1 immunostaining, cells were quantified as the mean number of labelled body cells in each hemisphere per brain slice. For each mouse, cells were manually counted as previously described.

No manipulations of individual image elements were performed.

3. STATISTICAL ANALYSIS

Data were analysed using the software IBM SPSS Statistics 23.0. We first checked the data for normality (Kolmogorov-Smirnov's test) and homoscedasticity (Levene's test). Then, we evaluated the differences between groups using t-Student's test, ANOVA or Chi-square analysis when appropriate. We evaluated the EPM results using an unpaired *t*-Student's test. For the analysis of the antinociceptive responses, assessed in tail immersion and hot-plate test (with *age* and *rearing* as variables), CPP score, somatic signs in withdrawal syndrome and the immunohistochemical results (with *rearing* and *treatment* as variables in all cases) two-way ANOVAs were calculated. We performed a paired *t*-Student's test to evaluate the differences between the time spent in the conditioned compartment during post-conditioning and pre-conditioning sessions. Chi-square analysis was used to evaluate differences in the percentage of animals that enter in the dark compartment in the passive avoidance test. To evaluate possible statistical differences in the tolerance to the effects induced by WIN55,212-2 (with *rearing*, *treatment* and *sessions* as variables), reversal learning and latency to scape in passive avoidance test (with *rearing*, *treatment* and *trials* as variables), we performed an ANOVA with repeated measurements. When applicable, pairwise comparisons were analysed with Bonferroni's correction. Statistical differences were found when $p < 0.05$.

4. RESULTS

A summary of the statistical analysis of the experiments is available in Supplementary Material, showing all significant and non-significant effects and comparisons.

4.1. MSEW promotes anxiety-related behaviour

We wanted to evaluate whether there were differences in anxiety-related behaviour between MSEW and SN animals at the age of PD56. To do so, we measured three different variables: percentage of time in open arms, percentage of entries in open arms and total number of entries. The *t*-Student's test revealed that MSEW animals spent lower percentage of time in open arms than SN group (unpaired *t* test $t_{28}=2.064$; $p < 0.05$, Figure 1B). However, no differences were found regarding the percentage of entries in open arms and the total number of entries (unpaired *t* test $t_{28}=1.132$ and $t_{28}=1.454$ respectively; $p > 0.05$, Figure 1C and D), indicating an absence of basal locomotor impairments. Thus, MSEW animals showed anxiety-related behaviours without basal locomotor deficits.

4.2. Influence of MSEW on the thermal nociceptive threshold

We conducted both tail immersion and hot-plate tests to study whether MSEW and SN mice differs in the pain threshold at two different ages, PD57 and PD84. The two-way ANOVA to evaluate the tail withdrawal showed neither *rearing* nor *age* effects ($p > 0.05$, Figure 1E). Regarding hot-plate test, we performed a two-way ANOVA for the latency to lick the paw, using *rearing* and *age* as variables, the analysis revealed that there is only a main effect of *age* ($F_{1,45}= 5.196$; $p < 0.05$, Figure 1F), without interaction between variables. The analysis revealed that animals from PD57 showed longer latencies in licking the paw than PD84 mice, independently the rearing. Additionally, when we analysed the latency to jump, two-way ANOVA revealed an interaction between *age* $\times$ *rearing* ($F_{1,45}= 4.292$; $p < 0.05$, Figure 1G), but no main effects of each variable. The *post-hoc* analysis revealed that male SN mice at PD57 showed longer latencies to jump than SN at PD84 ($p < 0.05$) and MSEW animals from the same age PD57 ($p < 0.01$). Altogether, the results indicate that younger animals, specially MSEW animals on PD57, show a decrease in the nociceptive threshold.

Figure 1 Anxiety-like behaviours and basal nociceptive responses in MSEW and SN mice. (A) Experimental schedule. Blue (SN) and yellow (MSEW) bars represent (B) the percentage of time spent in open arms, (C) the percentage of the entries in open arms and (D) the total number of entries in closed and open arms. SN (n=15) MSEW (n=16) (*t*-Student test: **p* < 0.05). Nociceptive responses evaluated in SN and MSEW at two different ages (PD 57 and 84): (E) the tail withdrawal in tail-immersion test, (F) latency to lick a paw and (G) latency to jump in hot-plate test (SN PD57, n=16; PD84 n=6; MSEW PD57 n=20; PD84 n=7) (two-way ANOVA, **p* < 0.05, ***p* < 0.01). All data are represented as the mean ± SEM.

4.3. WIN55-212 induces CPP in both MSEW and SN males

When comparing time spent in the compartment associated with WIN55,212-2 in pre-conditioning versus post-conditioning sessions, the *t*-Student's test shows that WIN55,212-2 (0.5mg/kg) induced CPP in both SN- ($t_{11}=3.761$, $p < 0.01$; Figure 2B) and MSEW mice ($t_{13}=3.022$, $p < 0.05$). In fact, the analysis of the scores using a two-way ANOVA revealed a *treatment effect* ($F_{1,48} = 8.992$, $p < 0.01$) indicating that the WIN55,212-2 (0.5mg/kg) treatment increased the time spent in the drug-paired compartment during the post-conditioning session in comparison with the pre-conditioning session in both MSEW and SN animals (Figure 2C). Thus, WIN55,212-2 induced conditioning place preference in both SN and MSEW animals.

