CB1 cannabinoid receptor-mediated aggressive behavior

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\textbf{Abstract}

This study examined the role of cannabinoid CB1 receptors (CB1r) in aggressive behavior. Social encounters took place in grouped and isolated mice lacking CB1r (CB1KO) and in wild-type (WT) littermates. Cognitive impulsivity was evaluated in the delayed reinforcement task (DRT). Gene expression analyses of monoamine oxidase A (MAO-A), catechol-o-methyltransferase (COMT), 5-hydroxytryptamine transporter (5-HTT) and 5-HT1B serotonergic receptor (5HT1Br) in the median and dorsal raphe nuclei (MnR and DR, respectively) and in the amygdala (AMY) were performed by real time-PCR. Double immunohistochemistry studies evaluated COMT and CB1r co-localization in the raphe nuclei and in the cortical (ACo), basomedian (BMA) and basolateral (BLA) amygdaloid nuclei. The behavioral effects of the CB1r agonist ACEA (1 and 2 mg/kg) on aggression were also evaluated in isolated OF1 mice. CB1KO mice housed in groups showed higher levels of offensive aggression. Isolation increased aggressive behavior only in WT. In grouped CB1KO mice COMT gene expression was significantly higher in the MnR and DR, while MAO-A gene expression was lower in the MnR. Gene expression of 5HT1Br, COMT and MAO-A was higher in the amygdala of CB1KO mice. The behavioral effects of the CB1 agonist ACEA (1 and 2 mg/kg) on aggression were also evaluated in isolated OF1 mice. CB1KO mice housed in groups showed higher levels of offensive aggression. Isolation increased aggressive behavior only in WT. In grouped CB1KO mice COMT gene expression was significantly higher in the MnR and DR, while MAO-A gene expression was lower in the MnR. Gene expression of 5HT1Br, COMT and MAO-A was higher in the amygdala of CB1KO mice. CB1r double-immunohistochemistry revealed cytoplasmic-labeled COMT-ir cells in the raphe nuclei and in the cortical (ACo), basomedian (BMA) and basolateral (BLA) amygdaloid nuclei. The behavioral effects of the CB1r agonist ACEA (1 and 2 mg/kg) significantly decreased the aggression levels of OF1 mice. These results suggest that CB1r plays an important role in social interaction and aggressive behavior.

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1. Introduction

Emotion is a highly complex behavior in response to various environmental stimuli. An appropriate emotional outcome requires fine-tuned neurotransmitter release processes and functional neuronal circuits. Therefore, maintaining a balanced signaling is highly important, especially in stressful situations. In recent years, the endocannabinoid system has been considered one of the endogenous control mechanisms of stress (Häring et al., 2012).

The CB1 receptor (CB1r), the most abundant cannabinoid receptor in the central nervous system, is expressed in the main brain areas, including the limbic system (hypothalamus, amygdala, habenula and cerebral cortex) regulating emotional and stress-related behavior (Herkenham et al., 1990; Katona et al., 2001; Marsicano et al., 2002; Mackie, 2005). CB1r are found primarily on presynaptic neurons and facilitate the inhibition of neurotransmitter release. Endocannabinoids also act as retrograde messengers in the central nervous system (Wilson and Nicoll, 2002; Mechoulam and Parker, 2013). Endocannabinoid retrograde control modulates the release of several neurotransmitters (monamines, cholecystokinin, glutamate and GABA) involved in emotional behavior (Melis et al., 2004; Jung et al., 2005; Mato et al., 2007; Aso et al., 2009).

Numerous experimental studies have shown that the endocannabinoid system is implicated in the control of emotional behavior by functionally altering CB1r (Valverde and Torrens, 2012). Moreover, experiments with CB1KO mice have revealed...
anxiogenic- and depressive-like phenotypes (Haller et al., 2002; Maccarrone et al., 2002; Urigüen et al., 2004). However, to our knowledge, only one study has evaluated the social and aggressive profile of CB1KO mice. The authors of this study reported that exposure to the resident-intruder procedure induced stronger aggressive responses in CB1KO mice than in wild type mice, although these differences were not observed in subsequent encounters (Martin et al., 2002).

