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Chronic periadolescent cannabinoid treatment enhances adult hippocampal PSA-NCAM expression in male Wistar rats but only has marginal effects on anxiety, learning and memory

A. Higuera-Matas [a], F. Botreau [a], M. Miguéns [a], N. Del Olmo [b], E. Borcel [a], L. Pérez-Álvarez [a], C. García-Lecumberri [a], E. Ambrosio [a,*]

[a] Psychobiology Department, Faculty of Psychology, UNED, 28040, Madrid, Spain
[b] Department of Pharmacology, Pharmaceutical Technology and Development, Univ. San Pablo CEU, P.O. Box 67, 28660 Boadilla, Madrid, Spain

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ABSTRACT

Pubertal and adolescent exposure to cannabinoids is associated with enduring alterations in anxiety and memory. However, periadolescence virtually remains unexplored. Here, we measured anxiety in the Elevated Plus Maze (EPM) in adult Wistar rats treated at periadolescence (P28–P38) with the cannabinoid agonist CP 55,940 (CP) (0.4 mg/kg; 2 ml/kg i.p., 1 daily injection), and we also defined their recognition memory in the novel object paradigm and spatial learning and memory in the water maze. Additionally, we measured the expression of hippocampal PSA-NCAM (Polysialic Acid-Neural Cell Adhesion Molecule) and long-term potentiation (LTP) as well as, given their role in mnemonic processing, the levels of plasma corticosterone and estradiol. We found that CP had no robust effects on anxiety or in recognition memory. In the water maze, only a slight decreased percentage of failed trials in the reference memory task and an improvement in an indirect index of attention were observed. However, we detected an up-regulation of hippocampal PSA-NCAM expression, only in CP-males, although this effect was not related to changes in LTP. No hormonal alterations were evident. Based on our data, minimal long-term effects on anxiety, learning and memory appear to result from cannabinoid exposure during the periadolescent period.

1. Introduction

During adolescence, a period of particular vulnerability to the effects of drugs of abuse (Viveros et al., 2005), marihuana is the most commonly used illicit drug (Gruber and Pope, 2002). During this period of development, substantial morphological and functional modifications are taking place in the brain and these changes are thought to support the emergence of adult cognitive processes (Chambers et al., 2003). For example, it has been shown that the number of the cannabinoid type-1 (CB1) receptors peaks during the peri-pubertal and periadolescent periods in the limbic forebrain before rapidly declining and reaching adult values (Rodriguez de Fonseca et al., 1993). These receptors are very abundant in the hippocampus (Herkenham et al., 1990), one of the regions comprising the limbic forebrain and mainly involved in learning and memory processes (Squire, 2004). Indeed, it has been reported that cannabinoids alter normal synaptic functioning in the hippocampus resulting in memory impairment (Mato et al., 2004; Robbe et al., 2006). Accordingly, it might be hypothesized that exposure to cannabinoids during puberty and adolescence would provoke changes in hippocampal function that would alter adult cognitive domains. In this regard, conflicting data have emerged on the residual effects of cannabinoids. Early studies in humans showed that chronic marihuana use before the age of 16 was correlated with impaired visual scanning task performance (Ehrenreich et al., 1999). Moreover, it was reported that marihuana exposure during adolescence is associated with a long-term deficit in working memory (Schwartz et al., 1989).

Animal studies in this field have shown that chronic cannabinoid exposure during adolescence impairs working memory when measured by the novel object recognition task in adult female rats; however, this impairment was not evident after chronic administration to adults (O'Shea et al., 2004). Similarly, an impaired working memory was observed in adult male rats treated in adolescence with the cannabinoid agonist WIN 55,212-2 (Schneider and Koch, 2003).

Periadolescence is also a period of great plasticity (Viveros et al., 2005); however, the studies focusing on the developmental effects of cannabinoids during this specific stage are scarce. For instance it was shown that a chronic treatment with the cannabinoid agonist WIN 55,512-2 from postnatal days 15 to 40 (P15–P40) did not induce any effect in recognition memory or operant learning when rats were

* Corresponding author. Psychobiology Department, Faculty of Psychology, UNED, C/ Juan del Rosal no. 10, 28040 Madrid, Spain. Tel.: +34 913987974; fax: +34 913986287.
E-mail address: eambrosio@psi.uned.es (E. Ambrosio).

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tested as adults. However, there was evidence of increased anxiety and haloperidol-reversible impairments in sensory-motor gating as revealed in the pre-pulse inhibition paradigm (Schneider et al., 2005).

Learning and memory have been demonstrated to be influenced by anxiety-related responses (Herrero et al., 2006), therefore it is also important to understand how early cannabinoid exposure may affect these.

Despite the growing literature on the residual effects of cannabinoids on learning, memory and anxiety, little is known about their underlying neural bases. Long-term potentiation (LTP) is the best studied form of synaptic plasticity in the hippocampus (Malenka, 2003; Malenka and Bear, 2004) and it is thought to support normal learning and memory (Lynch, 2004). Cannabinoids exert complex actions on the induction and maintenance of LTP. In this sense, chronic administration of THC provokes deficits in hippocampal LTP that are evident as early as three days after withdrawal (Hoffman et al., 2007). Indeed, when the long-term effects of prenatal chronic THC treatment were analyzed, hippocampal LTP was significantly reduced in adulthood in parallel with reduced levels of glutamate (Mereu et al., 2003). However, to our knowledge, the long-lasting effects of periadolescent cannabinoid exposure on hippocampal LTP have not yet been studied.

The phenomenon of LTP in part relies on the neural cell adhesion molecules (NCAM) (Luthl et al., 1994; Ronn et al., 1995) and its polysialylated form (PSA-NCAM) (Muller et al., 2000, 1996). The relationship of these molecules with the cannabinoid system is only just beginning to be elucidated, and it was recently reported that acute activation of CB1 cannabinoid receptors transiently diminished PSA-NCAM expression in the dentate gyrus of the hippocampus (Mackowiak et al., 2007).

