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Changes in Hippocampal Morphology and Neuroplasticity Induced by Adolescent THC Treatment are Associated With Cognitive Impairment in Adulthood

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ABSTRACT: Marijuana and hashish are the illicit drugs most frequently used by human adolescents. Given the continued neurodevelopment throughout adolescence, adolescents may be more vulnerable than adults to certain neural consequences of heavy marijuana use. This study aimed to assess whether an experimental model of adolescent chronic exposure to Δ^9-tetrahydrocannabinol (THC), may induce lasting effects on learning and memory. Adolescent rats have been treated with THC or its vehicle from 35 to 45 postnatal days (PND) and left undisturbed until their adulthood (75 PND) when aversive and spatial memory was assessed using the passive avoidance and radial maze tasks. No alteration was found in aversive memory, but in the radial maze THC pretreated animals exhibited a worse performance than vehicles, suggesting a deficit in spatial working memory. To correlate memory impairment to altered neuroplasticity, level of marker proteins was investigated in the hippocampus, the most relevant area mediating spatial memory. A significant decrease in the astroglial marker glial fibrillar acid protein was found as well as in pre- and postsynaptic protein expression (VAMP2, PSD95) and NMDA receptor levels in pretreated rats. To parallel these changes to alteration in dendritic morphology, Golgi-Cox staining was performed in the hippocampal dentate gyrus. Pretreated rats had a significantly lower total dendritic length and number than vehicles, as well as reduced spine density. Our data suggest that THC pretreated rats may establish less synaptic contacts and/or less efficient synaptic connections throughout the hippocampus and this could represent the molecular underpinning of the cognitive deficit induced by adolescent THC treatment. © 2009 Wiley-Liss, Inc.

KEY WORDS: cannabinoids; adolescence; spatial memory; synaptic markers; Golgi-Cox staining

INTRODUCTION

Cannabis use begins commonly in adolescence as youths aged 12–17 constitute about two thirds of the new cannabis users (SAMHSA, 2004). Given the continued neurodevelopment throughout adolescence, adolescents may be more vulnerable than adults to certain neural consequences of heavy marijuana use. Although development of overall brain size is largely complete by age 5 (Durston et al., 2001), specific structural and functional changes continue into adolescence, leading to greater cognitive efficiency. Gray matter volumes decrease, in part due to synaptic pruning as unnecessary neural connections are eliminated (Huttenlocher, 1990; Huttenlocher and Dabholkar, 1997; Durston et al., 2001), and white matter volumes increase as myelination progresses (Durston et al., 2001, Paus et al., 2001). Since the endocannabinoid system plays an important role during the different stages of brain development, as cannabinoids influence the release and action of different neurotransmitters and promote neurogenesis (Viveros et al., 2005), exposure to cannabinoids during this developmental phase may conceivably lead to subtle but lasting changes in the brain and behavior. Despite this prevalence of marijuana use during adolescence, few studies have examined its impact on adult behavior.

We recently demonstrated that Δ^9-tetrahydrocannabinol (THC) chronic administration in adolescent rats induces subtle but lasting alterations in the emotional circuit ending in depressive-like behavior in adulthood (Rubino et al., 2008). This effect was observed only in females, male rats presenting neither behavioral, nor biochemical parameters of depression. Beside the emotional profile, other brain functions could be altered by heavy marijuana exposure during adolescence, for example cognitive behavior. The acute effect of cannabis on learning and memory is well-known (Riedel and Davies, 2005; Ranganathan and D'souza, 2006), however literature regarding long-term cognitive effects of adolescent cannabinoid exposure is scarce and not always in accordance, depending on the compound administered, the treatment paradigm used and the cognitive task performed. Adolescent exposure to THC, the natural cannabinoid agonist, has been shown to induce both impaired memory (Quinn et al., 2008), and no lasting learning deficits in adult rats (Cha et al., 2006, 2007). However, when synthetic cannabinoid agonists were used in adolescent rats, impaired recognition memory was observed in adulthood (Schneider and Koch, 2003, 2007; O'shea et al., 2004, 2006).

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The current study therefore aimed to assess whether adolescent exposure to high THC doses may induce lasting effects on learning and memory in male rats. Different forms of memory (aversive and spatial) were assessed using passive avoidance and radial maze tasks. Besides the behavioral aspect, we were mainly interested in investigating the cellular and molecular underpinnings of the observed phenotype. To correlate cognitive impairments to altered neuroplasticity, level of proteins mainly involved in synaptic plasticity was investigated in the most relevant area for learning and memory, the hippocampus. Finally, to parallel changes in neuroplasticity to alteration in dendritic morphology, we performed Golgi-Cox staining.

MATERIALS AND METHODS

Drugs

THC, a generous gift from GW Pharmaceutical (Salisbury, UK), was dissolved in ethanol, cremophor, and saline (1:1:18).

Animals and Treatment

Male Sprague-Dawley rats (postnatal days, PND, 28) were obtained from Charles River, Calco, Italy. They were housed in groups of five in standard conditions of temperature and humidity under a 12-h light/12-h dark cycle with ad libitum access to food and water. Rats were allowed to acclimate in their new environment for 1 week before the start of the treatment. The treatment began at PND 35 and lasted until PND 45, as already published (Rubino et al., 2008). During this period, rats received increasing i.p. doses of THC twice a day (2.5 mg/kg PND 35–37; 5 mg/kg PND 38–41; 10 mg/kg PND 42–45) or its vehicle. Experiments were carried out in strict accordance with the guidelines released by the Italian Ministry of Health (DL 116/92 and DL 111/94-B), and the European Community directives regulating animal research (86/609/EEC). All efforts were made to minimize the number of animals used and their suffering.

