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RESEARCH ARTICLE

Long-term behavioral and biochemical effects of an ultra-low dose of Δ^9-tetrahydrocannabinol (THC): neuroprotection and ERK signaling

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Abstract We have previously reported that a single injection of an ultra-low dose of delta-9-tetrahydrocannabinol (THC; the psychoactive ingredient of marijuana) protected the brain from pentylenentetrazole (PTZ)-induced cognitive deficits when applied 1–7 days before or 1–3 days after the insult. In the present study we expanded the protective profile of THC by showing that it protected mice from cognitive deficits that were induced by a variety of other neuronal insults, including pentobarbital-induced deep anesthesia, repeated treatment with 3,4 methylenedioxymethamphetamine (MDMA; "ecstasy") and exposure to carbon monoxide. The protective effect of THC lasted for at least 7 weeks. The same ultra-low dose of THC (0.002 mg/kg, a dose that is 3–4 orders of magnitude lower than the doses that produce the known acute effects of the drug in mice) induced long-lasting (7 weeks) modifications of extracellular signal–regulated kinase (ERK) activity in the hippocampus, frontal cortex and cerebellum of the mice. The alterations in ERK activity paralleled changes in its activating enzyme MEK and its inactivating enzyme MKP-1. Furthermore, a single treatment with the low dose of THC elevated the level of pCREB (phosphorylated cAMP response element–binding protein) in the hippocampus and the level of BDNF (brain-derived neurotrophic factor) in the frontal cortex. These long-lasting effects indicate that a single treatment with an ultra-low dose of THC can modify brain plasticity and induce long-term behavioral and developmental effects in the brain.

Keywords Cannabinoid · Neuroprotection · Preconditioning · Cognitive deficit · Extracellular signal–regulated kinase (ERK)

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Introduction

Cannabis, though considered a mild, "soft" drug without long-lasting negative effects, has been shown to cause long-term cognitive deficits in chronic users manifested as impairment in attention, memory or executive functions (Solowij et al. 1995; Block 1996; Ehrenreich et al. 1999; McHale and Hunt 2008; Abdullaev et al. 2010; Battisti et al. 2010; Lundqvist 2010; Coullaut-Valera et al. 2011). A meta-analysis that examined the non-acute effects of cannabis concluded that there might be decrements in the ability to learn and remember new information in chronic users (Grant et al. 2003). Other studies showed emotional effects such as anxiety, depression (Reilly et al. 1998; Troisi et al. 1998; Bovasso 2001; Patton et al. 2002; Gruber et al. 2009) and lack of motivation (Kouri et al. 1995). fMRI studies indicated that even when cognitive deficits are not observed, chronic users compensate by mobilizing more neuronal resources in order to perform the required cognitive function (Kanayama et al. 2004; Jager et al. 2006). Furthermore, MRI studies showed a reduction in white and gray matter in the cerebellum of chronic

cannabis users (Cohen et al. 2012; Solowij et al. 2011). Studies with human subjects may inevitably be hampered by various confounding factors such as drug residues, abstinence effects or methodological drawbacks (Solowij et al. 2002; Grant et al. 2003; Fried et al. 2005), while animal studies enable better controlled studies. Indeed, consistent findings of long-lasting cognitive deficits were demonstrated following chronic exposure of laboratory animals to cannabinoid drugs. Chronic exposure of rats to Δ^9-tetrahydrocannabinol (THC) resulted in persistent reduction in maze learning (Fehr et al. 1976; Stiglick and Kalant 1982a, 1983) and in differential reinforcement responding (Stiglick and Kalant 1982b). Repeated exposure of rats to cannabinoid agonists during perinatal, adolescent or early adult ages produced long-lasting deficits in working memory and social interaction (O'Shea et al. 2006) as well as in spatial learning (Rubino et al. 2009) and caused anxiety and depression in rats (Rubino et al. 2008; Bambico et al. 2010). Morphological changes in the hippocampus of rats that were chronically treated with cannabinoids, including neuronal death and reduced synaptic density and dendritic length of pyramidal neurons, were also reported (Scallet et al. 1987; Landfield et al. 1988; Scallet 1991; Lawston et al. 2000; Rubino et al. 2009).