Figure 2. Rewarding properties of WIN 55, 212-2 (0.5mg/kg) in the conditioning place preference. (A) Experimental schedule. (B) Time spent in pre-conditioning (PreC) vs postconditioning (PostC) in all four experimental groups. (*t*-Student's test, $*p < 0.05$, $**p < 0.001$). (C) Vertical axis represents the place preference score calculated as the time spent in the conditioned compartment on the post-conditioning day minus the time in the same compartment on the pre-conditioning session. (Two-way ANOVA, $**p < 0.01$). Data are expressed as mean $\pm$ SEM ($n=11-14$). Maternal separation (MS), with early weaning (MSEW), postpartum day (PD), standard nest (SN).

4.4. WIN55,212- treatment impairs acquisition learning in left-right discrimination test

We conducted the reversal learning test to assay differences in spatial memory due to maternal separation and the cannabinoid treatment. We performed a three-way ANOVA (*treatment*, *rearing* and *trials* factors) for the acquisition trials that showed an

effect of *trials* ($F_{4,33}=6.17, p < 0.001$) and *treatment* $\times$ *trials* ($F_{4,33}=3.91, p < 0.01$; Figure 3B), without *rearing* or *treatment* effect. The *trials* effect revealed that all animals shortened the latencies to reach the platform throughout in comparison with the first trial ($p < 0.05$, all cases). Bonferroni's post-hoc analysis for *treatment* $\times$ *trials* interaction indicated that WIN55,212-2 treated animals exhibited longer latency to reach the escape platform than vehicle animals in the second trial ($p < 0.01$). By contrast, when we analysed the reversal trials, we did not find statistical differences due to either *rearing* or *treatment* conditions. Hence, WIN212,2 impaired acquisition phase in the left-right discrimination test regardless of the rearing.

4.5. WIN55,212-2 treatment impairs emotional memory in MSEW mice

We carried out a passive avoidance test to evaluate differences in emotional learning due to maternal separation and the cannabinoid treatment. The ANOVA with repeated measures indicated statistical differences due to *trials* ($F_{1,33}=44.08, p < 0.001$) and *rearing* main effects ($F_{1,33}=5.30, p < 0.05$), *treatment* $\times$ *trials* ($F_{1,33}=4.40, p < 0.05$), and *rearing* $\times$ *trials* ($F_{1,33}=5.52, p < 0.05$) interactions (Figure 3C). Additionally, the analysis revealed a trend in *treatment* ($F_{1,33}=4.40, p=0.057$), indicating that WIN55,212-2-treated animals tended to display shorter latencies to cross to dark compartment than Vehicle control animals.

The *post-hoc* analysis for *rearing* $\times$ *trials* indicated that SN animals remain more time in the lighter compartment than MSEW animals during retention trial before crossing to dark compartment ($p < 0.05$). The analysis for *treatment* $\times$ *trials* suggested that Vehicle-treated animals display longer latencies than WIN55,212-2-treated animals only in the retention trial ($p < 0.05$). Finally, the Chi-square test evidenced that there were differences in the percentage of animals that entered in the dark compartment during retention trial ($\chi^2=67.24, df=3; p < 0.001$), showing that all MSEW-WIN55,212-2 treated animals entered the dark compartment, whereas the rest of the groups showed that near the 50% of animals did not (Figure 3D, coloured bar). Therefore, WIN55,212-2 has a deep impact on emotional learning in MSEW animals.

Figure 3. WIN55,212-2 treatment in reversal learning and passive avoidance. (A) Experimental schedule. Effects of the cannabinoid (1mg/kg) or vehicle treatment received twice daily (B) in both phases (acquisition and reversal; $n=10/\text{group}$; ANOVA for repeated measurements, $**p < 0.01$ WIN55,212-2- vs vehicle-treated animals comparison) of left-right discrimination test and (C) both phases (acquisition and retention trials; $n=8-10/\text{group}$; two-way ANOVA, $*p < 0.05$, Vehicle vs WIN55,212-2 treatment comparison) of the passive avoidance test. (D) Percentage of animals that enter (coloured) / not enter (not coloured) in the black compartment during the retention trial of the passive avoidance test (Chi-square test, $***p < 0.001$). Maternal separation (MS) with early weaning (MSEW), postpartum day (PD), standard nest (SN).

4.6. WIN 55, 212-2 treatment increases the density of CB₁ expression in CA1 of hippocampus and BLA and MSEW increases the density of CB₁-containing cells in the cerebellum

The two-way ANOVA showed a *treatment* effect, indicating that the cannabinoid repeated treatment increased the CB₁ receptor expression in the CA1 hippocampal layer and in BLA ($F_{1,15}=5.05$; $p < 0.05$; $F_{1,15}= 5.25$; $p < 0.05$, respectively Figure 4A and B) in comparison with vehicle-treated animals. Moreover, the two-way ANOVA revealed a *rearing* main effect ($F_{1,15}=9.22$; $p < 0.01$) in the number of CB₁-positive Purkinje cells, suggesting that MSEW animals expressed higher number of CB₁-positive cells in the cerebellum than SN animals.