Behavioral Tests

Behavioral testing started at 75 days of age.

Passive avoidance

The step-through type passive avoidance task was used as previously described (Braida et al., 2004). Briefly, the apparatus consisted of two compartments, one light and one dark, connected via a sliding door. In the acquisition trial, each rat was placed in the light compartment and allowed to enter the dark compartment; the time (in s) taken to do so was recorded. Once the rat was in the dark compartment, the door was closed and an electric shock (0.8 mA for 5 s) delivered. The animal was then returned to the home cage. The retention trial was carried out 24 h after, by positioning the rat in the light compartment and recording the time taken to enter the dark compartment (retention latency). An increased retention latency indicates that the animal has learned the association between the shock and the dark compartment. During the retention trial, a cut-off time of 300 s was used.

Radial maze

Spatial memory, was studied in a eight-arm radial maze, as previously described (Sala et al., 1991). Briefly, the wooden maze had an octagonal center platform with eight arms separated by Plexiglas guillotine type doors. A food pellet, as reinforcer, was located at the end of each arm. Access to the arms was controlled by eight pneumatically operated sheet metal guillotine doors. Rats were kept at 85% of their free-feeding body weight for the duration of the study. After 3 days of free exploration, performance in the maze was tested by placing each animal on the platform of the maze with all the doors closed. After a 1 min acclimatization period, all the doors were simultaneously opened and each animal was trained to complete the maze until it either successfully visited all eight arms or 10 min had elapsed. During each session, spatial working memory was scored on the basis of the total number of errors and the number of correct choices before the first error and time to complete the maze. Total number of errors during the 16-day period was expressed as total area under the curve (AUC). Training, at the rate of one session/day, continued until the rats had fulfilled the criterion of entering seven different arms out of the first eight choices on five successive days.

Biochemical Studies

All the biochemical studies were performed on animals that underwent the behavioral tests.

[3H]MK801 receptor autoradiographic binding

Rats were decapitated and brains quickly removed. Twenty micron coronal sections were cut on a cryostat and thaw mounted on gelatin-coated slides. Sections were briefly dried at 30°C and stored at −80°C until they were processed for autoradiographic binding studies. NMDA receptor binding study was performed according to Newell et al. (2007). Autoradiograms were generated by exposing the dried sections for 4 weeks to Hyperfilm 3H (GE Healthcare, Milan, Italy).

Image analysis

Autoradiograms were analyzed using Image-Pro Plus 5.0 (MediaCybernetics, Silver Spring). Each area of both sides of the brain was traced with a mouse cursor using the Paxinos and Watson (2005) atlas as reference, and light transmittance was determined as the gray level. The gray level calculated after subtraction of the film background density was established within the linear range, determined using tritium standards (3H Microscales, GE Healthcare).

Immunoblotting

Rats were decapitated and brains quickly removed. The cerebral areas (prefrontal cortex and hippocampus) were obtained

within a few minutes by regional dissection on ice according to Heffner et al. (1980), and immediately frozen in liquid nitrogen and stored at −80°C until processing.

For total protein lysate, each brain region was homogenized in an appropriate volume of ice-cold Buffer A (10 mM Hepes pH 7.5, 1.5 mM MgCl2, 10 mM KCl, 2 mM DTT, 1 mM PMSF, 1 mM EDTA, 1 mM EGTA, 2 mM sodium orthovanadate, 50 mM NaF, 10 mM sodium pyrophosphate, 0.5% Triton, 5 μg/ml aprotinin, and 5 μg/ml leupeptin) and centrifuged at 13,000 rpm at 4°C for 3 min. The supernatant was used as total protein lysate and protein concentrations were determined according to the Micro-BCA assay kit (Pierce, Rockford, IL).

Equal amounts of protein from the lysate (5 μg for all blottings, except for the PSD95 and VAMP2 for which 20 μg were loaded) were resolved by 8–12% SDS-PAGE, blotted to polyvinylidene difluoride membrane, blocked for 2 h at room temperature in 5% dry skimmed milk in PBS before incubation overnight at 4°C with the primary antibody. Primary antibodies were: (I) polyclonal antisynaptophysin (1:5000; AbCam, Cambridge, UK); (II) monoclonal anti-PSD95 (1:2000; AbCam); (III) polyclonal antiglial fibrillar acid protein (GFAP) (1:2000; Sigma, Milan, Italy); (IV) monoclonal anti-βIII-tubulin (1:2000; Synaptic System, Gottingen, Germany) and (V) monoclonal anti-VAMP2 (1:1000; Synaptic System). Bound antibodies were detected with horseradish peroxidase linked antirabbit or anti- mouse antibody (1:5000; Chemicon International, Temecula, CA) for 1 h at room temperature and developed using an enhanced chemiluminescence reagent (GE Healthcare). To control for protein loading, the membranes were then stripped with Restore Western Blot Stripping Buffer (Pierce) for 15 min at 37°C and reprobed with a monoclonal antibody raised against β-actin (1:5000; Sigma). The relevant immunoreactive bands were quantified with scanning densitometry using Scion Image software. Expression of the proteins was normalized to that of β-actin and to allow comparison between different autoradiographic films, the density of the bands was expressed as a percentage of the average of control.