In contrast to the above reports, other studies have suggested that cannabinoids may have neuroprotective properties [for reviews see (Guzman et al. 2002; Mechoulam et al. 2002; Sarne and Mechoulam 2005; van der Stelt and Di Marzo 2005)]. Acute administration of cannabinoid agonists was found to protect against global and focal ischemic damage (Nagayama et al. 1999), against ouabain-induced excitotoxicity (van der Stelt et al. 2001a, b), against severe closed head injury (Panikashvili et al. 2001; Mauler et al. 2003) and against MDMA (3,4 methylenedioxymethamphetamine) neurotoxicity (Tourino et al. 2010). Other studies suggested neuroprotective effects of cannabinoids in neurodegenerative pathologies such as multiple sclerosis, Alzheimer's, Huntington's and Parkinson's diseases (Gowran et al. 2011; Sagredo et al. 2011). It was even suggested that the endogenous cannabinoid system has a physiological role in neuroprotection (Guzman et al. 2001; Mechoulam et al. 2002; Marsicano et al. 2003; Karanian et al. 2007).

The neuroprotective properties of cannabinoids are attributed, among other factors, to their ability to suppress voltage-dependent calcium channels (Mackie and Hille 1992) and consequently to attenuate the release of glutamate (Shen et al. 1996). In vitro findings, however, have shown that very low concentrations of cannabinoids can potentiate, rather than suppress, calcium entry into cells (Okada et al. 1992; Rubovitch et al. 2002), thus suggesting that very low doses of cannabinoid drugs may be neurotoxic in vivo. Indeed, in recent studies we have shown that a single extremely low dose of THC (0.002 mg/kg, a dose that is 3–4 orders of magnitude lower than the doses that produce the known acute effects of the drug in mice) significantly deteriorated the cognitive performance of mice that were tested 3 weeks and up to 5 months following the injection, in various behavioral assays that assess different aspects of learning and memory (Tselnicker et al. 2007; Senn et al. 2008; Amal et al. 2010). These findings led to the idea that this ultra-low dose of THC, which induces minor damage to the brain, may activate preconditioning and/or postconditioning mechanisms and thus will protect the brain from more severe insults (Sarne et al. 2011). Indeed, our recent findings support this assumption and show that treatment with extremely low doses of THC, several days before or after PTZ (pentylenentetrazole)-induced seizures, provides effective long-term cognitive neuroprotection (Assaf et al. 2011).

The long-lasting cognitive effects of the ultra-low dose of THC were found by us to be accompanied by a delayed (24 h) activation of ERK (extracellular signal–regulated kinase) in the cerebellum (Senn et al. 2008; Amal et al. 2010). ERK has been shown to have an important role in regulating many processes of cellular homeostasis, including both cell survival and death (Agell et al. 2002; Liou et al. 2003), and is considered a crucial component in the formation of long-term memory and for the strengthening of synaptic connectivity in a variety of behavioral processes (Fasano and Brambilla 2011). Several studies claim that ERK is involved in the beneficial effects of preconditioning in the ischemic brain (Choi et al. 2006; Jin et al. 2006), yet other studies claim that ERK promotes inflammation and oxidative stress (Noshita et al. 2002; Maddahi and Edvinsson 2011). A dual effect of ERK was also seen in stroke (Sawe et al. 2008). The dual activity of ERK makes it a suitable neuronal marker to investigate the long-lasting endogenous neuronal mechanism(s) that is modulated by the low dose of THC that induced both cognitive deficits (Tselnicker et al. 2007; Senn et al. 2008; Amal et al. 2010) and neuroprotection (Assaf et al. 2011).