Figure 4. WIN55,212-2 (1mg/kg) increases the expression of CB₁ in the CA1 hippocampal layer and BLA after a sub-chronic treatment.

Bar histogram showing the mean $\pm$ SEM the percentage of the area occupied by CB₁ in (A) CA1 of hippocampus and (B) basolateral amygdala (BLA) (n=4-5/group, 4 images per mouse). (C) Bar histogram showing the mean $\pm$ SEM the number of CB₁-containing cells in the cerebellar cortex. Statistical analysis (two-way ANOVA) applied independently to each area reveal significant differences due to *treatment* condition (*p < 0.05) in BLA and CA1 and a higher number of CB₁-positive cells in the cerebellum of MSEW animals. Basolateral amygdala (BLA), glomerular layer (gl), molecular layer (ml), Purkinje's cells (PC). Scale bar 50µm.

4.7.MSEW increases the number of Iba1-positive cells in BLA and cerebellum

To investigate the long-term impact of MSEW and WIN55,212-2 (1mg/kg, twice daily) administration on Iba1 microglial marker, we evaluated the Iba1-immunoreactive cells in the CA1 hippocampal layer, in BLA and cerebellum. Iba1 is a macrophages/microglial-specific calcium-binding protein involved in membrane ruffling and phagocytosis in activated microglia (Ohsawa et al., 2000). The two-way ANOVA showed a *rearing* effect, indicating that the MSEW directly increases the expression of Iba1 microglial marker in BLA and cerebellum ($F_{1,18}=7.408$; $p < 0.05$; $F_{1,18}=6.85$; $p < 0.05$, respectively Figure 5B and C) in comparison with SN animals. Thus, MSEW triggers Iba1-immunoreactivity in both BLA and cerebellum, without any effect of WIN55,212-2 treatment. When we evaluated the expression of Iba1-immunostained cells in the CA1 hippocampal layer, the two-way ANOVA revealed *rearing* $\times$ *treatment* interaction ($F_{1,18}=4.616$; $p < 0.05$). The *post-hoc* analysis indicated that MSEW-Vehicle

group showed higher Iba1-immunostained cells than SN-Vehicle group ($p < 0.05$; Figure 5A).

Figure 5. MSEW increases Iba1 immunolabelling in BLA and Cerebellum independently of the sub-chronic WIN55,212-2 (1mg/kg) treatment.

Bar histogram showing the mean $\pm$ SEM number of Iba1-immunoreactive (Iba1-ir) cells in (A) CA1 of hippocampus, (B) basolateral amygdala (BLA) and (C) cerebellum ($n=5-6$ /group) ($*p < 0.05$). Scale bar 200 μ m.

4.8. Tolerance to WIN 55, 212-2 effects

Locomotion: Three-way ANOVA shows a main effect of sessions ($F_{7,14} = 23.46$; $p < 0.001$), treatment ($F_{1,20} = 30.37$; $p < 0.001$), rearing ($F_{1,20} = 5.65$; $p < 0.05$) but also for session $\times$ treatment ($F_{7,14} = 7.87$; $p < 0.001$; Figure 6B) and session $\times$ rearing ($F_{7,14} = 4.95$; $p < 0.01$). As expected, WIN55,212-2-treated animals showed lower horizontal movements than vehicle-treated animals. Additionally, these data evidenced that MSEW displayed a reduced locomotion in comparison with SN mice. The *post-hoc* analysis revealed that SN animals showed an increased locomotion compared to MSEW animals on days 5 ($p < 0.01$) and 6 ($p < 0.05$). The Bonferroni's analysis suggested that WIN55,212-2-treated animals showed lower levels of locomotion on days 1am ($p < 0.001$), 2 am ($p < 0.001$), on day 4 ($p < 0.05$), 5 ($p < 0.001$) and 6 ($p < 0.01$) in comparison with vehicle-treated animals.

Figure 6. Effects of a repeated WIN55,212-2 (1mg/kg) treatment during tolerance schedule for different effects. (A) Experimental procedure schedule. Effects of the cannabinoid or vehicle treatment received twice daily on (B) locomotion, (C) percentage of maximum possible effect (MPE, %) in tail-immersion test and (D) variation in rectal temperature (value obtained before – value obtained 20 min after the WIN 55, 212-2 injection). Measurements were performed during the 6 days of treatment. am =measurement performed at 8.30h; pm= measurement performed at 17h. (E) Variation in rectal temperature after the first injection. Values are expressed as mean $\pm$ SEM. (three-way ANOVA for repeated measurements, $*p < 0.05$, $**p < 0.001$ $***p < 0.001$ for comparisons between WIN 55, 212-2-treated animals vs vehicle-treated animals; n=6/group). Maternal separation (MS), postpartum day (PD), standard nest (SN), perfusion (Perf), withdrawal syndrome (With S).

Tail withdrawal: The ANOVA for repeated measurements showed a main effect of treatment ($F_{1,20}=29.26$; $p < 0.001$), session ($F_{7,14}=10.83$; $p < 0.001$) and session $\times$ treatment ($F_{7,14}=7.05$; $p < 0.001$; Figure 6C). In this sense, the WIN55, 212-2 treatment increased the latency to withdraw the tail from the warm water, regardless of the rearing conditions. The Bonferroni's analysis showed that WIN55,212-2-treated animals differed

from vehicle-injected mice during all sessions ($p < 0.05$ in all cases) except on day 1pm, where we observed a trend towards significance ($p = 0.052$).