Male and female rats show different windows of vulnerability to the effects of cannabinoids, especially with regards to cognitive sequelae (O'Shea et al., 2006, 2004). Furthermore, given the influence of estrogens on learning and memory (Daniel, 2006; Daniel et al., 2005, 2006a,b), as well as the relationship between cannabinoids and the hypothalamic–pituitary–gonadal axis in females (Scorticati et al., 2004, 2003), we also decided to measure the levels of plasma estradiol in our animals in order to explore any estrogenic mechanisms influencing learning and memory in cannabinoid-exposed animals. Additionally, we analyzed corticosterone because it has been involved in memory formation (Gourley et al., 2009; Miranda et al., 2008; Sandi, 1998; Wang et al., 2008; Wiegert et al., 2008) and the hypothalamic–pituitary–adrenal axis is also affected by cannabinoid preexposure (Rubio et al., 1995).

According to the literature reviewed above, a deeper understanding of the neural correlates of periadolescent cannabinoid-induced emotional and cognitive alterations was required. Therefore, in order to replicate and extend the work of Schneider et al. (2005) we set out to study the emotional (anxiety responses in the EPM), cognitive (recognition memory in the novel object paradigm and water maze performance), neurophysiological (hippocampal LTP) and neurochemical (PSA-NCAM levels in the hippocampus) long-lasting effects of a chronic cannabinoid treatment during the periadolescent period (Higuera-Matas et al., 2008; Viveros et al., 2005).

2. Experimental procedures

2.1. Animals and drug administration

We used Wistar albino rats of both sexes and the experiments were carried out on the offspring of rats (from Harlan Interfauna Ibérica S.A., Barcelona, Spain) that were mated at our center approximately 2 weeks after their arrival (one male × one female). All animals were maintained at a constant temperature (20 °C) and in a reverse 12-h/12-h dark/light cycle (lights on at 20:00 h), with free access to food and water (commercial diet for rodents A04/A03; Panlab, Barcelona, Spain). On the day of birth (postnatal day 0), the litters were sex-balanced and culled to 10 ± 2 pups per dam. The animals were weaned at 22 days of age and housed in cages (3–4 rats per cage) according to sex and treatment (rats from each cage always were of the same sex and received the same treatment).

CP 55,940 (CP — Tocris, 0.4 mg/kg) or its corresponding vehicle (VH — ethanol:cremophor:saline, 1:1:18. Cremophor, Fluka BioChemiKa) was i.p. administered in a volume of 2 ml/kg once daily, from postnatal days 28 to 38 (11 injections). The dose of CP was chosen on the basis of our previous significant results on the long-term effects of a chronic periadolescent CP treatment (Higuera-Matas et al., 2008). We have chosen to administer the agonist during a periadolescent period because a notable increase in the number of CB1 binding sites is observed (Rodriguez de Fonseca et al., 1993), pointing to the active development of the endocannabinoid system at this time. Moreover periadolescent chronic cannabinoid treatment has been shown to induce behavioral disturbances in adult rats that, although less severe than those observed after adolescent cannabinoid administration, are still significant (Schneider et al., 2005).

Animals were housed individually when they reached P75, in order to have conditions that were as similar as possible to those used in our previous work (Higuera-Matas et al., 2008). Behavioral testing began 22 days after this manipulation, when all the acute effects of social isolation have elapsed. It must also be noted that if any residual effect of isolation remained during testing, the conditions would still be equivalent across all treatment groups.

All experimental procedures were carried out between 10:00 and 17:00 and three different batches of animals were used for the behavioral, biochemical and electrophysiological experiments. All animals were maintained and handled according to the European Union Laboratory Animal Care Rules (86/609/EEC Directive) and the "Principles of laboratory animal care" were strictly followed.

2.2. Behavioral tests

Experimental procedures began when rats from the first batch of animals were 97 days old [CP-females ($n = 11$), CP-males ($n = 9$), VH-females ($n = 10$), VH-males ($n = 8$)] with handling for three consecutive days. At P100, the rats were tested in the Elevated Plus Maze (EPM) under dim light. The EPM consists of two opposing open (45 × 10 cm) and closed arms (45 × 10 × 50 cm) that extend from a central platform (10 × 10 cm), elevated 65 cm above the floor. The rats were placed individually on the central platform facing one of the closed arms and were allowed to freely explore the maze for 5 min. The behavior of each rat was monitored using a video camera and the movements of the rats were automatically registered and analyzed with a computerized tracking system (Ethovision 3.0, Noldus IT, The Netherlands). Entry into an arm was defined as entry of all four paws into one arm. Hence, the time spent by rats in each kind of arm was recorded. The main index used to measure anxiety in the EPM was defined as follows: (% time spent in open arms) / (%time spent in open arms + %time spent in closed arms). This anxiety index is used to correct the differences in locomotor activity that may exist among groups. The behavioral analysis was completed by measuring the frequency and duration of the following behaviors: (1) head dipping, i.e., looking over the edge of an open arm or the central platform; (2) rearing, i.e., standing up on hind legs with or without pressing the front paws on the wall; (3) grooming and (4) freezing.

The day after the EPM test, the protocol to study recognition working memory began. The rats were habituated to the empty open field (100 × 100 × 40 cm) for 3 min over 2 days. In the acquisition phase, the rats were placed into the open field containing two identical objects that they had never seen, and they were allowed to remain in the arena until they explored the object for 30 s or for a total of 5 min (we considered that the rat was exploring the object whenever the animal sniffed, licked, chewed or moved vibrissae while

directing the nose towards and less than 1 cm from the object). The time spent by the rat exploring each object was recorded. Recognition memory was tested after 30 min in a 5 min test session, during which one of the objects used during training was replaced by a novel object. The objects were cleaned thoroughly between trials to ensure the absence of olfactory cues. Additionally, they had been tested before in naive rats to ensure an equivalent level of basal preference. The recognition index was calculated as the time spent exploring the new object minus the time spent exploring the old object divided by the total exploration time. An index with values higher than 0.2 is considered as indicative of good recognition (Vannucchi et al., 1997).

Four days after object recognition task, the animals were tested in a water maze as described in Cassel et al. (2007) with minor modifications. The Morris water maze used was a black circular pool (2 m diameter, 45 cm high) filled with water (30 cm depth at 25 °C) and divided into four quadrants. The pool was located in a room with numerous extra maze cues, and the behavior of the individual animals was monitored with a video camera and a computerized tracking system (Ethovision 3.0, Noldus IT). Different parameters of the rats' performance were analyzed, including: escape latency or total time needed by the rats to find the platform (s); total distance swum to reach the platform (cm), % time spent in each quadrant and swimming speed (cm/s). When differences in velocity were detected among groups, the distance to the platform rather than the escape latency was reported. As regards this and in order to use the most suitable parameter for each specific phase, we decided to analyze swimming speeds during each phase of the water maze task (i.e. reference memory, cued performance and working memory) rather than pooling together the speeds of each single trial. We first tested spatial reference memory, then cued performance and finally, spatial working memory.