In the present study we followed the long-term (7 weeks) effects of an ultra-low dose of THC on the entire ERK signaling system, including its upstream and downstream effectors, in different brain regions. We also searched for biochemical interactions between the insult-inducing agent (PTZ) and the protecting factor (THC). In order to explore how general the protective effect of THC is, we tested its ability to protect the mice from various different insults, including pentobarbital-induced deep anesthesia, repeated administration of MDMA ("ecstasy") and exposure to carbon monoxide.

Experimental procedures

Animals

The study was performed on male ICR mice, 8 weeks old, weighing 30–40 gr. The animals were housed 8–10 per cage in the Animal Care Facility at a temperature of 21 °C and a 14/10-h light/dark cycle, with free access to food and water. All behavioral experiments were performed during the light phase. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Tel-Aviv University.

Treatments

In order to reduce stress at the day of treatment, the mice were habituated by daily injections of saline, at least 2 times prior to the treatment. All drugs were injected intraperitoneally (i.p) in a volume of 0.1 ml/10 g body weight. Δ9-tetrahydrocannabinol (THC) (donated by NIDA, USA, and by Prof. Mechoulam, the Hebrew University, Jerusalem) was dissolved from a stock solution made in ethanol into a vehicle solution consisted of 1:1:18 ethanol/cremophor (Sigma–Aldrich)/saline and administered in a dose of 0.002 mg/kg. Pentylenentetrazole (PTZ) (Sigma-Aldrich) was dissolved in saline and administered in a dose of 60 mg/kg. Pentobarbital (pento) (Pental veterinary, CTS, Chemical industries) was dissolved in saline and administered in a dose of 100 mg/kg. This dose of pentobarbital caused no mortality, and the mice were asleep for about 8 h after the injection. MDMA (3,4 methylenedioxymethamphetamine) was dissolved in saline and injected daily throughout three consecutive days in a dose of 10 mg/kg. Mice were exposed to carbon monoxide (CO) in a tightly closed glass chamber for 12 s thrice with 45-min intervals between exposures.

Behavioral tests

Double-blind behavioral tests have been carried out 3–7 weeks after the experimental treatment (pento, CO or MDMA with or without THC). Each experiment consisted of 4 groups of treated mice and their matched controls. In order to carry out behavioral tests of treated and control mice on the same day and under the same conditions, experiments were repeated several times with small groups of mice, and results were combined for statistical analysis. Each group of mice underwent the three behavioral tests (see below), and representative experiments are shown.

Open field

Motor activity was tested in an open field that consisted of a black plastic arena (60 cm × 60 cm) whose floor was divided into 10 cm × 10 cm squares. Each mouse was introduced to the field, and the number of squares that it crossed during a 5-min session was recorded. The arena was cleaned with alcohol after each mouse.

The dry maze

The dry maze ("oasis test") is a land-based spatial learning assay that was designed to approximate the spatial learning demands required by the Morris water maze. The test was performed according to Clark et al. (2005), with modifications (Amal et al. 2010). The maze consisted of a white plastic arena (200 cm in diameter) with 20 wells arranged in 3 concentric circles. Each well contained 0.27 ml of water. The maze was situated in a room that contained various visual clues. The mice were allowed to drink freely only 1 h per day starting from 3 days before the experiment and all along the experiment. Each experiment comprised two parts. In the first part ("training stage") all the wells were filled with water. The mouse was introduced into the arena at different starting points, with its head turned toward the wall of the arena, and the time the mouse took to find a well and drink from it was recorded. The "training stage" consisted of 3 trials of 3 min each. On the second part of the experiment (the "test stage", trials 4–10), the mouse was introduced into the same arena, with only one of the 20 wells filled with water (the same single well in all the trials). Each mouse was introduced into the arena twice daily, and the arena was cleaned with alcohol after each trial. The time the mouse took to locate the single well that was filled with water and to drink from it was recorded. If the mouse failed to locate the well within 3 min, it was gently guided to the filled well.