The relation between cannabinoids and aggression was firstly studied in a series of studies in the 1970s, which highlighted how cannabis administration in stressful situations could cause or exacerbate aggression in rats, despite its effect being sedative as a rule. For example, aggressive behavior was elicited by rodents receiving chronic administration of cannabis sativa extract or THC after food, sleep or drug deprivation (Carlini and Gonzales, 1972; Carlini et al., 1976). Treatment of aggressive, electrically-shocked rats with cannabis increased the number of aggressive responses (Corder and Olson, 1972). Aggression was induced in group-housed rats by chronic administration of daily doses of THC, and even a single dose was found to produce an attack response in rats housed in isolation (Ueki et al., 1972). It has also been shown that the effects of the endocannabinoid anandamide depend on the dose employed; high doses reduce aggression in aggressive mice, while low doses increase aggressive responses in timid mice (Sulcova et al., 1998). A recent report revealed that intrauterine exposure to cannabis was associated with increased risk of aggressive behavior and attention problems as early as 18 months of age in girls, but not in boys (El Marroun et al., 2012), whereas the CB1r inverse agonist taranabant was found to produce irritability and anger/aggression when administered to obese and overweight patients (Proietto et al., 2010).

Aggressive signals, postures and acts are mechanisms by which animals obtain specific goals or defend themselves against threats or attacks (Miczek et al., 2002). These behaviors occur in individuals competing for food, water and other resources necessary for survival and reproduction (resident-intruder aggression), defending their territory or offspring (territorial and maternal aggression), or in response to frustration or fear (Miczek et al., 2001). On the other hand, aggression as a “trait” is the focus of genetic studies. While these aggressive traits are clearly polygenic, it is remarkable that several studies have found that an interaction between genotypes such as tryptophan hydroxylase 2 (TPH2), monoaminooxidase-A (MAO-A) and 5-hydroxytryptamine transporter (5-HTT) polymorphisms and environmental triggers such as social stress increases the likelihood of violent outbursts (Takahashi et al., 2012).

The purpose of the present study was to examine the role of CB1r in social and aggressive behavior. CB1KO mice were used to study the impulsivity trait in the delayed reinforcement task and social and aggressive behavior under group versus single housing conditions. Changes in the gene expression of 5-HTT, catechol-o-methyl-transferase (COMT), MAO-A and 5-HT1B serotonergic receptor (5HT1B) in specific brain nuclei were assessed using real time-PCR. CB1r and COMT co-localization in the median (MnR) and dorsal (DR) raphe nuclei and in the cortical (ACo), basomedial (BMA) and basolateral (BLA) amygdaloid nuclei was evaluated by confocal microscopy. The behavioral effects of a CB1r agonist (ACEA) on social activity and aggression were also evaluated.

2. Material and methods

2.1. Subjects

A total of 49 male CB1KO and 69 male WT mice, all 42 days of age, were used in the first experiment. All the mice were littermates (WT and CB1KO) to avoid the confusion factor of different nurturing conditions. Mice lacking the CB1 receptor were obtained as described previously (Ledent et al., 1999). Sixth generation heterozygote mice on a CD1 (Charles River, France) background were bred at our animal vivarium and homozygotes of the same generation were also employed in our experiments. For the second experiment, 82 OF1 male mice were acquired commercially from Charles River (Barcelona, Spain). The mice arrived at the laboratory at 42 days of age. All animals were housed under standard laboratory conditions: constant temperature (21 °C), a reversed light schedule (white lights off 0730–1930 h), and food and water available ad lib. except during behavioral testing. For the first experiment, half of the experimental animals were individually housed for 28 days in transparent plastic cages (24 × 13.5 × 13 cm). For the second experiment, 32 mice were housed in isolation under the same experimental conditions as in the first study. The remaining animals were housed in groups of four to be used as standard opponents or saline-treated grouped mice. OF1 mice were employed as they show aggressive behavior from the age of 6 weeks. This aggressiveness increases with age, isolation for 21 days has been shown to increase this behavior (Rodríguez-Arias et al., 1998, 1999).

All procedures involving the mice and their care complied with national, regional and local laws and regulations, and with European Community Council Directives (86/609/ECC, 24 November 1986).

2.2. Drug treatment

In the second experiment, mice were injected i.p. with a 0.01 ml/kg volume of the CB1 cannabinoid agonist Arachidonoyl-2-chloroethyllamide (ACEA) (Tocris Bioscience, Bristol, UK) at doses of 1 and 2 mg/kg. The control group was injected with physiological saline (0.9% NaCl), which was also used for dissolving the drug.