For reference memory testing, rats underwent a training schedule over 4 consecutive days with 4 consecutive trials per day. The platform (11 cm diameter) was placed in the middle of one of the quadrants (1.5 cm below the water surface), equidistant from the sidewall and the middle of the pool, and its location did not change during training. The duration of each trial was 60 s or until the rat found the platform. If the rat failed to escape within those 60 s, it was guided to the platform by the experimenter. Once the animals reached the platform, they were left on it for 20 s (even those that failed to locate it), they were then removed from the pool and the next trial began. Starting points were randomly assigned and they were different from trial to trial. Twenty-four hours after the last training day, the platform was removed and the rats were allowed to search for it for 60 s (probe test).

Two days after the reference memory test, rats were submitted to a visible platform test, where the platform location was signaled by a flag to test cued performance. A similar procedure to the one used for reference memory was followed, but this time the rats had two consecutive training days with four consecutive trials per day and no probe test. All the other parameters remained the same. The day after the last visible platform test session, the working memory procedure began. Training was carried out on 4 consecutive days with four consecutive trials per day. Each trial lasted 60 s or until the rat found the hidden platform, the location of which was changed every day but remained constant during the trials performed on each day. The start position also changed across trials and days. Here, data are presented as the averaged first, second, third and fourth trials of each consecutive day (i.e. [Trial1-Day1 + Trial1-Day2 + Trial1-Day3 + Trial1-Day4]/4, etc…). This index allows us to assess working memory by examining the changes in performance between trials 1 and 2.

2.3. Biochemical assays and hormone determinations

2.3.1. Tissue extraction

Tissue dissection was performed in the second batch of animals on P100 in males or on the first day of proestrous following P100 in females (CP-females $n=7$; CP-males $n=8$; VH-females $n=7$; VH-males $n=7$). Vaginal smears were collected from females and if positive for proestrous, the animal was quickly sacrificed. Otherwise, the rats were returned to their home cage and tested again on the following day until they were found to be in proestrous. We chose to select this phase of the oestrous cycle in order to have estradiol levels falling in the detection range of the RIA kit. Rats were sacrificed by rapid decapitation having previously been lightly anesthetized with isoflurane. The hippocampus was dissected out from each animal on ice and trunk blood was collected. The tissue samples were coded and stored at −80 °C until use. Blood was centrifuged (3000 rpm for 20 min, at 4 °C) and the plasma stored at −35 °C until the day of the RIA assay.

2.3.2. Synaptosome preparations

Crude synaptosomal pellets were obtained according to a protocol modified from Lynch and Voss (1991). In brief, the hippocampal tissue was homogenized with 16 strokes in ten volumes of ice-cold sucrose (0.32 M) and HEPES (5 mM) buffer that contained a cocktail of protease inhibitors (Roche, Spain) and centrifuged at 1000 *g* for 5 min. The supernatant was then centrifuged at 15 000 *g* for 15 min, and the pellet was resuspended in Krebs buffer containing protease inhibitors for future use. The protein concentration for each sample was estimated by the method of Bradford (1976).

2.3.3. Determination of corticosterone and estradiol levels

Plasma estradiol levels were measured with a RIA kit (DSL Diagnostics, USA). The intra-assay variability of the RIA ranged from 2.01% to 5.17%. The sensitivity of the assay (minimum detectable dose) was 11 pg/ml. Corticosterone concentration was also determined by a RIA kit (Coat-A-Count, Diagnostics Products Corporation; CA, USA) with an intra-assay variability ranging from 3.1% to 4.5% and a sensitivity of 5.7 ng/ml.

2.3.4. ELISA

The accumulation of PSA-NCAM was quantified by enzyme-linked immunoabsorbent assays (ELISAs) according to a previously described protocol (Merino et al., 2000). Flat bottom 96 well microplates were allowed to adsorb a coating solution (Na_2CO_3 0.1 M $NaHCO_3$, 0.1 M) for 2 h at room temperature. The solution was removed and 50 µl of the pellet from the samples was added at a concentration of 10 µg/ml to each well of the ELISA plates. The plates were incubated overnight at 4 °C and then washed three times with 1 M phosphate-buffered saline (PBS) containing 0.05% Tween 20, pH 7.4. Additional binding sites were blocked with BSA (3%) for 2 h at room temperature, and the wells were rinsed three times as described above before incubating them with 50 µl aliquots of the corresponding first antibody for 20–24 h at 4 °C. After again washing the wells, 50 µl aliquots of peroxidase conjugated second antibody were added and left for 2 h. Subsequently, 50 µl of citrate buffer (50 mM Na_2HPO_4, 25 mM citric acid, pH 4.5) containing o-phenylene diamine (1 mg/ml) and H_2O_2 (0.06%, added just before use) were placed in each well and the peroxidase was allowed to react for 10 min at room temperature. The reaction was terminated by the addition of 50 µl of 10 M H_2SO_4 to each well and the optical density was determined by measuring absorbance at 492 nm with a Microplate Reader (DigiScan Reader V3.0 and DigiWIN software Program; ASYS Hitech GmbH, Austria). A monoclonal antibody was used (clone 2-2B AbCys, France) and this mouse IgM antibody (diluted 1:2000) was recognised by a peroxidase (l chain) conjugated IgM anti-mouse secondary antibody used at a 1:1000 dilution (Sigma-Aldrich, Spain).