Object recognition test

The test utilizes the tendency of mice to explore novel objects. The assay consisted of two parts: a familiarization session and a test session. Twenty-four hour before the test, the mice were allowed to explore the black plastic arena without objects for 5 min. During the first session, the mice were left to explore for 5 min two identical objects that were located 10 cm from the side walls. Twenty-four hour later, the mice were introduced to the arena for the test session in which one of the familiar objects was replaced by a novel object. Exploration was defined as directing the nose of the mouse to the object at a distance of $\leq$1 cm and/or touching the object with the nose. The time spent by the mouse in exploring each object was recorded for 5 min, and relative time of exploration was calculated as the time spent exploring the object over total exploration time for both objects. The arena and the objects were cleaned with alcohol after each trial.

Biochemical procedures

Tissue preparation

Mice were killed by cervical dislocation after completing the behavioral tests, 7 weeks after treatment. The brain was removed and the cerebellum, hippocampus, frontal cortex and occipital cortex were dissected out and immediately frozen in liquid nitrogen and stored at −80 °C. Each sample was homogenized in 200–400 µl of ice-cold extraction buffer containing 10 mM potassium phosphate, pH 7.5, 10 mM MgCl2, 5 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 2 mM DTT, 1 % Triton X-100, 50 mM β-glycerophosphate, 0.5 % protease inhibitor cocktail (Sigma–Aldrich) and 1 % phosphatase inhibitor cocktail 1 (Sigma–Aldrich). The samples were homogenized by hand in small glass homogenizers, and the homogenates were centrifuged at 15,000×g at 4 °C for 10 min. The supernatants were stored at −80 °C. Protein concentration was determined with the Bradford Reagent (Sigma–Aldrich).

Western blotting

Homogenate aliquots containing 60 µg of total protein made in sample buffer [50 mM Tris (pH 6.8), 10 % glycerol, 5 % mercaptoethanol, 2 % SDS and bromophenol blue (Sigma-Aldrich)] were loaded and separated on 10 % SDS–polyacrylamide gels and then transferred to nitrocellulose membranes (Whatman, Schleicher & Schuell, Dassel, Germany). The membranes were blocked in 10 mM Tris–HCl (pH 7.4), 135 mM NaCl and 0.1 % Tween-20 (TTBS) containing 5 % fat-free milk powder for 1 h at room temperature and then incubated overnight at 4 °C with either a mouse monoclonal antibody against phosho(Tyr 204)-ERK1/2 (1:600; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or a rabbit monoclonal antibody against p-CREB (1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or a rabbit monoclonal antibody against BDNF (1:400; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The membranes were then washed and incubated for 60 min at room temperature with horseradish peroxidase-labeled goat anti-mouse antibody (1:10,000; Santa Cruz Biotechnology Inc.) or goat anti-rabbit (1:10,000; Santa Cruz Biotechnology Inc.) and developed using an enhanced chemiluminescence (ECL) reagent. To determine total ERK1/2 levels, the membranes were stripped by 10-min incubation in 0.1 M NaOH containing 0.2 % SDS and reprobed for 60 min in room temperature with a rabbit polyclonal antibody raised against ERK (1:1,000; Santa Cruz Biotechnology Inc.) and then washed and incubated for 60 min at room temperature with horseradish peroxidase-labeled goat anti-rabbit antibody (1:10,000; Santa Cruz Biotechnology Inc.). The films were scanned, and the optical density of the relevant band was analyzed using TINA2.07 software. To allow comparison between different films, the optical density of the bands was expressed relative to the average of control samples in each film.

Data analysis

All results are presented as mean ± SEM. Comparison of two groups was carried out by Student's t test and multiple comparisons by analysis of variance (ANOVA) followed by Tukey's post hoc test. Level of significance was set at $p \leq 0.05$.