Rectal temperature: The ANOVA for repeated measurements to analyse the changes in body temperature revealed a main effect of *sessions* ($F_{7,14} = 33.06$; $p < 0.001$; Figure 6D), *treatment* ($F_{1,20} = 192.62$; $p < 0.001$), *rearing* ($F_{1,20} = 15.58$, $p < 0.001$) but also for *session* $\times$ *treatment* ($F_{7,14} = 22.7$; $p < 0.001$; Figure 6D), *session* $\times$ *rearing* ($F_{7,14} = 3.75$; $p < 0.05$) and *rearing* $\times$ *treatment* ($F_{7,14} = 7.71$; $p < 0.05$). The *rearing* effect suggested that MSEW animals decreased more the body temperature than SN animals. Additionally, the *treatment* effect indicated that WIN 55, 212-2-treated animals decreased the body temperature with higher amplitude than control mice. The *post-hoc* with Bonferroni's correction for *session* $\times$ *treatment* showed that WIN 55, 212-2 treated mice differed from control mice on session 1 am ($p < 0.001$), 1 pm ($p < 0.001$), 2 am ($p < 0.01$), 2 pm ($p < 0.001$), 3 am ($p < 0.001$) and 6 am ($p < 0.05$). The *post-hoc* analysis for *rearing* $\times$ *treatment* revealed that both MSEW- ($p < 0.001$) and SN-WIN55, 212-2-treated animals ($p < 0.001$) showed a decrease of body temperature in comparison with vehicle-animals. Finally, the Bonferroni's test indicated that MSEW mice treated with WIN55,212-2 were more affected than SN WIN55,212-2-treated mice ($p < 0.001$).

When we analysed the acute effect of WIN55,212-2, the two-way ANOVA yielded a significant effect of *treatment* ($F_{1,20} = 15.22$, $p < 0.001$), *rearing* ($F_{1,20} = 156.3$; $p < 0.001$) and *rearing* $\times$ *treatment* interaction ($F_{1,20} = 22.09$; $p < 0.001$; Figure 5E). The *rearing* effect indicates that MSEW animals showed higher rectal temperature differences than SN mice. The *post-hoc* for *session* $\times$ *treatment* indicated that SN- and MSEW-WIN55, 212-2-treated mice decreased their temperature more than their respective control group ($p < 0.001$, in both cases). Moreover, the rectal temperature difference was more exacerbated in MSEW-WIN 55, 212-2 than SN-WIN55,212-2 treated animals ($p < 0.001$).

4.9. Withdrawal syndrome induced by a repeated WIN 55, 212-2 treatment

Somatic signs: We found a *rearing* effect in front paw tremor ($F_{1,19} = 4.79$, $p < 0.05$, Figure 7I) and a trend toward significance in ataxia ($F_{1,19} = 3.35$, $p = 0.083$; Figure 7A). The results revealed that MSEW animals showed higher front paw tremor behaviour than SN after the withdrawal precipitation. The two-way ANOVA revealed a main *treatment* effect in ataxia ($F_{1,19} = 34.48$, $p < 0.001$; Figure 7A), piloerection ($F_{1,19} = 47.07$,

$p < 0.001$; Figure 7B), hunched-back posture ($F_{1,19}=175.75$, $p < 0.001$; Figure 7C),
 mastication ($F_{1,19}=30.86$, $p < 0.001$; Figure 7D), tremor ($F_{1,19}=22.17$, $p < 0.001$; Figure
 7E), basal activity ($F_{1,19}= 32.75$, $p < 0.001$; Figure 7F), ptosis ($F_{1,19}=32.94$, $p < 0.001$;
 Figure 7G), wet dog shakes ($F_{1,19}=15.74$, $p < 0.001$; Figure 7J), abdominal twist ($F_{1,19}=$
 12.29 , $p < 0.001$; Figure 7H), front paw tremor ($F_{1,19}=17.46$, $p < 0.001$; Figure 7I) and
 scratch ($F_{1,19}= 13.95$, $p < 0.001$; data not shown). In this sense, whilst WIN55,212-2
 promotes the expression of the major of these signs in comparison with vehicle-
 administered animals, the basal activity is deteriorated significantly by cannabinoid
 repeated treatment following SR141716A precipitation. Moreover, we observed an
 interaction between *treatment* $\times$ *rearing* for hunched-back posture ($F_{1,19}=6.25$, $p < 0.05$;
 Figure 7A) and front paw tremor ($F_{1,19}=4.43$, $p < 0.05$; Figure 7I). The *post-hoc* analysis
 for hunched-back posture behaviour revealed that both MSEW-WIN55,212-2 ($p < 0.001$)
 and SN-WIN55,212-2 ($p < 0.001$) mice showed higher score in hunched-back posture
 than MSEW Vehicle- and SN Vehicle-treated animals, respectively. The *post-hoc*
 analysis for front paw tremor behaviour suggested that WIN55,212-2 only precipitated
 front paw tremor in SN animals, showing differences with SN-Vehicle treated animals (p
 < 0.001).