Morphological Studies

Golgi impregnation

The modified Golgi-Cox procedure of Ramon-Moliner (1958) was used to stain the brains with slight modifications (Bartesaghi and Ravasi, 1999). After embedding in 8% celloidin, the blocks were cut along the sagittal plane in slices 80 μm thick. Sections were mounted with DPX on glass slides in serial order.

The granule cells were classified in superficial or deep based on their position within the granule layer. Neuron morphometry was analyzed in 100 granule cells of adult animals. Only well-impregnated neurons were chosen for the histological analysis. For each animal 10 granule cells were drawn, according to the aforementioned conditions. The spines on the dendrites of the granule cells were counted using a 100× oil immersion objective lens.

Measurements

The following stereology system was used: (i) light microscope (Leitz) equipped with a motorized stage and focus control system; (ii) color digital videocamera attached to the microscope; (iii) Image Pro Plus (Media Cybernetics) with the StagePro module for controlling the motorized stage in the x, y and z directions, as primary software. A dedicated software ("Mappa Neuroni" version 3.21.00, Immagini and Computer, Milan, Italy) was used to trace granule cell dendritic arbor from live images (magnification: 500×) acquired with the video camera, and at the end of the drawing session the software automatically provides the measurements of the dendritic branching processes reported below (measurements 1–4).

1. Mean number of branches of different order
2. Total number of branches
3. Mean length of branches of each order
4. Total dendritic length
5. Soma circumference
6. Spine density. Spine density values were obtained from dendritic segments proximal and distal to the cell body. For each neuron eight segments were analyzed (four proximal and four distal). For each animal, spines were counted in four neurons. These neurons belonged to the population used for measurements 1–5. Spine density was expressed as number of spines per 30 μm dendrite.

Statistical Analysis

Behavioral data were expressed as mean ± SEM and analyzed by Student's *t*-test, one way analysis of variance (ANOVA) for repeated measures followed by Tukey's post hoc comparisons, or two way ANOVA followed by Bonferroni test. The Chi-square test was used for nonparametric results. The AUC of the total number of errors to complete the maze, was calculated. Biochemical data were mean ± SEM and analyzed by Student's *t*-test. All statistical analyses were done using software Prism 4 (GraphPad, Software Inc., San Diego, CA). Pearson's correlation coefficients were calculated to determine relationships between behavior and biochemical variables. Statistical significance was taken as $P \leq 0.05$.

RESULTS

Behavioral Findings

Passive avoidance task

The result in the passive avoidance task is shown in Figure 1. Two-way ANOVA showed significant overall effects of time (Pre-Post) ($F_{1,36} = 58.02$ $P < 0.0001$) but not of treatment or interaction (treatment × time). The posthoc analysis showed that the training latency was not different between vehicle and THC pretreated group whereas the mean retention time during

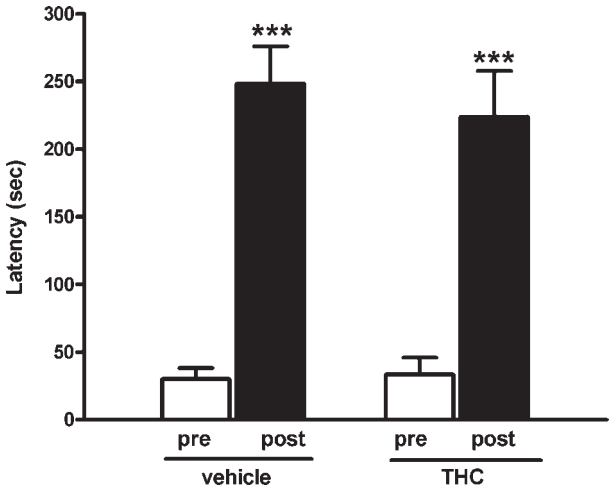

FIGURE 1. Effect of pretreatment with THC during adolescence in male rats in the passive avoidance task. Data are expressed as mean ± SEM step-through latency during PRE and POST. At least $n = 10$ for each group. $^{***}P < 0.001$ as compared to the corresponding PRE conditioning (ANOVA followed by Tukey's test).

testing, carried out 24 h afterwards, was significantly increased in both groups. The number of animals with reduced re-enter latency was quite similar in THC group compared to vehicle (26 vs. 20%), so no significant difference was obtained.

Radial maze

Radial maze performance, in terms of mean total number of errors, days to reach the criterion and percentage of animals that reached the criterion over 16 days, is shown in Figure 2. Two-way ANOVA showed significant overall effects of treatment ($F_{1,288} = 5.70$ $P < 0.01$) and time ($F_{15,288} = 4.00$ $P < 0.0001$), while no significant interaction (treatment × time) was obtained.

Vehicle group exhibited a better performance, in comparison with THC-pretreated animals, as shown by decreased number of errors starting from day 10 (Fig. 2). The calculated AUC (Fig. 2, inset) revealed a significant increase of this parameter in THC-pretreated animals in comparison with vehicle ($P < 0.05$). Considering the number of days taken to reach the criterion (Fig. 2), THC-pretreated group needed significantly more days than vehicles ($P < 0.05$, Student's t-test), and accordingly, the percentage of rats reaching the criterion within 16 days in THC-pretreated group was lower than in vehicles (Fig. 2, $P < 0.05$, Chi-square test).