Results

An ultra-low dose of THC protects against different insults that cause cognitive deficits

In our previous study (Assaf et al. 2011), we have shown that an ultra-low dose of THC applied 1–7 days before or 1–3 days after pentylenentetrazole (PTZ) protected against cognitive deficits that were induced by PTZ. In order to test how common the protective effect of THC was, we introduced the mice, in the present study, to three different other insults that caused cognitive deficits. First we tested whether THC, applied 24 h before pentobarbital (pento, 100 mg/kg in saline), protected against the cognitive damage of the hypnotic drug. The experiment consisted of 4 groups of mice (control, THC, pento and THC + pento) with 8–10 mice per group. The mice were tested in the object recognition assay 3 weeks after the treatment (Fig. 1). In the test session of the object recognition assay, control mice significantly preferred the new object, as expected (t test, $p < 0.05$). THC-treated mice did not show behavioral deficits and similarly preferred the new object (t test, $p < 0.05$). Mice treated with pento failed to dissociate between the two objects and did not prefer the new object. However, mice that had been treated with THC 24 h before pento preferred significantly the new object (t test, $p < 0.05$) and behaved similar to the control group.

Next we exposed mice to 100 % carbon monoxide (CO) and evaluated their behavior 3 weeks later. The experiment consisted of 4 groups of mice: control, mice exposed to CO, mice injected with THC and mice exposed to CO 48 h after THC (14–21 mice per group). The mice were evaluated 3 weeks later by the dry maze test. As depicted in Fig. 2a, in the "training stage" (trials 1–3), when all the wells were filled with water, the thirsty mice rapidly learned to drink out of the wells and there was no difference between the performance of the four groups. In the

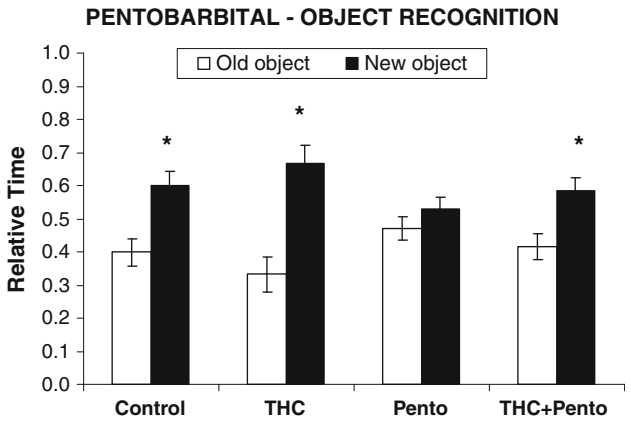

Fig. 1 Administration of an ultra-low dose of THC 24 h before pentobarbital protects the mice from pentobarbital-induced cognitive damage, as measured by the object recognition test. Control mice were injected with vehicle and 24 h later with saline; THC mice were injected with 0.002 mg/kg of THC in vehicle and 24 h later with saline; pento mice were injected with vehicle and 24 h later with pentobarbital (100 mg/kg in saline); and THC + pento mice were injected with THC and 24 h later with pentobarbital ($n = 8$–10 in each group). The mice were tested 3 weeks after treatment. Relative time (exploration time of the new object divided by total exploration time of both objects) is presented as mean ± SEM. *Asterisk* indicates a significant difference ($p < 0.05$, t test)