2.4. Long-term potentiation studies in hippocampal slices

When rats from the third batch were between P100 and P110 (CP-females $n=7$; CP-males $n=4$; VH-females $n=6$; VH-males $n=6$),

they were quickly decapitated and their brains were removed and dropped into bubbling (95% O_2 and 5% CO_2) ice-cold Krebs–Ringer bicarbonate (KRB) solution containing (in mM): 109 NaCl, 2.5 KCl, 1 KH_2PO_4, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 26.2 $NaHCO_3$ and 11 glucose. Transverse slices (400 μm) were cut with a manual tissue chopper from the dorsal portion of each hippocampus and placed in a humidified interface chamber at room temperature (20–25 °C) as described previously (del Olmo et al., 2000, 2003). After 2 h incubation, the slices were transferred to the submersion recording chamber that was continuously perfused with a standard KRB solution at 1.8–2 ml/min rate. Field excitatory postsynaptic potentials (fEPSPs) were recorded in the CA1 stratum radiatum with tungsten electrodes (1 MΩ) and evoked by stimulating Schaffer collateral–commisural fibres with biphasic electrical pulses (30–70 μA; 100 μs; 0.033 or 0.066 Hz) delivered through bipolar tungsten insulated microelectrodes (0.5 MΩ). The recording electrode was connected to an AI-402 amplifier (Axon Instruments, USA) attached to a CyberAmp 380 signal conditioner (Axon Instruments, USA) and electrical pulses were supplied by a pulse generator Master 8 (AMPI, Israel). Evoked responses were digitized at 25–50 Hz using a Digidata 1322A (Axon Instruments, USA) and stored on a Pentium IV IBM compatible computer using pCLAMP 9.0 software (Axon Instruments, USA). The synaptic strength was assessed by measuring the initial slope of the fEPSP that was analyzed by means of pCLAMP 9.0 software. Data were normalized with respect to the mean values of the responses obtained from each animal at the 20 min baseline period. After obtaining stable synaptic responses for at least 20 min, the hippocampal slice was tetanized with three trains of a 100 Hz pulse lasting for 1 s and 100 μs with an interval of 20 s (high frequency stimulation, HFS). A single slice from each separate animal was considered as $n=1$ and all electrophysiological experiments were carried out at 31–32 °C.

2.5. Statistical analysis

For the EPM, object recognition task, electrophysiology, hormone determinations (RIAs) and ELISAs; ANOVAs were used to analyze differences among groups. Two independent variables were assessed: SEX (male or female) and TREATMENT (CP or vehicle). Simple effects analysis was used to examine interactions and *t*-tests were applied in some cases to analyze specific group differences. For the water maze data, a mixed ANOVA was run with SEX and TREATMENT as the between-subjects factors and in the reference memory and visible platform tasks, DAYS was used as the within-subjects factor (with each day comprising the average of all the trials of that day), while TRIAL was the within-subjects factor in the working memory task (comprising the averaged first, second, third and fourth trials of each consecutive day — see above).

Non-parametric statistics (Kruskal–Wallis *H* test) were applied whenever the basic assumptions of ANOVA (normality and homogeneity of variances) were not met. The Greenhouse–Geisser correction for the degrees of freedom was applied when non-spherical mixed ANOVA was detected.

3. Results

3.1. EPM

No differences were found in the anxiety index between males and females (effect of SEX: $F_{1,34}=2.010$ $p=0.165$), CP and VH animals (effect of TREATMENT: $F_{1,34}=2.036$ $p=0.163$) and no SEX×TREATMENT interaction was detected ($F_{1,34}=0.011$ $p=0.919$) (Fig. 1A). A significant effect of the TREATMENT factor was observed on the time spent in the closed arms ($H_1=4.011$ $p<0.05$), with CP animals spending significantly less time compared to VH rats (Fig. 1B). In contrast, no other differences were found in the time spent in the remaining areas of the EPM (center: $F_{1,34}=0.136$ $p=0.715$ for SEX; $F_{1,34}=3.018$ $p=0.091$ for TREATMENT; $F_{1,34}=0.037$ $p=0.848$ for SEX×TREATMENT interaction; open arms: $F_{1,34}=2.176$ $p=0.149$ for SEX; $F_{1,34}=1.314$ $p=0.260$ for TREATMENT and $F_{1,34}=0.00$ $p=0.997$ for SEX×TREATMENT interaction). We detected a significant effect of TREATMENT on the frequency of grooming behavior ($F_{1,34}=6.816$ $p<0.01$), whereby CP animals showing less frequent grooming behavior (mean ± SEM: CP = 0.348 ± 0.195; VH = 1.088 ± 0.205) (data not illustrated). No significant differences were detected in the frequency of any of the other behaviors studied (head dipping: $F_{1,34}=3.554$ $p=0.068$ for SEX; $F_{1,34}=1.104$ $p=0.301$ for TREATMENT and $F_{1,34}=0.006$ $p=0.941$ for SEX×TREATMENT interaction; rearings: $F_{1,34}=0.376$ $p=0.544$ for SEX; $F_{1,34}=0.635$ $p=0.431$ for TREATMENT and $F_{1,34}=0.252$ $p=0.619$ for SEX×TREATMENT interaction).

3.2. Novel object

When non-spatial working memory was analyzed with the novel object test, no significant effects of any of the factors were found in the recognition index ($F_{1,32}=0.028$ $p=0.868$ for SEX; $F_{1,32}=0.472$ $p=0.497$ for TREATMENT and $F_{1,32}=0.139$ $p=0.712$ for SEX×TREATMENT interaction) (male-CP = 0.59 ± 0.06; male-VH = 0.57 ± 0.04; female-CP = 0.59 ± 0.03; female-VH = 0.54 ± 0.04) (data not illustrated).

3.3. Water maze

3.3.1. Reference memory

A significant effect of SEX was found in the swimming speed in this task ($F_{1,34}=4.496$ $p<0.05$), so we will report the path distance to the platform instead of the escape latency. We found a significant effect of DAYS ($F_{3,102}=59.299$ $p<0.01$) in the cumulative path distance to the platform, with significant differences between days 1 and 2, ($p<0.01$ using a Bonferroni-adjusted α level). These data show that animals reached their plateau at days 3 and 4, so we can conclude that they effectively learned the task. We also found a DAYS×SEX interaction ($F_{3,102}=3.656$ $p<0.01$). More specifically, male rats outperformed females as demonstrated by the significant effect of SEX on days 3 ($F_{1,34}=5.089$, $p<0.05$) and 4 ($F_{1,34}=5.570$ $p<0.05$) of training. Indeed, male rats travelled a shorter cumulative distance to the platform, which demonstrated better learning (Fig. 2A). There were no other significant effects on the main measures of behavior. However, when we looked at the percentage of failed trials per day we found that CP-treated animals significantly failed in less trials than the VH controls as revealed by the significant effect of TREATMENT ($F_{1,34}=5.577$ $p<0.05$; $mean_{FailedTrialsCP}=27.321$ SEM = 3.534; $mean_{FailedTrialsVH}=39.453$ SEM = 3.729) (Fig. 2B).