Biochemical Findings

To examine whether cognitive impairment could be associated with altered neuroplasticity, quantitative immunoblotting was employed to determine the level of βIII-tubulin, GFAP, synaptophysin, VAMP2 and post synaptic density protein 95 (PSD95) in the hippocampus, the main brain region modulating spatial memory.

βIII-tubulin was evaluated as a neuronal marker, as in the adult central nervous system (CNS), the distribution of class III β-tubulin is almost exclusively neuron specific (Svendsen et al., 2001; Dràberova et al., 2008). Exposure to THC did not modify the level of βIII-tubulin (Fig. 3).

GFAP represents the principal 8–9 nm intermediate filament in mature astrocytes of the CNS (Xu et al., 1999). Levels of this protein were significantly decreased in the hippocampus of THC-pretreated rats ($P < 0.01$ between THC-pretreated and vehicle, Fig. 3).

Synaptophysin is an abundant component of the transmitter vesicle membrane, and is commonly used as a presynaptic marker (Sudhof and Jahn, 1991; Janz et al., 1999). Adolescent exposure to THC did not modify the levels of synaptophysin (Fig. 3).

VAMP2/synaptobrevin is a short and very abundant synaptic vesicle protein that interacts with syntaxin-1 and SNAP-25 constituting the SNARE complex, the biochemical intermediate essential for vesicular transport and/or fusion processes (Sollner et al., 1993; Deàk et al., 2006). As shown in Figure 3 adolescent exposure to THC significantly reduced the expression of VAMP2 ($P < 0.05$).

PSD95 is a scaffolding protein localized to the postsynaptic density of asymmetric synapses (Hunt et al., 1996; Glantz et al.; 2007), that anchors receptors and downstream signaling

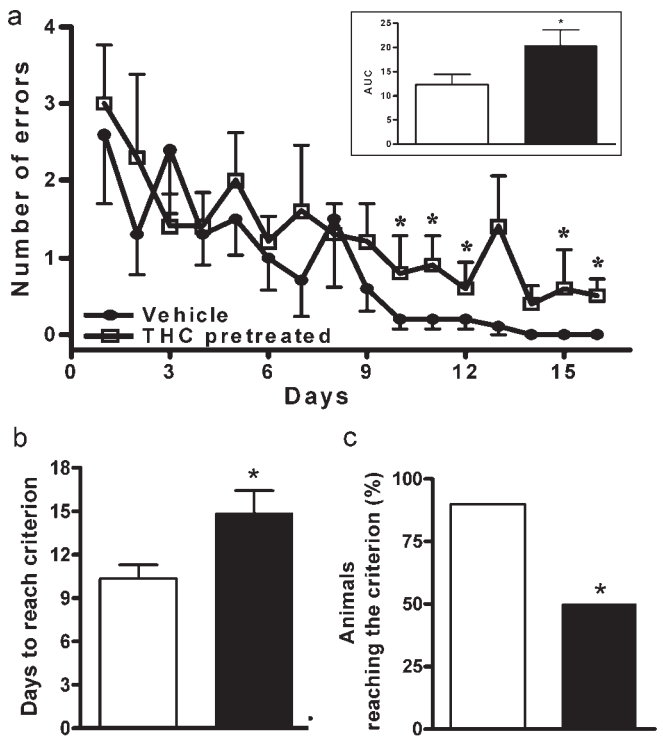

FIGURE 2. Effect of pretreatment with THC or vehicle during adolescence in male rats on radial maze performance, evaluated for 10-min daily sessions, starting from day 1 to 16, on mean ± SEM of total number of errors (a) and corresponding AUC (inset); (b) number of days (mean ± SEM) taken to reach the criterion; (c) percentage of animals reaching the criterion at day 16th. At least $n = 10$ for each group. $^{*}P < 0.05$ as compared with corresponding vehicle group.