2.3. Delayed reinforcement task

Evaluation of delay discounting was carried out in twelve modular operant chambers (Panlab, Barcelona, Spain) each of which was placed inside an isolation box (containing a fan and a light) equipped with a chamber light, two levers, one feeder device with a magazine to release food pellets (20 mg Dextrose precision rodent pellets, Bio-Serv, Frenchtown, NJ, USA), one stimulus light and a buzzer. In the training phase, each session began with the chamber light on and the lever press switch off. One lever delivered one food pellet (immediate lever), whereas the other lever delivered three food pellets combined with a 0.5 s stimulus light and 0.5 s, 2850 Hz, 85 dB buzzer (delayed lever). Following food delivery, a 30 s time-out period (signaled with the chamber light off) was established, during which additional pressing of either lever was recorded (no pellets were released). After this 30 s time-out period, the chamber light was turned on, indicating the start of the intertrial interval (ITI). The next trial was initiated when the subject was observed to be waiting in front of the lever press. All mice underwent one 30-min session per day. The maximum number of trials that an animal could complete during the training phase (in the case of it responding immediately after the end of each time-out period) was 60. The length of the training phase depended on the time needed to achieve the following learning task criteria (maximum 3 days): (1) reaching ~75% preference for the delayed lever; 2) > 10 reinforced trials per session and 3) < 20% deviation in the number of reinforced trials. Once these criteria were fulfilled, mice underwent the test phase during which there was a delay between pressing the lever and the delivery of three pellets. During this period, the stimulus light (not the 0.5 s buzzer) was turned on and additional pressing of either lever was recorded (without consequence). The delay was fixed and progressively increased over subsequent days (0, 6, 12, 18, 24, 30, 42, 54, 66, 78, 90 s). Changes in the percentage of preference for the delayed lever in relation to different delays (cognitive impulsivity) were analyzed.

2.4. Social encounters

This test consisted of confronting an experimental animal in a neutral cage (61 × 30.5 × 36 cm) for 10 min following 1 min of adaptation prior to the encounter. Standard opponents were rendered temporarily anosmic by intranasal lavage with a 4% zinc sulfate solution on the day before testing (Smothery et al., 1986). This kind of mouse induces an attack reaction in its opponent but does not outwardly provoke or defend itself, since it cannot perceive a pheromone that is present in the urine of the experimental animals. It elicits aggressive behavior in mice with a normal sense of smell (Brain et al., 1981; Mugford and Nowell, 1970).

Behavior was videotaped under white illumination. The videotapes were subsequently analyzed using a custom-developed program (Brain et al., 1989) that facilitates the estimation of times allocated to different broad functional categories of behavior — non-social exploration, social investigation, threat, attack, avoidance/flight and defensive/submissive — each of which is characterized by a series of different postures and elements. A more detailed description can be found in Rodríguez-Arias et al. (1998). In addition, latency and unit of threat and attack (total duration of attack/number of attacks) were evaluated in all the experiments.

2.5. Real time-PCR analyses

Gene expression studies were carried out in selected brain regions of CB1KO and WT mice and focused on the main targets involved in the regulation of aggression
and social behavior. Mice were killed and their brains were removed from the skull and frozen over dry ice. Coronal brain sections (500 μm) beginning at plates 19–20 (Paxinos and Franklin, 2001) were obtained in a cryostat (−10 °C). The amygdala (AMY containing ACO, BMA, and BLA). MmNoR and DR were microdissected according to a modification of the Palkovits method (Palkovits, 1983, as previously described (García-Gutiérrez et al., 2010)). Total RNA was isolated from brain tissue micro-punches using Trizol reagent (Invitrogen, Madrid, Spain) and subsequently reverse transcribed to cDNA. Quantitative analysis of the relative abundance of 5-HTT (Mm00435391_m1), COMT (Mm00514377_m1), MAO-A (Mm00558004_m1) and 5HT1B (Mm00439377_s1) gene expression was performed using the ABI PRISM 7700 Sequence Detector System. All reagents were obtained from Life technologies (Foster City, CA) and the manufacturer’s protocols were followed. The reference gene was 18S rRNA, detected using Taqman ribosomal RNA control reagents. All primer–probe combinations were optimized and validated for relative quantification of gene expression. In brief, data for each target gene were normalized to the endogenous reference gene, and the fold change in target gene mRNA abundance was determined using the 2^(-AΔCt) method (Schmittgen et al., 2000). This quantification method involves comparing the Ct values of the samples of interest with a control or calibrator, such as a non-treated sample or RNA from normal tissue. The Ct values of both the calibrator and the samples of interest are normalized to an appropriate endogenous housekeeping gene (18S rRNA). Total RNA was isolated from brain tissue micro-dissected according to the Palkovits method (Palkovits, 1983, as previously described (García-Gutiérrez et al., 2010)).