During the probe test, no effects of any factors were detected on the swimming speed. The ANOVA did not show any significant effect in the % of time spent in the quadrant where the platform was located (effect of SEX: $F_{1,34}=0.497$ $p=0.486$; effect of TREATMENT: $F_{1,34}=0.334$ $p=0.567$; SEX×TREATMENT interaction: $F_{1,34}=0.341$ $p=0.563$).

3.3.2. Visible platform cued task

Swimming speed was not affected by SEX, TREATMENT or their interaction. We found a significant effect of DAYS in this task ($F_{1,34}=24.841$ $p<0.01$), showing that the latency to find the platform was lower on the second day as compared to the first day ($p<0.01$). There was also a significant effect of SEX and TREATMENT when analyzed as between-subjects factors ($F_{1,34}=9.534$ $p<0.01$ for SEX and $F_{1,34}=4.544$ $p<0.05$ for TREATMENT). However, when this issue was investigated in more detail, these effects were due to a significantly higher latency of VH-females on the first day ($t_{19}=2.304$ $p<0.05$), while no differences could be found on the second day among groups (Fig. 2C). This would also explain the difference in performance between days. The ANOVA did not show any interaction effects of TREATMENT

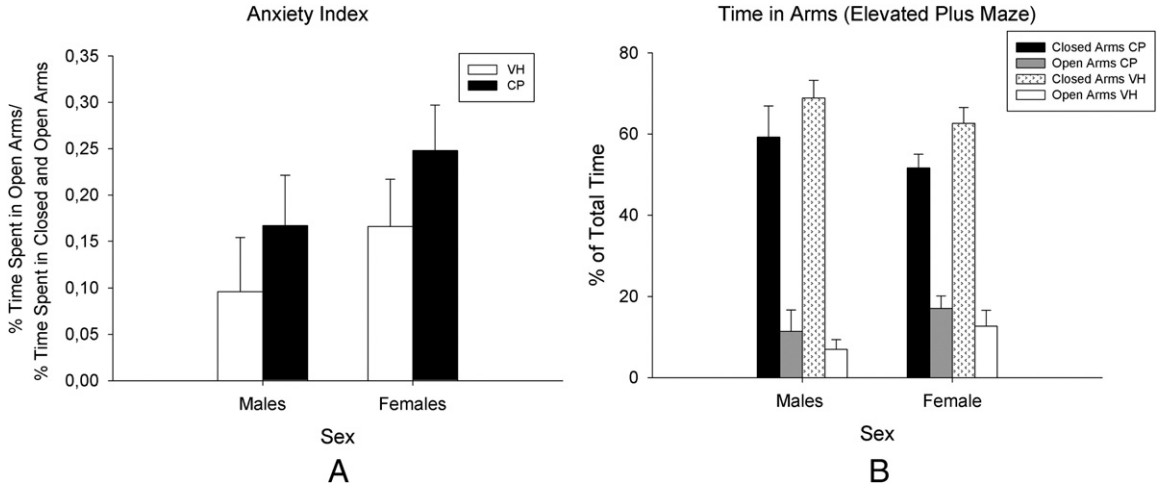

Fig. 1. Elevated Plus Maze: (A) Anxiety index in the EPM. No effects were observed due to any of the factors studied. (B) The figure shows the mean percentage of time spent by the rats in each kind of arm of the EPM. A significant effect of TREATMENT was found in the percentage of time spent in the closed arms ($p<0.05$). CP-treated rats spent less time in the closed arms, perhaps showing a more pronounced exploratory behavior.

($F_{1,34}=0.746$ $p=0.394$) or of the SEX ($F_{1,34}=1.940$ $p=0.173$) of the animals with DAYS.

3.3.3. Working memory

We detected a significant effect of SEX ($F_{1,34}=28.141$ $p<0.01$) on the swimming speed and therefore we will report the path distance to the platform. There was a significant effect of the within-subjects factor TRIAL in the path distance to the platform ($F_{3,102}=43.202$ $p<0.01$). We observed a significant decrease in the averaged trials 2 compared to the averaged trials 1 ($p<0.01$ for each comparison using a Bonferroni-corrected α level); however no differences were evident between the averaged trials 3 and 4, pointing to a stable performance