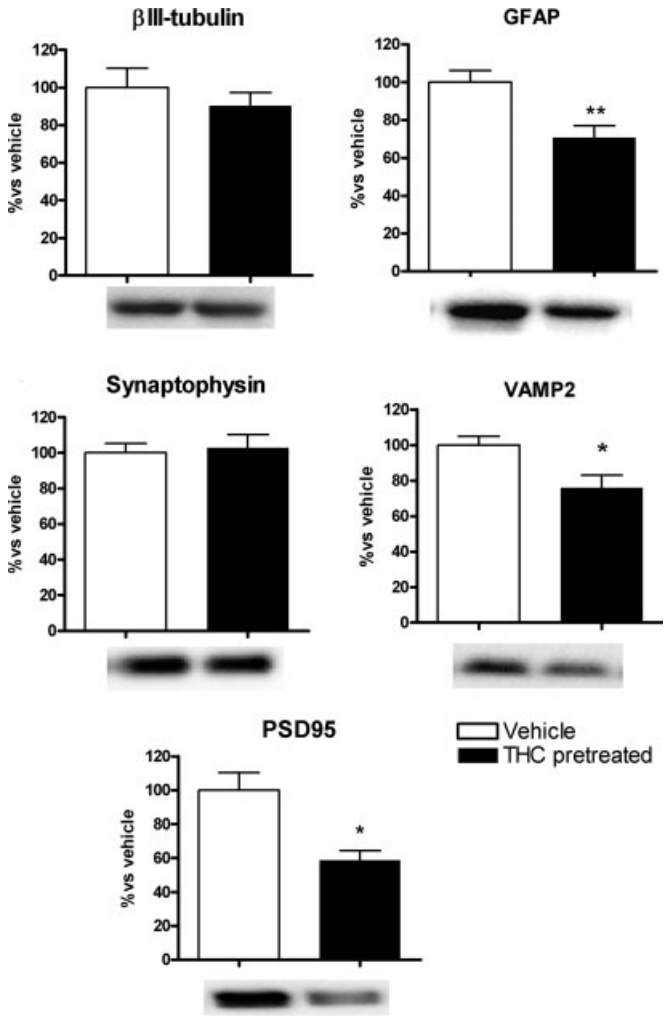

FIGURE 3. Effect of THC pretreatment during adolescence on betaIII-tubulin, GFAP, synaptophysin, VAMP2 and PSD95 protein levels in the hippocampus. In densitometric analysis, the results are expressed as the percentage of protein immunoreactivity vs. vehicle and are the mean ± SEM of at least four animals. Asterisks indicate significant differences between the vehicle group and the THC pretreated group (*$P < 0.05$, **$P < 0.01$, Student's *t*-test).

molecules to the postsynaptic density (Kim and Sheng, 2004) and is widely used as a marker for synaptic sites (Okabe et al., 1999). THC pre-exposure induced a significant reduction in PSD95 immunoreactivity in adult hippocampus ($P < 0.05$ between THC pretreated and vehicle, Fig. 3). Perhaps, the most important role for PSD95 is to anchor and organize the NMDA receptor and other synaptic proteins in the postsynaptic density (Sheng and Pak, 1999; Kim and Sheng, 2004) and interactions between PSD95 and the NMDA receptor may be important for synaptic plasticity, determining the size and strength of synapses (Hata and Takai, 1999). Thus we finally evaluated NMDA receptor levels on coronal brain sections from adult rats exposed to THC during adolescence by autoradiographic binding studies with [3H]MK801. Rats pretreated with THC showed a slight but significant reduction in receptor binding levels specifically in the hippocampus ($P < 0.05$, ↓13%, Table I), without affecting NMDA levels in amygdala, chosen as a control area (Table I). A more detailed analysis revealed that NMDA receptor levels were decreased in all subregions of hippocampus ($P < 0.01$, ↓15% CA1; $P < 0.05$, ↓17% CA2; $P < 0.01$, ↓15% CA3; $P < 0.05$, ↓11% DG). Regardless the slight alterations, a significant correlation exists between behavioral performance and NMDA receptor content, as rats having higher NMDA receptor density made significantly fewer errors in the radial maze (Pearson's correlation coefficient $r = -0.9475$, $P = 0.0041$).

No significant alterations were observed in these same markers of neuroplasticity when they were measured in the prefrontal cortex (data not shown).

Morphological Findings

Considering behavioral and biochemical results and the well known concept that hippocampus is the most relevant brain region for spatial memory (Olton et al., 1979; Morris et al., 1982; Rawlins and Olton, 1982; McGregor et al., 2004; Bird and Burgess, 2008) the morphological analysis was performed only in the hippocampus. The dentate granule cells (Fig. 4) are the first element of the trisynaptic circuit formed by the perforant path fibers from the entorhinal cortex-dentate granule cells-field CA3 pyramidal neurons-field CA1 pyramidal neurons. Processing of signals by this circuit is thought to be essential for the establishment of long-term memory (Amaral and Witter, 1995; Guidi et al., 2006), therefore adolescent exposure to THC may conceivably have induced alterations in the morphology of these cells. For this reason we performed a detailed morphological analysis of dendritic structure in the granule cells of the dentate gyrus (DG), using the modified Golgi-Cox procedure to stain the brain. As shown in Figure 4 THC pretreated rats had a significantly lower total dendritic length than vehicle ($P < 0.05$). This alteration was accompanied by reduction in total dendritic number ($P < 0.05$). Again, a significant correlation exists between total dendritic length and behavioral

TABLE 1.

Effect of THC Pretreatment During Adolescence on NMDA Receptor Expression in the Subregions of the Hippocampus

	NMDA receptor expression		
	Vehicle	THC pretreated	
Hippocampus	197.9 ± 4.6	171.6 ± 3.6*	↓13%
CA1	275.5 ± 6.8	234.5 ± 5.3**	↓15%
CA2	179 ± 7.0	148.2 ± 3.5*	↓17%
CA3	154 ± 3.2	131.3 ± 3.1**	↓15%
Dentate gyrus	169.2 ± 4.1	150.6 ± 3.1*	↓11%
Amygdala	74.11 ± 4.4	83.75 ± 4.1	

In densitometric analysis, the results are expressed as fmol/mg tissue and are the mean ± SEM of at least three animals (three sections/animal). Asterisks indicate significant differences between the control group and the THC-pretreated group (*$P < 0.05$, **$P < 0.01$).