2.6. Immunohistochemistry-confocal microscopy

Three WT mice were perfused for 15 min with 4% paraformaldehyde and 0.002% CaCl2 in 0.1M phosphate buffer (PB; pH: 7.3–7.4), after which their brains were removed and stored in 0.05% azide in PB saline (PBS-Az) at 4 °C. Floating coronal sections (80 μm-thick) were obtained using a vibratome at −2.12 to −2.80 mm from Bregma, containing ACO, BMA and BLA and at −7.64 to −8.30 from Bregma, containing DR and MmNoR. They were distributed in 8 parallel series and stored in PBS-Az at 4 °C. Series 1 was Nissl-stained and mounted for conventional light microscopy (LM). Series 2 was incubated overnight with monoclonal mouse anti-COMT (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C and for 50 min with horse biotinylated anti-mouse antibody (1:250, Vector, Burlingame, CA) at room temperature. Series 3 was incubated in 5% normal goat serum, 1% bovine serum albumin (BSA; Sigma, St. Louis, MO) and 0.05% Tween 20 in PBS for 1 h. Sections were then incubated overnight with rabbit anti-C14 (1:750, Sigma Chemical, St. Louis, MI) or mouse anti-C14 (1:1000, Santa Cruz Biotechnology) in 5% normal goat serum, 1% BSA and 0.05% Tween 20 in PBS at 4 °C. Series 4 was incubated in 5% normal goat serum, 1% BSA and 0.05% Tween 20 in PBS for 1 h. Sections were then incubated overnight with rabbit anti-C14 (1:3000, Sigma Chemical, St. Louis, MI) or mouse anti-C14 (1:1000, Santa Cruz Biotechnology) in 5% normal goat serum, 1% BSA and 0.05% Tween 20 in PBS at 4 °C. Sections were then incubated for 90 min with rabbit anti-C14 (1:750, Cayman Chemical, MI) or mouse anti-C14 (1:100, Santa Cruz Biotechnology) in 1% BSA and 0.05% Tween 20 in PBS for 1 h. Sections were then incubated with ABC (Vector, Burlingame, CA) at room temperature. Sections were then incubated with a biotinylated anti-rabbit antibody (1:200, Vector, Burlingame, CA) at room temperature. Fluorescent stained sections were mounted using ProLong Gold (Molecular Probes, Eugene, OR) or goat rhodamine red-X anti-rabbit (1:1500, Molecular Probes) at room temperature. For double and triple staining, the second and third antibodies were used after washing to remove the first.[5] Sections were then processed with an ABC (Vector) kit, mounted with DAB-stained and mounted for LM. Series 1 was Nissl-stained and mounted for conventional light microscopy (LM). Series 2 was incubated overnight with anti-COMT (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C and for 50 min with horse biotinylated anti-mouse antibody (1:250, Vector, Burlingame, CA) at room temperature. Series 3 was incubated in 5% normal goat serum, 1% BSA and 0.05% Tween 20 in PBS for 1 h. Sections were then incubated overnight with rabbit anti-C14 (1:750, Sigma Chemical, MI) or mouse anti-C14 (1:1000, Santa Cruz Biotechnology) in 5% normal goat serum, 1% BSA and 0.05% Tween 20 in PBS at 4 °C. Series 4 was incubated in 5% normal goat serum, 1% BSA and 0.05% Tween 20 in PBS for 1 h. Sections were then incubated overnight with rabbit anti-C14 (1:3000, Sigma Chemical, St. Louis, MI) or mouse anti-C14 (1:1000, Santa Cruz Biotechnology) in 5% normal goat serum, 1% BSA and 0.05% Tween 20 in PBS at 4 °C. Sections were then incubated for 90 min with rabbit anti-C14 (1:750, Cayman Chemical, MI) or mouse anti-C14 (1:100, Santa Cruz Biotechnology) in 1% BSA and 0.05% Tween 20 in PBS for 1 h. Sections were then incubated with ABC (Vector, Burlingame, CA) at room temperature. Sections were then incubated with a biotinylated anti-rabbit antibody (1:200, Vector, Burlingame, CA) at room temperature. Fluorescent stained sections were mounted using ProLong Gold (Molecular Probes, Eugene, OR) or goat rhodamine red-X anti-rabbit (1:1500, Molecular Probes) at room temperature. For double and triple staining, the second and third antibodies were used after washing to remove the first.

2.7. Statistical analyses

In the first study, the data of the social encounter experiments were analyzed using a two-way ANOVA with two between variables: Housing (with two levels: grouped or isolation) and Genetics (with two levels: wild type and CB1KO). A Bonferroni’s test was used to carry out post hoc comparisons. A two-way ANOVA with repeated measures followed by a Student–Newman–Keuls’s test was performed to compare WT and CB1KO mice at different time points in the delayed reinforcement task. Student t-test analyses were carried out for comparison of gene expression in CB1KO vs. WT mice. Quantitative data of de-convoluted images were analyzed by means of one-way ANOVA. Significant differences between means were identified by a Tukey’s test. In the second study, the data for each of the behaviors studied in the social encounter experiments were analyzed using a one-way ANOVA with one between variable: Treatment (with four levels). A Bonferroni’s test was used to carry out post hoc comparisons. Values of p < 0.05 were considered statistically significant.