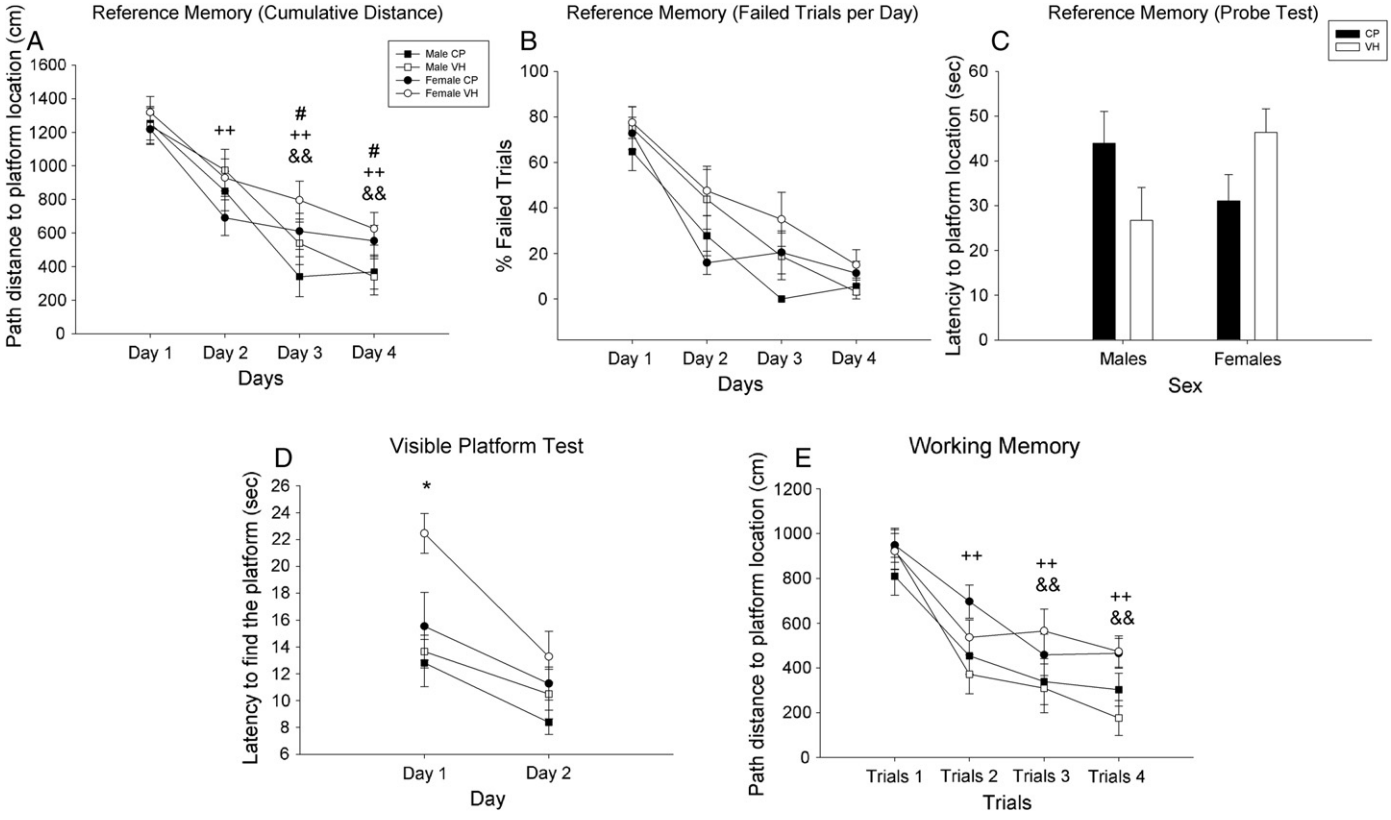

Fig. 2. Water maze tasks: (A) Cumulative path distance to platform location in the reference memory task. The mean distance in each subsequent trial was significantly lower than that observed in trial 1. Trials 3 and 4 were comparable. This indicates an asymptotic performance beginning in trial 3, which shows that animals effectively learned the task. ++Significant difference as compared to trial 1 ($p<0.01$). &&Significant difference as compared to trial 2 ($p<0.01$). #Significant difference between males and females ($p<0.05$). (B) % of failed trials per day. A general effect of the treatment is observed ($p<0.05$) with CP-exposed animals exhibiting a lower percentage of failed trials. This could point to a slight improvement in reference memory. (C) Latency to reach the visible platform in the 2 days of the task. A significant effect of TREATMENT and SEX was found. However, when we analyzed this effect in more depth we could see that the differences were specifically due to a significant difference between CP and VH females on the first day of training. *Significant difference with CP-females ($p<0.05$). (D) Working memory. The path distance in the working memory task is represented. A general SEX effect is observed. ++Significant difference as compared to trial 1 ($p<0.01$). &&Significant difference as compared to trial 2 ($p<0.01$).

of the task (Fig. 2D). Male rats performed better than females in this task as shown by the shorter average distance travelled to the platform (significant effect of SEX $F_{1,34}=9.945$ $p<0.01$). No other significant effect of any factor was found in the path distance ($F_{3,102}=0.953$ $p=0.385$ for TRIAL×SEX interaction; $F_{3,102}=1.158$ $p=0.318$ for TRIAL×TREATMENT interaction and $F_{3,102}=0.948$ $p=0.387$ for TRIAL×SEX×TREATMENT interaction).

3.4. Plasma estradiol and corticosterone levels

A significant effect of SEX on plasma estradiol levels was detected ($F_{1,25}=12.706$ $p<0.01$). As expected, female rats displayed higher levels of this hormone than male rats (see Table 1). However, no significant differences were found among our groups due to the TREATMENT ($F_{1,25}=0.354$ $p=0.557$) or SEX×TREATMENT interaction ($F_{1,25}=0.354$, $p=0.682$). With regard to corticosterone, a significant effect of SEX was found ($H_1=14.750$ $p<0.01$), showing the females' higher levels of corticosterone than the males. However no significant effect of TREATMENT was obtained ($H_1=0.190$ $p=0.663$) or of the SEX×TREATMENT interaction ($F_{1,25}=0.01$ $p=0.663$).

3.5. PSA-NCAM

Although no overall effects were detected when we analyzed PSA-NCAM in the hippocampus, we found a small but significant increment in PSA-NCAM in CP-males when compared to their VH controls ($t_{12}=2.735$ $p<0.05$: Fig. 3). No significant differences were obtained in the females ($t_{12}=0.563$ $p=0.584$).

3.6. Hippocampal long-term potentiation

The LTP induced by HFS was similar in all four groups of rats, with no differences due to SEX ($F_{1,13}=0.310$ $p=0.587$), TREATMENT ($F_{1,13}=0.490$ $p=0.496$) or the interaction of these two factors ($F_{1,13}=0.542$ $p=0.475$) (Fig. 4).

4. Discussion

In this report we have found that a chronic periadolescent cannabinoid treatment only produces a marginal effect on adult anxiety (anxiolysis, as revealed by the reduced frequency of grooming behavior and decreased time in closed arms in the EPM). Like recognition memory when measured in the novel object paradigm, adult performance in the water maze was not generally affected by the treatment. We only detected a significant effect of the treatment in the percentage of failed trials which were fewer in CP-exposed animals. Male rats outperform females, both in the reference and working memory versions of the task. Interestingly, in the visible platform version of the water maze we observed some evidence that might indicate altered attention processing. However, there was a greater accumulation of PSA-NCAM in the hippocampus of adult males exposed to CP during periadolescence than in controls, which was not related to changes in hippocampal LTP. Cannabinoid exposure did not affect plasma corticosterone or estradiol levels.

Table 1
Plasma corticosterone (ng/ml) and estradiol (pg/ml) concentrations.