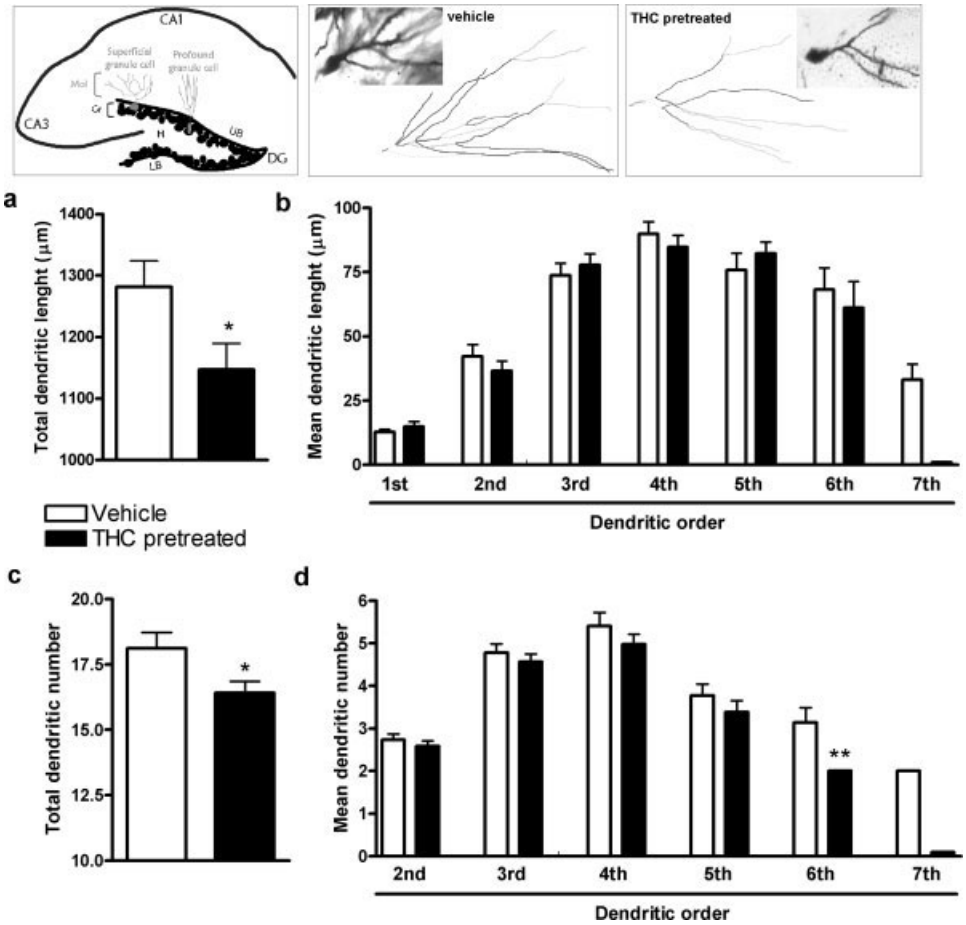

FIGURE 4. Upper panel: Camera lucida drawing of subregions and layers of the hippocampus. Abbreviations: DG, dentate gyrus; Gr, granule cell layer; H, hilus; LB, lower blade; Mol, molecular layer; UB, upper blade. Photomicrograph and software reconstruction of a Golgi Cox-stained granule cell in a vehicle animal and a THC pretreated animal. Lower panel: effect of THC pretreatment during adolescence on dentate gyrus morphology in terms of total dendritic length (a), mean dendritic length of branches of different order (b), total dendritic number (c), and mean dendritic number of branches of different order (d). The results are expressed as the mean ± SEM of five animals per group. Asterisks indicate significant differences between the control group and the THC pretreated group (*$P < 0.05$, **$P < 0.01$, Student's *t*-test).

performance (Pearson's correlation coefficient $r = -0.8155$, $P = 0.007$). Moreover a significant decrease in the mean number of dendrites of sixth order ($P < 0.01$) was found with a 25% reduction in the percentage of neurons having sixth order dendrites. None of THC pretreated animals had seventh order dendrites, whereas these were present in some neurons from vehicle animals. Quantitative analysis of spine density per 30 µm of dendrite revealed that there were significantly fewer spines in the THC pretreated group both on the proximal ($P < 0.0001$) and distal ($P < 0.01$) dendritic tree (Fig. 5). No differences were found in the soma circumference, neither between deep and superficial neurons (data not shown).

DISCUSSION

The main finding of our work is that chronic exposure to heavy THC during adolescence produced residual impairment in spatial working memory in adult animals paralleled by reduced levels of markers of neuroplasticity in the hippocampus and morphological alterations in the DG. We applied a protocol of heavy THC exposure since it has been reported the availability of more potent varieties of cannabis, termed sinsemilla or skunk (NIDA, 2005) that are 25 times stronger than the cannabis strains sold a decade ago. Although population consuming these strains does not represent the majority, it is increasingly evident that teenagers requiring drug treatment belong to the one smoking highly potent cannabis strains (Owen, 2007).