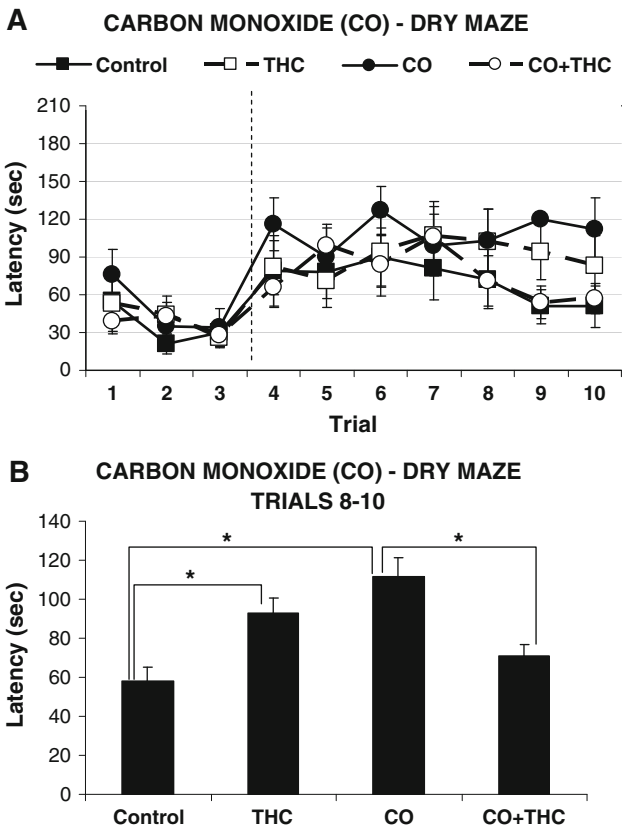

Fig. 2 Administration of an ultra-low dose of THC 48 h before exposure to CO protects the mice from CO-induced cognitive damage, as measured by the dry maze. The results were obtained from three different experiments with similar results. Each experiment consisted of 4 groups of mice ($n = 4$–10 per group in each individual experiment and $n = 14$–21 in the combined experiment presented here). The control group was injected with vehicle, the THC group was injected with 0.002 mg/kg THC, the CO group was injected with vehicle and exposed to CO 48 h later, and the THC + CO group was injected with THC and exposed to CO 48 h later. The mice were tested 3 weeks after treatment. The time the mouse took to reach the well and drink the water was measured ("latency"). **a** Performance curve of the four groups along the two stages of the test (see Methods). **b** The average time it took each group to find the well that was filled with water at the final part of the test (trials 8–10). Two-way (**a**) and a one-way (**b**) ANOVA indicated significant effects of treatment (for details see text). *Asterisk* indicates a significant ($p < 0.05$) difference between groups (Tukey post hoc)

second part (the "test stage"; trials 4–10), when only one well was filled with water and the mice had to learn and remember its location, the four groups diverged and a two-way ANOVA indicated a significant difference between the groups [$F(3,489) = 8.974$, $p < 0.05$]. It took a significantly longer time for the CO group to find the single full well than for the control group (Tukey's post hoc test, $p < 0.05$). The THC group also spent more time finding the full well than the control group, but this difference was not statistically significant. Mice that had been injected with THC 48 h before the exposure to CO behaved almost as the control group and found the full well significantly faster than the mice treated with CO (Tukey $p < 0.05$). Figure 2b presents the mean latency of the four groups at steady state (trials 8–10). One-way ANOVA revealed a significant effect of treatment [$F(3,209) = 8.621$, $p < 0.05$] with a significant difference between CO and control, THC and control, and CO and THC + CO groups (Tukey $p < 0.05$), but no difference was found between THC + CO and control mice. The results indicate that treating the mice with an ultra-low dose (0.002 mg/kg) of THC 48 h before their exposure to CO protected the mice from the cognitive deficit that was induced by CO.

In the next experiment we injected the mice with 3,4 methylenedioxymeth-amphetamine (MDMA; 10 mg/kg in saline) once a day for 3 consecutive days and tested whether THC can protect against the cognitive damage that was caused by MDMA. In preliminary experiments we found no protection when THC was injected either 24 h before or 24 h after MDMA (data not shown) and so decided to inject the mice with THC both 24 h before and 24 h after MDMA. The experiment consisted of 4 groups of mice (control, THC, MDMA and THC + MDMA + THC; 17 mice per group) that were examined for cognitive deficits 3 weeks following the last exposure to MDMA. Figure 3 depicts the second session of the object recognition test, when one of the objects in the arena was replaced by a new object. Control mice significantly preferred the new object, as expected (t test, $p < 0.05$). In contrast, neither MDMA-

treated nor THC-treated mice showed a significant preference for the new object. However, mice that were treated with THC before and after MDMA preferred the new object significantly (t test, $p < 0.05$), similarly to the control group (see "Discussion").