Finally, we found a *treatment* effect in the global score ($F_{1,19}= 96.30$, $p < 0.001$;
 Figure 7E), indicating that the expression of most somatic signs was due to the
 WIN55,212-2 treatment.

649 *Figure 7. Effects of SR141716A (10mg/kg) after sub-chronic WIN55,212-2 or vehicle*
650 *administration. Data are expressed as mean $\pm$ SEM (n=6/group) of counted and checked physical*
651 *withdrawal signs during 45 min immediately after SR141716A injection in all groups of animals:*
652 *(A)Ataxia, (B) Piloerection, (C) Hunched-back posture; (D) Mastication, (E) Tremor (F) Activity*
653 *(G)Ptosis, ; (H)Twist = abdominal twist; (I) Pw tremor = front paw tremor; (J) Wet dog shakes*
654 *and (K) Global score (two-way ANOVA; *** p < 0.001 for comparison between WIN 55, 212-2-*
655 *treated animals vs vehicle-treated animals; a*** p < 0.001, for the comparison between SN-*
656 *WIN55,212-2 vs SN-Vehicle; b*** p < 0.001, for the comparison between MSEW WIN 55,212-*
657 *2-treated animals vs MSEW-Vehicle mice; # p < 0.05, for the comparisons between MSEW vs SN*
658 *animals)*

659 *4.10. MSEW reduces the density of CB₁ in CA1 of hippocampus and in BLA after*
660 *a single SR141617 administration*

661 We evaluated the density of CB₁ after a single SR141617 administration in all
662 four groups. The two-way ANOVA showed a *rearing* effect ($F_{1,15} = 5.96$; $p < 0.001$) and
663 *rearing* $\times$ *treatment* interaction ($F_{1,15} = 4.62$; $p < 0.05$; Figure 8A) in CA1 of hippocampus.
664 The *rearing* effect suggested a substantial CB₁ reduction in the CA1 of hippocampus of
665 MSEW compared to SN mice after SR141716A injection. By contrast, the *post-hoc*
666 analysis revealed that the percentage of area occupied by CB₁ is dramatically reduced in
667 MSEW treated with the cannabinoid in comparison with SN ($p < 0.01$), after antagonist
668 injection. When we analysed the expression of CB₁ in BLA, we observed a *rearing* effect

($F_{1,15} = 9.42$; $p < 0.001$), suggesting that the percentage of area occupied by CB₁ is higher in SN than MSEW group (Figure 8B). By contrast, the CB₁ positive cells in cerebellum were not statistically different independently of the rearing conditions (Figure 8C).

Figure 8. MSEW reduces the expression of the CB₁ in the CA1 of hippocampus and basolateral amygdala in MSEW animals after SR141617A injection.

Bar histogram showing the mean $\pm$ SEM the percentage of the area occupied by CB₁ in (A) CA1 of hippocampus and (B) basolateral amygdala (BLA) ($n=4-5$ /group, 4 images per mouse). (C) Bar histogram showing the mean $\pm$ SEM the number of CB₁-positive cells in the cerebellar cortex. Statistical analysis (two-way ANOVA) applied independently to each area reveal significant differences due to *rearing* condition ($p < 0.05$) in BLA and CA1, and a decreasing of the CB₁ expression in the MSEW-WIN in comparison with SN-WIN animals ($**p < 0.01$) in CA1 of hippocampus. Basolateral amygdala (BLA), glomerular layer (gl), molecular layer (ml), Purkinje's cells (PC). Scale bar 50 μ m.

5. DISCUSSION

MSEW could be a vulnerability factor for the later use of drugs of abuse, including cannabinoids. In this study, we evaluated the long-term effects of maternal neglect during early-life on the effects of the synthetic cannabinoid agonist WIN55,212-2, the development of tolerance to different responses, the physical withdrawal syndrome after SR141716A administration and the impact on emotional memory after a sub-chronic cannabinoid treatment. We have also examined changes of CB₁ expression possibly involving complementary mechanisms such as long-term microglial alterations as we

shown differences in Iba1-positive cells in different brain areas. In fact, this is the first study that proposes that the early-life adversity could alter emotional memory, in addition to the development of tolerance and physical dependence after a cannabinoid repeated treatment.

Our results demonstrated that WIN55,212-2 (0.5mg/kg) produced rewarding effects in the CPP in both SN and MSEW groups. To our knowledge, this is the first study that evaluates the influence of maternal separation on the rewarding effects of cannabinoids. Most studies have failed to report cannabinoids rewarding responses in a great variety of paradigms including CPP (Cheer et al., 2000; Kubilius et al., 2018; Pandolfo et al., 2009) and intracranial self-stimulation (Vlachou et al., 2005), probably due to the dose-dependent biphasic effect of some cannabinoids (Spiller et al., 2019). This biphasic effect could explain the mild enhancement of rewarding effects at low doses but no effects or even aversive effects at higher doses of some cannabinoids (Spiller et al., 2019). Moreover, there are contradictory results observed when evaluating the reinforcing effects of cannabinoids using the intravenous self-administration paradigm. Although some authors have achieved cannabinoid intravenous self-administration, (Justinova et al., 2013; Mendizábal et al., 2006; Spencer et al., 2018), they observed lower rates of nose-pokes in comparison to alcohol-vehicle solution (Spencer et al., 2018) or to other drugs of abuse (Halladay et al., 2020; Luján et al., 2018). By contrast, other authors recently demonstrated that inhaled Δ^9 -THC potentiates reinforcing properties, improving the rates of cannabinoid self-administration or intracranial self-stimulation in ecological models (Freels et al., 2020; Ravula et al., 2019). However, none of these previous studies were performed in models of early-stressed animals.