3. Results

3.1. 1st experiment

3.1.1. Social encounters

Grouped CB1KO mice spent more time engaged in Threat behavior (F1,178 = 7.488; p < 0.01), devoting more time to each threat episode (Unit of Threat) (F1,178 = 41.893; p < 0.001) and performing the first threat sooner (F1,178 = 6.591; p < 0.01) than grouped WT mice (p < 0.001 in all cases) (Fig. 1). No differences were observed among isolated mice.

Similarly, group-housed CB1KO mice spent more time engaged in Attack behavior (F1,178 = 3.515; p < 0.05) (Fig. 1) and devoted more time to each attack (Unit of Attack) (F1,178 = 22.754; p < 0.001) than grouped WT mice (p < 0.05 and p < 0.001, respectively). Isolation increased attack only in WT animals (p < 0.01 for attack and p < 0.001 for unit of attack), as no differences were observed in CB1KO mice with respect to housing conditions. All grouped mice, both WT and CB1KO (F1,78 = 5.557; p < 0.01), took longer to perform the first attack than single-housed animals (p < 0.01).

Grouped CB1KO mice spent less time engaged in Non-Social Interaction (Table 1) (F1,178 = 19.310; p < 0.001) than grouped WT mice (p < 0.01), and isolated CB1KO spent more time engaged in this behavior than their single-housed WT counterparts (p < 0.001). Isolation reduced the time spent by WT in Non-Social Exploration (p < 0.001), whereas no significant differences were observed among CB1KO mice. Isolated CB1KO mice spent less time engaged in Social Investigation (Fig. 1) (F1,178 = 2.862; p < 0.05) than isolated WT (p < 0.01). Grouped CB1KO spent more time in Avoidance and Fear behaviors (Table 1) (F1,178 = 16.82; p < 0.001) than WT (p < 0.001) and isolated CB1KO mice (p < 0.01).

3.1.2. Impulsivity level

The delayed discounting rate (change in the percentage of preference for the delayed lever) was assessed in WT and CB1KO mice. Since no delay was imposed on test day 1, no differences were found between WT and CB1KO, which started with similar preference levels. On subsequent days, the discounting rate was similar between genotypes when delays of 6–30 s were imposed. Differences between the percentage of preference values reached statistical significance between the sixth and last delay (Fig. 2): two-way RM ANOVA followed by Student–Newman–Keuls method; genotype: F1,110 = 1.349, p = 0.261; delay: F1,110 = 61.427, p < 0.001; genotype × delay interaction: F1,110 = 3.522, p < 0.001.

3.1.3. 5-HTT, COMT, MAO-A and 5-HT1B gene expression analyses in WT and CB1KO mice

Real-time-PCR analysis revealed that MAO-A gene expression was increased in the AMY (Student t-test: t = −2.130, 18 d.f., p = 0.049), unchanged in the MmR (Student t-test: t = 0.0421, 18 d.f.,
p = 0.967) and decreased in the DR (Student t-test: t = 3.504, 18 d.f., p = 0.003) of CB1KO mice. COMT gene expression was significantly higher in the three brain regions of CB1KO mice (Student t-test: AMY, t = −3.280, 18 d.f., p = 0.005; MnR, t = −2.000, 18 d.f., p = 0.063; DR, t = −2.673, 18 d.f., p = 0.017). No differences were detected in 5-HTT gene expression in the MnR (Student t-test, t = 0.456, 18 d.f., p = 0.654) and DR (Student t-test, t = 0.803, 18 d.f., p = 0.433) between WT and CB1KO mice. 5HT1B gene expression in the amygdala was significantly higher in CB1KO mice than in WT mice (Student t-test, t = −3.835, 18 d.f., p = 0.001) (Fig. 3A, B and C).

3.1.4. Co-localization studies of CB1r and COMT in the amygdaloid and raphe nuclei

COMT and CB1r double-immunohistochemistry showed cytoplasmic-labeled COMT-ir cells in both raphe (MnR and DR) and amygdaloid (Aco, Bma and Bla) nuclei (Figs. 4 and 5). However, CB1r immunolabeling was only observed in the amygdaloid nuclei and it was localized in axons and processes of the neuropile (Fig. 4A2, B2, C2 and D2). COMT immunolabeling was more intense in cells of BLA and MnR, while it was weaker in BMA, Aco and DR. CB1r immunolabeling was circumscribed very specifically to BLA: no immunolabeled processes or buttons were observed in the adjacent neuropile, though scattered COMT-ir cells were present (Fig. 4; asterisks in A2 and arrow in A3). The density of CB1r-ir buttons (on average, 472.8 buttons/10,000 μm²) was significantly higher in BLA than in BMA and Aco (229.5 and 207.9 buttons/10,000 μm², respectively; p < 0.001) (Fig. 6A). In addition, the percentage of CB1r-ir buttons adjacent to COMT-ir somata was significantly higher in BLA (17.4%) than in BMA and Aco nuclei (5.4% and 6.1%, respectively; p < 0.001) (Fig. 6B). No significant differences were found between the mean density (p = 0.946) or percentage of perisomatic CB1r-ir buttons (p = 0.917) in BMA and Aco nuclei (Fig. 6).