	Corticosterone		Estradiol	
	VH	CP	VH	CP
Males	280.97 ± 30.95	333.99 ± 10.10	10.90 ± 1.31	10.42 ± 1.71
Females[a]	498.62 ± 41.61	552.30 ± 63.19	21.30 ± 4.32	18.66 ± 2.35

Table shows the mean ± standard error of the mean of corticosterone and estradiol plasma concentrations. There was a general effect of the SEX variable showing that females had higher plasma concentrations of both hormones than males.
[a] Significant effect of SEX ($p<0.01$).

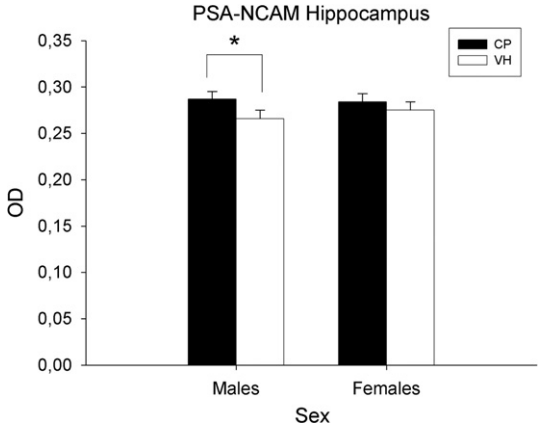

Fig. 3. Levels of PSA-NCAM in the hippocampus revealed by ELISA. CP-males have significantly higher expression of this protein in their hippocampus. *Significant difference as compared to VH ($p<0.05$).

There is little information available regarding the long-term effects of cannabinoid exposure and the relevant data that have been published are somewhat controversial (Rubino et al., 2008). As far as anxiety is concerned, human epidemiology studies suggest an association between adolescent use of cannabis and the subsequent development of anxiety disorders (Hayatbakhsh et al., 2007). However, the data obtained from experimental animals are unclear and different results have been reported depending on the agonist used and the specific period when cannabinoid treatment is administered. Accordingly, chronic treatment with the cannabinoid agonist CP 55,940 from P35 to P45 induces a decrease in the level of emotionality/anxiety in the open field test and the EPM (Biscaia et al., 2003). Additionally, if CP treatment is expanded (P30–P51) rats exhibit less anxious behavior in the social interaction test (O'Shea et al., 2006, 2004). Interestingly, when THC was administered chronically during the same period as in Biscaia's report (P35–P45), no long-lasting consequences on adult anxiety were observed (Rubino et al., 2008). These divergent findings may reflect the different potencies of the agonists (CP being a full agonist while THC acts as a partial agonist) or the specific maturational stage of the endocannabinoid and related neutransmission systems. Therefore, it seems that the time period and the agonist used in the experiments play a crucial role in explaining the results obtained in each set of experiments. In our case, while we used CP 55,940 as the agonist (same as in Biscaia's work), it was administered earlier (P28–P38) and did not produce any robust change in anxiety responses. However, since the rats that received CP 55,940 spent less time in the closed arms of the EPM and exhibited less frequent grooming behavior, it could be argued that a subtle modulation of basal anxiety-like behavior may have occurred; nonetheless, because corticosterone levels were not affected by cannabinoid exposure, this effect on adult anxiety is likely to be weak. Interestingly, periadolescent administration of a standard (1.2 mg/kg) dose of WIN 55,512-2 provoked a higher anxiety when rats were tested as adults as revealed by the reduced number of rearings and less time spent in the center of an open field (Schneider et al., 2005). Again, the discrepancies between this report and our results (a slight anxiolysis in our experiments and increased anxiety in Schneider's experiments) could be due to the different agonists used or the duration of the treatment (P15–P40 in Schneider's experiments and P28–P38 in our case).

Another possible explanation for the different results obtained in the work described above relies on the time elapsed between the end of the chronic treatment and the beginning of behavioral testing. For instance, in the experiments by O'Shea et al. the test was performed 23 days after the treatment (O'Shea et al., 2004) while in Biscaia's report 37 days lapsed between treatment and testing (Biscaia et al., 2003) and in our case, tests began 59 days after the treatment.

It may also be suggested that the effects found in the above-mentioned studies could be task-dependent, given that similar results

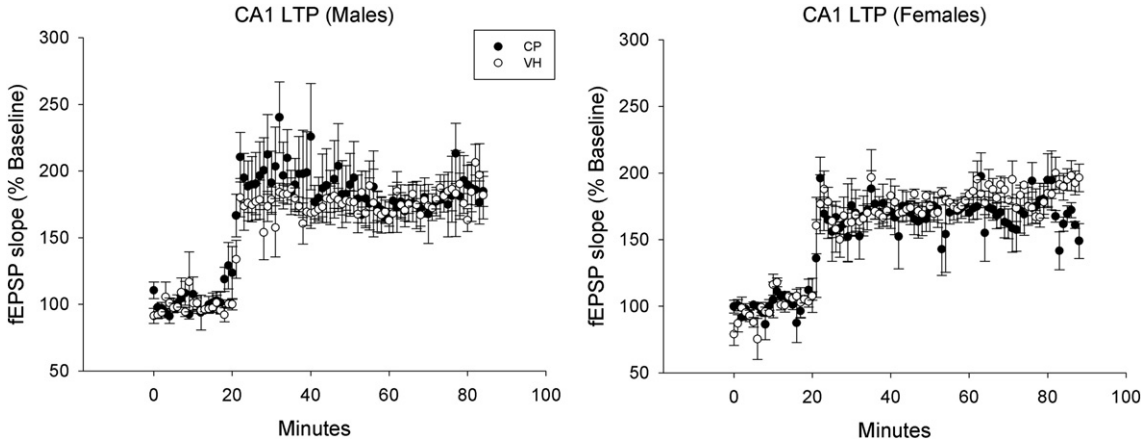

Fig. 4. Hippocampal LTP in male and female rats exposed to CP or VH. Neither SEX nor TREATMENT affected adult CA1-LTP.

have been obtained with the social interaction test and the EPM (Biscaia et al., 2003; Rubino et al., 2008) but a lack of effect was reported in the emergence test (O'Shea et al., 2006). Moreover, while the EPM was used in our experiments, Schneider et al. studied anxiety with an open field.