Our data strongly contrast with previous results obtained in our laboratory using alcohol and cocaine. MSEW mice displayed a reduction of alcohol-induced CPP but increasing alcohol consumption in a free-access paradigm (Portero-Tresserra et al., 2018). MSEW males also consumed more cocaine during adulthood in a self-administration procedure, suggesting a higher vulnerability to develop drug seeking-behaviour due to a decreased drug sensitivity in MSEW male mice (Castro-Zavala et al., 2020). Changes in drug-seeking behaviour were also documented in other maternally deprived rodent models, using MDMA (Llorente-Berzal et al., 2013) or cocaine (O'Connor et al., 2015),

probably due to the differences in the reinforcing strength of the drugs used in these studies.

We evaluated the implications of the cannabinoid repeated treatment (WIN55,212-2 1mg/kg i.p., twice daily) in learning process, because it is known that CB₁ activation mediates cannabinoid induced memory deficits (Wise et al., 2009). In these experiments, we observed that WIN55,212-2 impaired spatial and emotional learning, respectively. In the left-right discrimination experiment we detected minor alterations in spatial memory due to the fact that WIN55,212-2 treatment induced longer latencies to reach the escape platform only in the second trial, whereas the rest of trials vehicle and WIN55,212-2 groups behaved in a similar way. Some authors found that spatial (radial water maze test) and non-associative learning (novel-object recognition) were impaired through CB₁ activation by WIN55,212-2 (0.3mg/kg) (Galanopoulos et al., 2014) and Δ^9 -THC (10mg/kg) treatment, probably by acting into GABAergic hippocampal interneurons through glutamatergic signalling (Puighermanal et al., 2009). The possible mild memory impairment that we observed could be mediated by CB₁ increased expression in hippocampal CA1. In fact, the implication of memory mediated by CB₁ signalling is clear since it was demonstrated that AM251 CB₁ antagonist (0.5mg/kg, 3 weeks) counteracts memory deficits observed in a phencyclidine animal model of schizophrenia (Guidali et al., 2011), and also restores the levels of anandamide and 2-arachidonoylglycerol in prefrontal regions.

Our anatomical data in CA1 hippocampal layer strongly contrast with previous studies in maternally deprived animals, chronically treated during adolescence with the cannabinoid agonist CP 55,940 (0.4mg/kg) (López-Gallardo et al., 2012). The authors found that CB₁ receptor density in CA1 of hippocampus was higher in maternal separated male rats than control group (López-Gallardo et al., 2012). These differences with our study could be due to age, cannabinoid agonist doses as well as the time-point of experimental conditions of the study. By contrast, in agreement with our anatomical results, Bortolato et al., (2010) found an enhanced expression of CB₁ receptor in limbic areas such as prefrontal cortex, BLA, CA1 hippocampal region and perirhinal cortex, in response to a neurotoxic dose of methamphetamine. In line with this, CB₁ receptor activation mediates the kainic acid excitotoxicity and the anandamide levels increase after the kainic acid injection, which have a neuroprotective role against neurotoxicity

(Marsicano et al., 2003). Thus, we cannot discard that the treatment used in the present study (WIN55,212-2 1mg/kg i.p.; twice daily, for 5 days) could display a neurotoxic effect, altering the CB₁ receptor density in CA1 and the endocannabinoid system, and impairing the left-right discrimination. However, the higher expression of Iba1 microglial marker in MSEW-Vehicle animals did not result in greater spatial memory impairments, at least under our experimental conditions.

Despite the discrepancies regarding the implication of WIN55-212,2 in the spatial and working memories, the role of cannabinoids in emotional learning seems to be clear. WIN55,212-2 disrupted the emotional reactivity in both SN and MSEW animals, as previously reported (Burstein et al., 2018; Hasanein and Teimuri Far, 2015; Shiri et al., 2017). In this sense, WIN55,212-2-treated groups displayed shorter latencies during retention trial (Figure 3C), which could be related with an increase of CB₁ receptor density in the BLA (Figure 4B), as also occurs in CA1 hippocampal region. It is known that this type of fear memories are processed by amygdaloid circuits (Aincy et al., 2018; Choi et al., 2010), since the hyperexcitability of BLA principal neurons is associated to emotional disorders in both rodents and humans (Prager et al., 2016; Senn et al., 2014), which could be mediated by CB₁ receptor on inhibitory GABAergic cells (Llorente-Berzal et al., 2015). Thus, abnormal CB₁ signalling induced by WIN55,212-2 associated with a greater Iba1-positive cell in the BLA could explain the emotional memory impairments observed in both SN-WIN55,212,2 and MSEW-WIN55-212,2 animals.