3.2. 2nd experiment

3.2.1. Social encounters in OF1 mice: effect of the CB1r agonistacea

Administration of the CB1r agonist AEA increased aggressive behaviors (Fig. 7 and Table 2). Isolated animals spent more time engaged in Threat behaviors (F(3,38) = 10.964; p < 0.001) than grouped (p < 0.001) or single-housed animals treated with AEA (p < 0.05). Similarly, the Unit of Threat (F(3,38) = 8.852; p < 0.001) was longer in isolated animals treated with saline or the lowest AEA dose than grouped animals (p < 0.001) or isolated animals treated with the highest AEA dose (p < 0.01). The group receiving the highest dose of AEA also presented a shorter Unit of Threat than the isolated saline-treated group (p < 0.01). These isolated-treated group spent more time in Attack (F(3,38) = 3.041; p < 0.05) and presented a longer Unit of Attack (F(3,38) = 3.055; p < 0.05) than grouped mice (p < 0.05). AEA-treated mice did not show differences in attack behaviors with respect to saline-treated mice housed in groups or in isolation. Non-Social exploration (F(3,38) = 3.711; p < 0.02) was less pronounced in isolated mice treated with saline than in those housed in groups (p < 0.01).

4. Discussion

The results of our studies suggest that CB1r play a relevant role in the regulation of aggressive behavior. This assumption is supported by the following observations: 1) Group-housed CB1KO mice showed more aggression than their WT counterparts, spending more time engaged in threat and attack behaviors, exhibiting their first aggressive behavior sooner, and showing longer aggressive interactions during the social interaction test; 2) CB1KO are more impulsive than WT mice; and 3) administration of the CB1r agonist AEA to single-housed aggressive mice significantly decreased their aggressive behavior.

Group-housed CB1KO mice displayed higher levels of aggressive behavior during the social interaction test than their WT mice (which lacked this kind of behavior). The social interaction test confronts an experimental mouse with a standard opponent that does not provoke aggression in the experimental mouse. Thus, under these conditions, WT do not behave aggressively whereas CB1KO mice display an offensive aggression by threatening and attacking the standard opponent in absence of specific provocation. Despite their higher level of aggression, group-housed CB1KO mice presented more similar non-aggressive social interactions to their conspecifics than did WT mice. However, after isolation, an increase in these social contacts was observed in WT but not in CB1KO mice. Grouped CB1KO mice also presented avoidance and flee behaviors that were not observed in WT animals. Some authors consider avoidance and flee behaviors to be panic-like attack behaviors (Blanchard and Blanchard, 1999; Estelles et al., 2004, 2005) that are in accordance with the high anxiety associated with these mice (Haller et al., 2002; Maccarrone et al., 2002; Martin et al., 2002; Ugurgen et al., 2004). Thus, group-housed CB1KO mice presented a particular behavioral profile characterized by elevated aggression and flee behaviors, not modified by isolation, which, on the other
hand, strongly affected the behavior of WT mice. Long-term social isolation in mice induces offensive and aggressive behavior (Malick, 1979; Valzelli, 1985) accompanied by hypofunction of the serotonergic system (Bickerdike et al., 1993; Rilke et al., 1998) and increases in the level of endogenous opiates (Sudakov et al., 2003; Broseta et al., 2005).

The impulsivity trait has been closely associated with aggression. For example, early impulsivity and aggression-related disorders are thought to predict later aggressive and criminal behavior (Farrington, 1989). For example, young children with a difficult temperament who lack behavioral control are likely to have externalizing behavior problems, aggressive and impulsive characteristics, to engage in violent offenses during adolescence, and to be diagnosed with antisocial personality disorders in adulthood (Raine et al., 1998). A neural circuit composed among other regions of the amygdala has been implicated in emotion regulation. Functional or structural abnormalities in one or more of these regions or in the interconnections between them may increase susceptibility to impulsive aggression and violence (Davidson, 2000). In the present study a link between impulsivity and aggression has also been observed. CB1KO mice, besides exhibiting higher aggression, showed an impulsive phenotype in the delayed discounting rate task. Latency to perform the first aggressive behavior can also be regarded as a measure of impulsivity or inhibitory control, since increased attack latencies indicate an inhibition of the tendency to initiate an aggressive encounter (Coppens et al., 2012). Accordingly, CB1KO mice showed significantly shorter latencies to initiate aggression whether housed in groups or individually. In addition, as impulsivity is a characteristic associated with enhanced reactive aggression in humans (Cherek et al., 2002), decreased 5-HT activity is associated with enhanced impulsivity in both animals and humans (Cherek et al., 2002; Mobini et al., 2000; Ho et al., 2002). No other transmitter system has been as consistently implicated in the neurobiological mechanisms that mediate impulsive aggressive behavior as 5-HT (Miczek et al., 2002, 2007; Takahashi et al., 2012). A great number of studies have related impulsive, hostile and violent behavior to a 5-HT deficiency (for review see Takahashi et al., 2012). Accordingly, selective serotonin reuptake inhibitors (SSRIs) reduce impulsive aggression in healthy subjects with high aggression scores (Berman et al., 2009). However, despite the effective antiaggressive action of SSRIs, no differences in the concentration of 5-HTT in either the DR or MnR were observed when we compared CB1KO and WT mice.