BEHAVIORAL FINDINGS

Adult rats exposed to THC during adolescence succeeded in simple passive avoidance learning, but showed a slight but significant impairment in learning behavior when facing complex

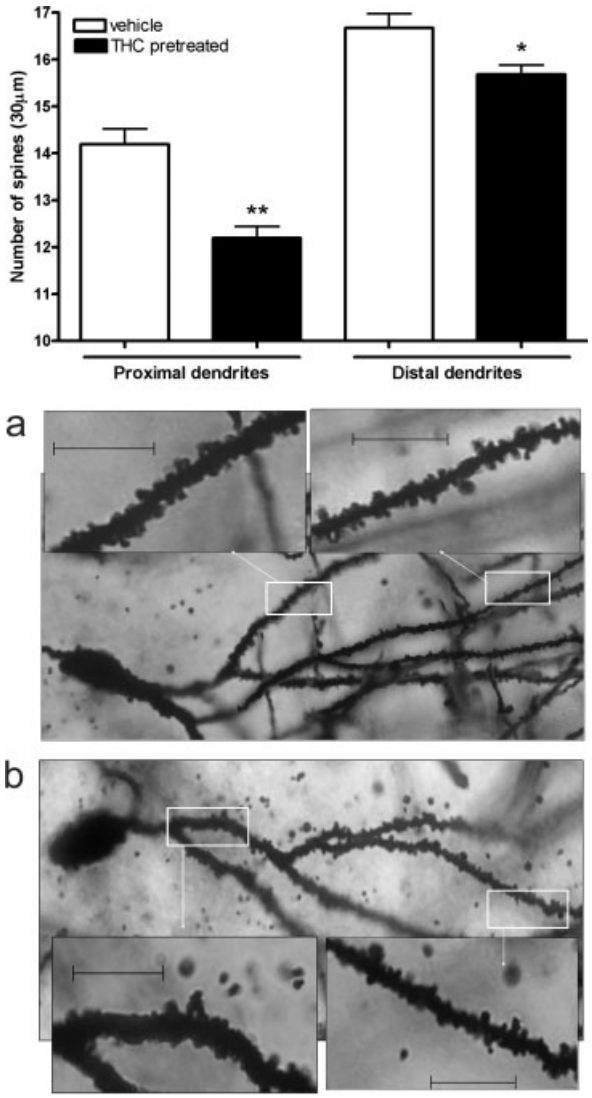

FIGURE 5. Effect of THC pretreatment during adolescence on dentate gyrus morphology in terms of spine density (number of spines per 30 μm) at proximal and distal levels of the dendritic tree (Upper panel). The lower panels are magnifications of proximal and distal dendritic regions in a vehicle animal (a) and a THC pretreated animal (b) (bars = 10 μm). The results are expressed as the mean ± SEM of five animals per group. Asterisks indicate significant differences between the control group and the THC pretreated group (*$P < 0.05$, **$P < 0.01$, Student's *t*-test).

learning paradigms, such as the radial maze. Indeed, in control animals spatial memory was consolidated over the 16-day trial period, with animals showing consistently fewer or even no performance errors as the trial progressed. This progressive improvement was not so clearly evident in animals treated with THC during adolescence. In contrast, Cha (2006, 2007) found no long-lasting significant effects on either spatial or nonspatial learning in rats that had been previously exposed to THC. The different treatment paradigm could account for this discrepancy since lower THC doses were used in Cha's study, suggesting that THC could be expected to affect performance only when administered with a heavier protocol. However, the finding of poorer memory performance in adult animals treated in adolescence with cannabinoid agonists was already reported by other authors (Schneider and Koch, 2003; O'shea et al., 2004, 2006; Quinn et al., 2008). Interestingly, adult chronic cannabinoid treatment had no long-lasting effects on cognitive function (Schneider and Kock, 2003; Quinn et al., 2008) supporting the idea of adolescents being specifically vulnerable to enduring adverse effects of cannabinoids. Literature in humans too, offers consistent evidence of greater neurocognitive deficits among marijuana users who initiated use early in adolescence, and suggests that the adolescent brain may be particularly vulnerable to the influence of heavy marijuana use (Pope et al., 2001; Jacobsen et al., 2004; for review see Solowij and Battisti, 2008, and Schweinsburg et al., 2008).

Summarizing, adolescent exposure to THC produced residual impairment in spatial working memory in adult rats. The observation that the same rats were not impaired in the passive avoidance task does not appear to be attributable to increased anxiety, since male rats treated with the same protocol during adolescence did not show any emotional alteration (Rubino et al., 2008). Considering the behavioral results and the well known concept that hippocampus is the most relevant brain region for spatial memory (Olton et al., 1979; Morris et al., 1982; Rawlins and Olton, 1982; McGregor et al., 2004; Bird and Burgess, 2008) we decided to perform the biochemical and morphological analysis only in this region.

BIOCHEMICAL FINDINGS

It is well documented that complex brain functions, such as learning and memory, require structural changes, involving either the production of new synapses or a reorganization of existing synapses (Bailey and Kandel, 1993; Moser, 1999) including alterations in proteins which are dynamically regulated at pre- and postsynaptic sites. Thus we tried to correlate cognitive impairments induced by adolescent exposure to THC to altered neuroplasticity in the hippocampus, by monitoring levels of both presynaptic and postsynaptic marker proteins. Since astrocytes play an active role in synaptic signaling (Carmignoto, 2000), the astrocytic component was also monitored by GFAP levels evaluation, in comparison to the neuronal component (βIII tubulin levels).