Additionally, we found that MSEW animals showed shorter latencies to enter the dark compartment in the passive avoidance test, indicating retardation in learning process. Indeed, data revealed that memory deficit is more severe in MSEW-WIN55,212 treated animals since 100% of the animals of this group crossed to dark compartment of passive-avoidance box in comparison with the rest of the experimental groups (50% animals; Figure 3D). Some authors found that the mitogen-activated protein kinases / extracellular signal-regulated kinase (MAPK/Erk) phosphorylation, which is directly involved in the anxiety and learning (Novaes et al., 2017), is increased in the amygdala of maternally separated rodents (Nishinaka et al., 2016; Park et al., 2014). In fact, both depressed-like rats (Eriksson et al., 2012) and depressed patients (Clark et al., 2009) show a memory decline. Our laboratory has previously demonstrated that MSEW is a reliable model that reproduces some aspects of psychiatric disorders due to early-life adversities, since these

animals show depressive-like behaviour, anxiety-like behaviour (Figure 1B) (Gracia-Rubio et al., 2016) and deficits in social interaction (Portero-Tresserra et al., 2018). The sustained stress during MSEW results in a higher expression of Iba1 that could contribute to depressive- and anxiety-related behaviours, as well as impairments in emotional fear memory. Moreover, amygdala-dependent memory disturbances were previously related with higher expression of neuroinflammation markers, such as Iba1 (Spencer et al., 2017). Our results support that higher Iba1-positive cells present in the BLA of MSEW mice could be involved in emotional memory deficits observed in passive avoidance test. Thus, the present study shows that MSEW males suffered from a decline in emotional memory. Previous findings in our laboratory demonstrated that MSEW animals showed alterations in serotonergic (Gracia-Rubio et al., 2016) and glutamatergic neurotransmission (Castro-Zavala et al., 2020), neuroinflammatory markers (Gracia-Rubio et al., 2016) and downregulation of endocannabinoid system (Portero-Tresserra et al., 2018).

Interestingly, 24 h after the last cannabinoid injection of the repeated WIN55,212-2 treatment, we observed anatomical changes in the density of CB₁ receptor in hippocampus, BLA and cerebellum between SN and MSEW animals. We selected these areas because a previous study demonstrated that microinjections of the SR141716A CB₁ antagonist directly into these areas, after a repeated cannabinoid treatment, were able to promote a physical withdrawal syndrome (Castañé et al., 2004). After the repeated cannabinoid administration, both SN and MSEW WIN55,212-2-treated animals showed an increase of CB₁ receptor expression in CA1 hippocampal layer and in BLA in comparison with vehicle control groups (Figure 4A and B). An early study suggested that chronic administration of other cannabinoids, such as Δ^9 -THC or anandamide (3mg/kg of body weight of rats, using a single i.p. injection during 5 days) increased the density of cannabinoid receptors in both hippocampus and cerebellum (Romero et al., 1995), increasing also their maximum binding capacity (B_{max}). Moreover, a recent study found similar anatomical results, showing a CB₁ protein level reduction in CA1 but an increase in BLA after 10 days of WIN55,212-2 treatment (1.2mg/kg of body weight of rats, one daily injection) (Sbarski and Akirav, 2018).

Furthermore, the number of Purkinje CB₁-positive cells expressed in the cerebellum was higher in MSEW mice than SN ones, regardless of the treatment administered (Figure 4C). We found that early-life stress promoted an increase in

Purkinje CB₁-containing cells and an induction of higher levels of Iba1-immunoreactive cells in MSEW animals (Figure 5C). In fact, the CB₁ regulation seems to be necessary for the control of cerebellar neuroinflammation, since global lack of CB₁ receptors in knock-out mice induces the expression of some pro-inflammatory factors in this brain area, such as cyclooxygenase-2, tumour necrosis factor α (TNF α) and interleukin-10 (Cutando et al., 2013). Previously, other authors described increases of CB₁ mRNA and protein levels after stress exposure, indicating a main role of CB₁ in stress-induced excitotoxicity and neuroinflammation (Zoppi et al., 2011), which could be mediated by the phosphorylation of p38 mitogen-activated protein kinase cascade (Derkinderen et al., 2001). In agreement with our results, maternal separation could promote an enhancement of neuroinflammatory responses, increasing Iba1-positive cells, as well as cytokines (IL-1 β and TNF α) levels in the hippocampus and prefrontal cortex (Ferle et al., 2020), as our results seem to demonstrate in the present study and supported also by our previous studies (Gracia-Rubio et al., 2016).

Moreover, we investigated whether MSEW could trigger differences in the tolerance to different responses promoted by the agonist WIN55,212-2. In this way, we observed a reduction of the baseline thermal nociceptive response in basal conditions of the MSEW animals only in the latency to jump in the hot-plate test (Figure 1C), whereas no changes were observed in the tail-immersion or in the latency to licking paw in the hot-plate test, as other authors documented recently (Melchior et al., 2018). These observations are supported by the lack of significant differences in the percentage of maximum possible response in SN- and MSEW-vehicle-treated animals during the evaluation of the tolerance to the antinociceptive effects of WIN55,212-2. In fact, the differences observed in maximum possible response were only revealed after the WIN55,212-2 (1mg/kg, i.p.) repeated treatment, independently of the rearing conditions.