5-HT in the mammalian central nervous system derives mainly from DR and MnR. Inhibition of the metabolism of monoamines renders 5-HT and other monoamines more available in the brain. However, CB1KO mice seem to better-metabolize 5-HT, as they show higher levels of COMT in DR and MnR and also in AMY. In a recent study by Ginsberg et al. (2011), resident aggressive mice showed a significant upregulation of COMT expression in several brain regions. MAO-A was also increased in AMY, although no changes or decreases were detected in DR or MnR. Inhibition of MAO-A correlates with reduced aggression and foot shock-induced...
aggression in isolated male mice, probably as a result of increased 5-HT levels (for review see Nelson and Chiavegatto, 2001). An increase of COMT and MAO-A in the amygdala may reduce 5-HT levels, which could be related with the elevated concentration of 5-HT1B observed in CB1KO mice. COMT immunolabeling was seen in the cytoplasm of immunoreactive cells and less frequently in processes emerging from their soma (arrows in B1 and C1). In contrast, CB1r immunolabeling can only be seen in processes (mostly axons) and buttons that are COMT immunonegative. Note that the specificity of CB1r immunolabeling defines the BLA limits. Asterisks point to CB1r immunonegative adjacent neuropile to BLA. Note that COMT-ir cells are present (arrow in A3) in these CB1r immunonegative regions. The intensity of cytoplasmic COMT immunolabeling is weaker in BMA and ACo nuclei than in BLA (compare B1 with C1 and D1). Similarly, the density of CB1r-ir buttons and processes is also weaker in BMA and ACo nuclei (compare B2 with C2 and D2). Same scale for each set of confocal images.

Our results suggest that the CB1r plays a relevant role in inhibiting aggression, as mice lacking this receptor behave aggressively. Accordingly, administration of the CB1r agonist ACEA significantly reduced aggressive behavior in highly aggressive mice.

Fig. 4. Double immunolabeling for COMT and CB1r in the amygdaloid nuclei of WT mice. Confocal photomicrographs showing immunolabeling for COMT (green labeling in A1, B1, C1 and D1) and CB1r (red labeling in A2, B2, C2 and D2) in BLA (A1–B3), BMA (C1–C3) and ACo (D1–D3). Low power confocal A1–A3 images show the average overlay of 4 consecutive confocal images (2 μm-thick each). Double labeling (yellow labeling in A3, B3, C3 and D3) indicates that COMT does not co-localize with CB1r in the amygdaloid nuclei. COMT immunolabeling was seen in the cytoplasm of immunoreactive cells and less frequently in processes emerging from their soma (arrows in B1 and C1). In contrast, CB1r immunolabeling can only be seen in processes (mostly axons) and buttons that are COMT immunonegative. Note that the specificity of CB1r immunolabeling defines the BLA limits. Asterisks point to CB1r immunonegative adjacent neuropile to BLA. Note that COMT-ir cells are present (arrow in A3) in these CB1r immunonegative regions. The intensity of cytoplasmic COMT immunolabeling is weaker in BMA and ACo nuclei than in BLA (compare B1 with C1 and D1). Similarly, the density of CB1r-ir buttons and processes is also weaker in BMA and ACo nuclei (compare B2 with C2 and D2). Same scale for each set of confocal images.
Isolated OF1 mice were used in the second part of our study, since this strain of mice displays higher levels of aggression after isolation; indeed, these animals have been used to evaluate the anti-aggressive effects of other drugs, such as DAr antagonists (Rodríguez-Arias et al., 1998). Acute administration of the CB1r agonist effectively decreased aggression; there was a significant reduction in threat, the mean time of each threat interaction and the mean time of each attack after administration of 2 mg/kg of ACEA. These results suggest that activation of CB1r regulates the function of 5-HT neurons in key brain regions such as the amygdala, resulting in the reduction of aggressive behavior, as we can observe in single-housed OF1 mice. The results of our immunohistochemical experiments support this hypothesis, since they revealed a high density of CB1r-ir buttons in BLA (but lower in BMA and ACo nuclei) from which 17.4% were perisomatic to COMT-ir somata (while this percentage dropped to 5.4% and 6.1% in BMA and ACo nuclei). The possibility that these buttons establish active synapses needs to be confirmed by electron microscopy and electrophysiology; nevertheless, our confocal data show that CB1r are located in presynaptic synaptic buttons and strongly suggest that they regulate the release of excitatory neurotransmitters, thus affecting the function of COMT-ir target cells. It is important to note that immunohistochemistry experiments were focused on the amygdaloid nuclei since despite there were cytoplasmic-labeled COMT-ir cells in both raphe and amygdaloid nuclei, CB1r immunolabeling was observed in these raphe nuclei. Same scale for B.