Interestingly, decreases in presynaptic (VAMP2) and postsynaptic (PSD95) proteins were found in the hippocampus of THC pretrated rats, together with a reduction in GFAP. VAMP2 is critically involved in synaptic vesicle fusion and neurotransmitter exocytosis (Wojcik and Brose, 2007). It has been shown that synapses deficient in VAMP2 exhibit a 10-fold decrease in spontaneous synaptic vesicle fusion and a similar decrease in sucrose-triggered fusion, but a more than 100-fold decrease in Ca^{2+}-induced fusion (Schoch et al., 2001), suggesting that VAMP2 is not required for synaptic fusion as such, but is essential for a normal rate of fusion upon stimulation (Schoch et al., 2001). Therefore, the decrease we observed in

hippocampal VAMP2 levels of THC pre-exposed animals might impact the efficacy of synaptic transmission.

Adult rats pre-exposed to THC also showed reduced PSD95 levels in the hippocampus. PSD95 mutant mice exhibit defects in synaptic plasticity and spatial learning despite the proper synaptic localization of NMDA receptors (Migaud et al., 1998). These results suggest that interactions between NMDA receptors and PSD95 family proteins are not required for synaptic targeting of NMDA receptors, instead these interactions are important in coupling the NMDA receptor to pathways that control bidirectional synaptic plasticity and learning (Migaud et al., 1998). Our finding of reduced hippocampal PSD95 in rats with impaired spatial memory further confirms the key role of this protein in synaptic events underlying cognitive functions.

Due to the close link between PSD95 and NMDA receptors, we then checked NMDA receptor level in the hippocampus of adult rats. The slight but significant reduction in the hippocampal NMDA receptor level in THC pre-exposed rats together with the decreased PSD95 expression suggest a decrease in NMDA receptor function and strengthen the hypothesis that hippocampal-dependent spatial learning in rats depends on the integrity of the glutamate receptor functionality (Davis et al., 1992).

Finally, we found a significant reduction in hippocampal GFAP levels in adult rats pre-exposed to THC. Recent works on glial cell physiology have revealed that glial cells, and astrocytes in particular, are much more actively involved in brain information processing than previously thought. Astroglia promote the synaptic efficiency of individual neurons (Boehler et al., 2007) and increasing evidence support a dynamic role for astroglia in normal and dysfunctional brains (Mong and Blustein, 2006; Seifert et al., 2006). Astrocytes play a key role in glutamate neurotransmission (Hertz and Zielke, 2004) and GFAP is critical for the dynamic response of astrocytes to neuronal signals (Weinstein et al., 1991). Therefore, the decrease in GFAP we observed could participate to the altered hippocampal neuroplasticity associated to cognitive deficit.

As a whole these data point to a slight but significant long-lasting impairment in concerted structural and functional plasticity of both neurons and glia in the hippocampus of THC pre-exposed animals, that could perturb the formation of spatial memory.

MORPHOLOGICAL FINDINGS

Since the DG plays an important role in learning and memory by processing and representing spatial information (Kesner, 2007) we tested whether chronic THC in adolescence might result in morphological changes in dentate granule cells. THC pretreated animals showed a significant change in dendrite morphology, having less dendrites in terms of both number and length, that fits well with the biochemical and behavioral picture obtained in these animals. Moreover, a significant decrease in spine density was also found. In apparent contrast with our results, Kolb et al. (2006) found that prior exposure to THC increased the length of the dendrites and the number of dendritic branches in the shell of the nucleus accumbens and in the medial prefrontal cortex, but not in the hippocampus. This disagreement might be due to different THC doses (low vs. high) but mainly to the different period of treatment (adulthood vs. adolescence) used in the two studies. In fact during adolescence brain is still developing and characterized by strong neuronal plasticity, with sprouting and pruning of synapses, myelinization of nerve fibers, changes in neurotransmitter concentrations and their receptor levels in brain areas essential for behavioral and cognitive functions (Rice and Barone, 2000). On the other hand, it was already reported that non stimulant drugs of abuse, such as opiates, are able to reduce total dendritic length limited to higher order dendritic branches when administered during brain developmental period (Ricalde and Hammer, 1990). Moreover Robinson et al. (2002) demonstrated that morphine persistently reduced the density of dendritic spines in granule cells of DG, suggesting a reorganization of brain circuits by the drug. Alterations in dendritic spine density can be considered evidence of changes in patterns of synaptic connectivity. It has been shown that, in adult animals, as the number of dendritic spines increases, so does the number of synaptic contacts (Harris and Kater, 1994). Furthermore, several studies have shown that increased dendritic spine density determined using Golgi-Cox staining is accompanied by an increased number of synapses per neuron assessed with electron microscopy (Woolley et al., 1990; Woolley and McEwen, 1992; Kolb et al., 1998). On these basis we might hypothesize that the picture we found in the DG morphology could imply a reduction in synaptic connectivity induced by adolescent exposure to THC associated with impaired neuronal plasticity, which in turn may contribute to cognitive deficits. Indeed it has been already speculated that preventing the formation of new synapses may contribute to the impairment of memory produced by cannabinoids (Kim and Thayer, 2001).

CONCLUSIONS

Adolescent exposure to THC induced spatial memory deficits in adult animals whose molecular underpinnings could be the establishment of less synaptic contacts and/or less efficient synaptic connections throughout the hippocampus. However, we did not investigate whether a similar picture was produced as a long-term consequence after chronic THC treatment in adult rats, and this would be extremely relevant in order to demonstrate whether adolescence represents a more vulnerable period than adulthood for THC long-term effects. Experiments are now in progress in our laboratory to assess this important issue, although literature from both animals and humans seems to suggest that adolescent heavy cannabis exposure could be associated with long-term impaired memory function.

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