The effects of MSEW in locomotion were present after the cannabinoid repeated treatment. MSEW animals displayed a reduction in locomotion in comparison with SN counterparts, probably due to that the MSEW-WIN55,212-2 group did not restore a complete basal locomotion as it happened with the vehicle-treated animals (Figure 4B). Indeed, differences in locomotor activity between MSEW-WIN55,212 and SN-WIN55,212 groups are in line with previous results by Macrì and Laviola, (2004) using WIN55,212 at higher dose (2mg/kg), whereas a low-dose of WIN55,212 (0.1mg/kg)

enhanced hyperlocomotion in non-stressed rats (Enayatfard et al., 2013). Otherwise, a murine model of chronic stress showed an increased locomotion that was only revealed under bright light conditions (Strekalova et al., 2005). We could not discard the possibility that the low illumination conditions of our experiment could be masking the MSEW-related effects on locomotion, acting as a possible confounding factor.

In addition, when we analysed the rectal temperature variation and we observed that MSEW WIN55,212-2-treated mice were more affected than SN-WIN55,212-2 group (Figure 6D and 6E). Indeed, the MSEW-WIN55,212-2 group showed decreasing temperatures throughout sessions, probably due to thermoregulatory compensation impairments yielded by early-life stress (Melo et al., 2018). Thus, these results indirectly support that repeated WIN55,212-2 treatment produces deeper effects on MSEW animals, probably due to their higher vulnerability to the effects of this cannabinoid. We hypothesized that MSEW animals could show impairments in the desensitization of CB₁ pathway, as also occurs in a model of S426A/S430A mutant mice with a delayed tolerance to hypothermia and tail-flick analgesia responses after Δ^9 -THC repeated treatment (Morgan et al., 2014; Nealon et al., 2019). Both mutations lead to a nonphosphorylatable CB₁ receptor, unable to be desensitized by the GRK/ β -arrestin pathway (Nealon et al., 2019). Nevertheless, these animals finally developed tolerance using an alternative signalling pathway, as also may be occurring in our MSEW WIN55,212-2-treated group. Thus, the MSEW-WIN55,212-2 mice may show abnormalities in the restoration of CB₁ receptor signalling.

When physical dependence was precipitated by a systemic SR141716A injection (10mg/kg, i.p.) in mice administered with a repeated WIN55,212-2 treatment, we observed that the CB₁ antagonist receptor was not able to promote somatic signs *per se*, neither in SN- nor in MSEW-vehicle group. By contrast, we observed a dramatic expression of physical withdrawal symptoms in cannabinoid-treated animals, as previously documented in mice and rhesus monkeys (Castañé et al., 2004; Stewart and McMahon, 2010; Wilkerson et al., 2019). Among the somatic signs observed, we found wet dog shakes, ataxia, hunched-back posture, piloerection, ataxia, lack of activity and front paw tremor (Figure 6). These results suggest that, although MSEW-WIN55,212-2 animals showed deeper effects than SN during tolerance, they express the somatic signs in a similar way during physical withdrawal syndrome. By contrast, animals chronically

treated with WIN55,212-2 and after withdrawal syndrome, reduced the expression of CB₁ in both CA1 of hippocampus and BLA as a consequence of early-life stress. We propose that MSEW animals show impairment in the CB₁ recruitment process, which causes an intense decrease of the receptor expression in BLA and CA1, after the SR141716 administration. Indeed, our laboratory has demonstrated that MSEW animals exhibit a downregulation of different endocannabinoid ligands in discrete brain areas (Portero-Tresserra et al., 2018) that could explain the abnormalities observed in CB₁ receptor restoration. Nevertheless, in the present study, the total number of CB₁ immunoreactive cells in granular layer of cerebellum remained unaltered. Although we did not find changes in the number of CB₁-positive Purkinje cells, we could not discard changes in the affinity of their binding sites (Romero et al., 1995). Contrasting with the results obtained before the antagonist injection, SR141716A injected animals show no differences in the number of CB₁-positive Purkinje cells, which could indicate a possible compensatory mechanism that increases the number of CB₁-positive cells in the cerebellum.

In summary, our results propose that although MSEW does not apparently alter the rewarding effects of cannabinoids, it could lead to a delay in the tolerance to some of the effects displayed by WIN55,212-2 by modulating the expression of CB₁ receptors after a long-term brain exposition to cannabinoids. Additionally, the cannabinoid WIN55,212-2 agonist treatment clearly affects more the amygdala-dependent memory in animals with early-life adversities, probably accompanied by an increase of MSEW-induced Iba1-positive cells in BLA. Likewise, the increased number of CB₁-positive Purkinje cells and the Iba1-immunoreactive microglia could be reflecting a neuroinflammatory process in MSEW animals. By contrast, when physical dependence is precipitated, CB₁-positive fibres decrease in the BLA and hippocampus of MSEW mice, probably due to an abnormal restoration of CB₁ receptor levels. In this sense, our study shed light on how CB₁ signalling is altered as a consequence of early-life adversities and how these alterations could promote differences in vulnerability to cannabinoid effects.

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CONTRIBUTORS

A.M.S. and O.V. were responsible for the study concept and design. A.M.S., A.G.B, A.C.Z. and L.A.Z. carried out the experimental studies. A.M.S. A.G.B. and O.V. participated in the interpretation of findings. A.M.S and O.V. drafted the manuscript. All authors critically reviewed the content and approved the final version for publication.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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