Fig. 5. Immunolabeling for COMT in NLR of WT mice. Photomicrographs showing COMT immunolabeling in DR (A) and Mnr (B). COMT immunolabeling can be seen mostly in the cytoplasm and in some processes emerging from it. The intensity of cytoplasmic immunolabeling in COMT-ir cells was stronger in Mnr than in DR (compare immunolabeling in A and B). No immunoreactivity for CB1r was found in these raphe nuclei. Same scale for B.

Fig. 6. Histograms showing mean density (A) and percentage of perisomatic (B) de-convoluted CB1r-ir buttons in BLA, BMA and ACo. Both mean density and percentage of perisomatic CB1r-ir buttons in amygdaloid nuclei was significantly higher in BLA. No differences were found between BMA and ACo nuclei. Error bars (±SD); no significant differences (n.s.); *p < 0.001 (n = 12 for each nuclei).

Fig. 7. Means of accumulated times (in seconds) with ±SEM for Threat (black) and Attack (white) behaviors during the social interaction test in grouped and isolated adult OF1 mice treated with saline or ACEA (1 or 2 mg/kg). Differences with respect to saline-treated grouped mice *p < 0.05; ***p < 0.001. Differences with respect to saline-treated isolated mice +p < 0.05.

Table 2

<table>
<thead>
<tr>
<th>Category</th>
<th>Grouped Saline</th>
<th>Isolated Saline</th>
<th>ACEA 1 Saline</th>
<th>ACEA 2 Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-social Exploration</strong></td>
<td>417 ± 17</td>
<td>338 ± 22**</td>
<td>395 ± 17</td>
<td>397 ± 15</td>
</tr>
<tr>
<td>Social Investigation</td>
<td>98 ± 12</td>
<td>118 ± 34</td>
<td>90 ± 18</td>
<td>92 ± 10</td>
</tr>
<tr>
<td>Latency of Threat</td>
<td>263 ± 31</td>
<td>162 ± 32</td>
<td>277 ± 42</td>
<td>240 ± 25</td>
</tr>
<tr>
<td>Unit of Threat</td>
<td>0.04 ± 0.1</td>
<td>2.1 ± 0.2***</td>
<td>1.3 ± 0.5***</td>
<td>0.8 ± 0.2**</td>
</tr>
<tr>
<td>Latency of Attack</td>
<td>275 ± 27</td>
<td>205 ± 39</td>
<td>303 ± 42</td>
<td>223 ± 34</td>
</tr>
<tr>
<td>Unit of Attack</td>
<td>0.1 ± 0.1</td>
<td>3.1 ± 0.8*</td>
<td>1.8 ± 1</td>
<td>1.7 ± 0.7</td>
</tr>
<tr>
<td>Avoidance/Flee</td>
<td>0 ± 0</td>
<td>1 ± 1.1</td>
<td>0 ± 0</td>
<td>0.5 ± 0.6</td>
</tr>
</tbody>
</table>

Means of accumulated times (s) with ±SEM allocated to different categories of spontaneous behavior of grouped and isolated adult mice treated with saline or ACEA (1 or 2 mg/kg) during the social interaction test. Differences with respect to saline-treated grouped mice **p < 0.01; ***p < 0.001. Differences with respect to saline-treated isolated mice +p < 0.01. Differences with respect to ACEA 2-treated isolated mice ++p < 0.01.
only in processes and buttons of the amygdaloid nuclei. Interest-
ingly, a previous study proposed that the endocannabinoid system exerts a tonic/constitutive regulation of 5-HT neurons in DR, probably through a CB1r-mediated mechanism (Mendinéu and Pineda, 2009).

In conclusion, our findings support an important role for CB1r in social interaction and aggressive behavior. Pharmacological manipulation of this receptor deserves further investigation as a potential target in the management of aggression-related psychiatric disorders.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2013.07.013.

References

M. Rodríguez-Arias et al. / Neuropharmacology 75 (2013) 172–180