With regards to cognitive function, the data available seem to be more consistent. Human studies dealing with the developmental effects of cannabis use on adult cognition are rare, but it has been shown that adolescent cannabis use might have residual deleterious effects on adult attention (Ehrenreich et al., 1999; Solowij and Michie, 2007) and working memory (Schwartz et al., 1989). Experimental data show that adolescent cannabinoid treatment induces differential effects on adult learning and memory processes depending on the agonist used, the treatment regime and the task employed to assess the animal's cognitive performance (Rubino et al., 2008). In this sense, adolescent exposure to increasing doses of CP 55,940 leads to impaired working memory in adult rats as measured in the object recognition paradigm (O'Shea et al., 2006, 2004). Similarly, chronic treatment with the cannabinoid agonist WIN 55,512-2 during adolescence provokes deficits in adult object recognition memory, while this was not the case if the treatment was administered in adulthood (Schneider and Koch, 2003, 2007). Interestingly, THC exposure in adolescence does not appear to provoke any consistent long-lasting impairment in water maze performance, neither in adult males nor females (Cha et al., 2007, 2006), although it does affect working memory measured with the novel object paradigm (Quinn et al., 2007). Under our conditions, there is no evidence for long-lasting impairment in water maze performance as a consequence of periadolescent cannabinoid exposure, confirming to some extent these earlier findings (Cha et al., 2007, 2006). However, we did detect a marginal effect on adult reference memory as revealed by the fewer failed trials of CP animals, nonetheless, the main indexes of water maze performance were not affected.

It is noteworthy that the shorter latency of CP-treated females in finding the platform in the visible platform task on the first day disappears on the second day, possibly indicating a change in attention rather than an improvement in sensory-motor capacities in this group of animals. It might be argued that when having to pay attention to novel stimuli, such as the flag signaling the location of the platform during the first day of the test, CP-treated female rats perform better. However, this effect might not be evident when the novelty of the situation disappears, for example on the second day of the task. Given the intrinsic limitations of the water maze to examine these cognitive processes, this issue should be further explored with more standardized tasks to study attention.

Interestingly, we found no deficits in adult working memory in both the water maze and the novel object test. The failure to identify an impairment in working memory is quite surprising given that it has consistently been shown that chronic THC administration during adolescence impairs working memory in a persistent manner (O'Shea et al., 2006, 2004; Quinn et al., 2007). This discrepancy may be due to methodological issues, particularly with regards to the novel object paradigm and in relation to the inter-trial interval between the first and second exposure to the objects. We have used a short interval (30 min) while in the aforementioned studies this interval ranged from 1 to 48 h. Thus, it seems that the impairment induced by cannabinoids could be so subtle that it is only noticeable when longer and more demanding intervals are introduced. With an easier task, like the one used here, chronic CP 55,940 administration does not seem to produce any alteration and interestingly, this lack of effect is also relevant to spatial working memory as measured in the water maze. It is noteworthy however, that our main interest when employing the novel object task was to assess non-spatial working memory and because longer intervals are more likely to require reference memory we decided to restrict our analysis to this short interval. With regards to the significant sex differences found in spatial reference and working memory, the fact that males outperform females is consistent with previous data in rats, although the influence of strain and training protocols should not be disregarded (Jonasson, 2005).

The only biochemical effect observed with our treatment, was the enhanced expression of PSA-NCAM in the hippocampus of adult males that received CP during periadolescence. Indeed, since the total amount of NCAM and its 180, 140 and 120 isoforms, were not altered by the treatment or between the sexes (unpublished observations), it seems that these increased PSA-NCAM levels are due to a higher polysialylation of NCAM. This is quite interesting since it has been reported that acute CB1 receptor activation in adult animals transiently decreases PSA-NCAM expression in the dentate gyrus of the hippocampus (Mackowiak et al., 2007). Thus, the influence of the cannabinoid system on PSA-NCAM expression appears to depend on the age, the sex and the type of cannabinoid exposure (acute or chronic).

Despite the up-regulation of hippocampal PSA-NCAM in CP-males, there was no enhanced LTP in the CA1 field of the hippocampus in any of the animals exposed to the agonist. Given that we have not performed a detailed anatomical analysis of PSA-NCAM expression in the hippocampus of our animals, we cannot rule out the possibility that the abovementioned up-regulations in PSA-NCAM expression could be circumscribed to other hippocampal areas different from the CA1 field (such as the dentate gyrus, as described in Mackowiak's report (Mackowiak et al., 2007)). This could explain to some extent the lack of effect of the chronic treatment on CA1-LTP. Nonetheless, although PSA-NCAM is important in the induction of LTP, this molecule is not the only participant in the plasticity processes; therefore, an additional orchestrated change in other plasticity-

related systems, such as the glutamatergic, could also be necessary for an LTP alteration following CB1 agonist exposure. In this respect, the only study to our knowledge aimed at ascertaining the long-term effects of chronic THC exposure on adult LTP (Mereu et al., 2003), prenatal exposure to THC produced memory deficits linked to dysfunction of hippocampal LTP and glutamate release. According to these data, it would again seem that the time of cannabinoid exposure and the potency of the agonist employed are key factors to explain the long-term physiological and behavioral effects of adolescent cannabinoid exposure.

Taken together, our results show that exposing animals to the cannabinoid agonist CP from P28 to P38 does not produce a significant deficit in adult reference or working memory. There is increased hippocampal PSA-NCAM expression in CP-males, but this was not related to changes in CA1 synaptic plasticity (LTP). Anxiety responses of CP-treated animals were comparable to those of the controls, although a slight anxiolysis was suggested by the reduced percentage of time spent in the closed arms of the EPM and the less frequent grooming behavior in CP-treated rats. No changes in adult plasma corticosterone are induced by exposure to CP and the levels of estradiol are also unaffected by the treatment, both facts being consistent with the lack of robust alterations in cognition or emotion. We suggest that the long-term effects of cannabinoids depend on the potency of the agonist employed and more importantly, the specific time frame in which cannabinoid exposure takes place. The enduring and pervasive effects of cannabinoids seem then to be restricted to the pubertal and adolescent stages, but not periadolescence. Moreover, it should also be kept in mind that our behavioral, electrophysiological and biochemical analyses were performed within a specific time frame (beginning at P100) and that other phases of the adulthood period should also be tested.

In summing up, further research is needed to clarify the precise windows of vulnerability to the deleterious effects of cannabinoids on neural, cognitive and emotional development.

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