In all the above-described experiments, the various insults (pento, CO and MDMA) as well as THC failed to modify motor activity of the treated mice, as was measured by the open field test, suggesting no motor deficits that could interfere with the cognitive tests.

An ultra-low dose of THC induces long-term modifications in the ERK/MAPK signaling system

Our next goal was to study the long-term biochemical effects of a single injection of THC in various brain areas. We have previously shown that the ultra-low dose of THC caused an increase in ERK2 activity (phosphorylation of ERK) in the cerebellum, 24 h after the injection (Senn et al. 2008), followed by its decline 7 weeks later (Assaf et al. 2011). In the current study we tested whether there are any long-term (7 weeks) changes in ERK2 activity in other brain regions that are known to be related to memory and learning. In the frontal cortex and the hippocampus, ERK2 phosphorylation rose significantly by 22 % and by 19 %, respectively, in THC-treated mice compared to their matched controls ($n = 40$ per group, t test, $p < 0.05$) (Fig. 4). In the same mice, ERK2 phosphorylation in the cerebellum declined significantly by 27 % (t test, $p < 0.05$), and there was no alteration in ERK2 phosphorylation in the occipital cortex (Fig. 4). It is worthwhile noting that there were no changes in total ERK in any of these brain regions, indicating the augmentation or suppression of ERK activity without affecting its synthesis or degradation.

We then tested whether the decline in ERK activity in the cerebellum is due to the decline in the activity of its upstream kinase MEK or due to an elevation in its specific phosphatase MKP-1. We found that 7 weeks after a single injection of the ultra-low dose of THC, there was a non-significant decrease in MEK phosphorylation by 21 % as well as a significant decrease in MKP-1 by 36 % ($n = 8$ per group, t test, $p < 0.05$) (Fig. 5a), indicating the suppression of the entire ERK/MAPK system. We also found that ERK2 activation in the hippocampus was accompanied by a nonsignificant elevation in phosphorylated MEK by 38 % and a significant elevation of MKP-1 by 15 % ($n = 8$ per group, t test, $p < 0.05$), indicating the activation of the entire ERK/MAPK system in this brain region (Fig. 5b).

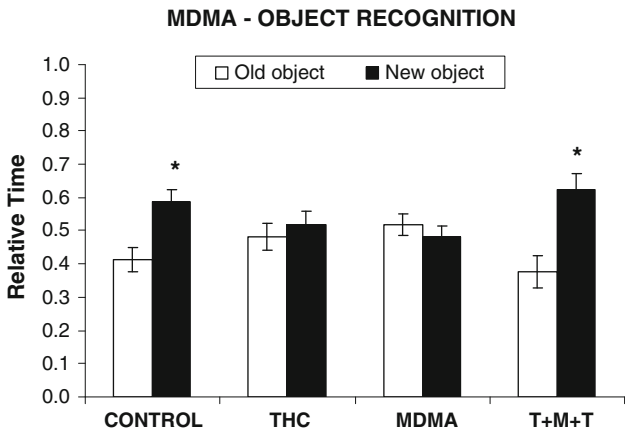

Fig. 3 Administration of an ultra-low dose of THC 24 h before and 24 h after MDMA protects the mice from MDMA-induced cognitive damage, as measured by the object recognition test. The results were obtained from two different experiments ($n = 8$–10 per group in each experiment) with similar results. All mice were injected once daily for 5 consecutive days. The control group was injected with vehicle, saline X3 and vehicle. The THC group was injected with THC (0.002 mg/kg in vehicle), saline X3 and THC. The MDMA group was injected with vehicle, MDMA (10 mg/kg in saline) X3 and vehicle. The THC + MDMA + THC group was injected with THC, MDMA X3 and THC. The mice were tested 3 weeks after treatment. The relative time (exploration time of the new object divided by total exploration time of both objects) is presented as mean ± SEM. *Asterisk* indicates a significant difference ($p < 0.05$, t test)

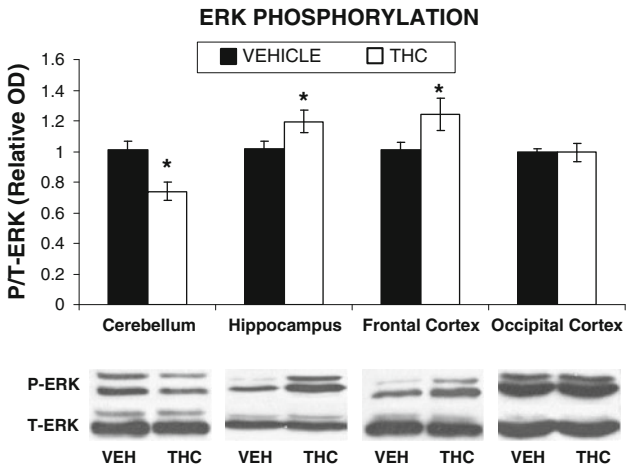

Fig. 4 An ultra-low dose of THC induces long-term changes in the phosphorylation of ERK2 in different brain regions. Mice were injected with either THC (0.002 mg/kg in vehicle) or vehicle, and the levels of phosphorylated ERK2 (P-ERK) and of total ERK2 (T-ERK) in the cerebellum, hippocampus, frontal cortex and occipital cortex were determined by densitometric analysis of Western blots, 7 weeks following the treatment. The experiments were performed 5 times, each time with 8 THC-treated mice and 8 matched controls. All the experiments showed the same results, though not always statistically significant. The histogram presents the combined results of 40 THC-treated mice and their 40 matched controls. The optical density of the band in each film was standardized to the average of controls in the same film. Results (relative OD) are presented as mean ± SEM. *Asterisk* indicates a significant difference ($p < 0.05$, t test). Lower panel: representative blots of the four different brain regions

We next looked for changes in proteins downstream to ERK that are known to be related to the formation of long-term memory and to neuroprotection. We found a significant elevation of pCREB (phosphorylated cAMP response element–binding protein) by 20 % in the hippocampus ($n = 32$ per group, t test, $p < 0.05$) but no change in its level in the frontal cortex. On the other hand, there was a significant elevation of BDNF (brain-derived neurotrophic factor) by 34 % in the frontal cortex ($n = 40$ per group, t test, $p < 0.05$) but no change in its level in the hippocampus. Figure 6 depicts the elevation of pCREB and of BDNF in the hippocampus and the frontal cortex, respectively, 7 weeks after a single injection of 0.002 mg/kg of THC.

Biochemical interaction between THC and PTZ

The behavioral interaction between THC and PTZ, in which THC protected the mice from the cognitive damage that was induced by PTZ (Assaf et al. 2011), led us to look for possible biochemical interactions between these two drugs. The experiment consisted of 4 groups of mice, 16 mice per group: control, THC (0.002 mg/kg), PTZ (60 mg/kg) and THC applied 24 h before PTZ. The mice underwent the behavioral tests on weeks 4–6 and then were killed 7 weeks after the treatment. In the hippocampus, THC caused a significant elevation of 35 % in phosphorylated ERK2 compared to the control group (t test, $p < 0.05$) (Fig. 7). PTZ caused a nonsignificant elevation of 14 % in phosphorylated ERK2. When THC was applied 24 h before PTZ, the level of phosphorylated ERK2 was significantly lower than the level in the PTZ group by 27 % (t test, $p < 0.05$), than the level in the THC group by 39 % (t test, $p < 0.05$) and than the level in the control group by 17 % (Fig. 7). We then calculated the expected additive effect of the two drugs on ERK activity that summed up to an elevation of 50 % compared to control (see last column in Fig. 7). There was a clear and significant difference between the observed and the expected effect of THC and PTZ when applied together (see "Discussion").

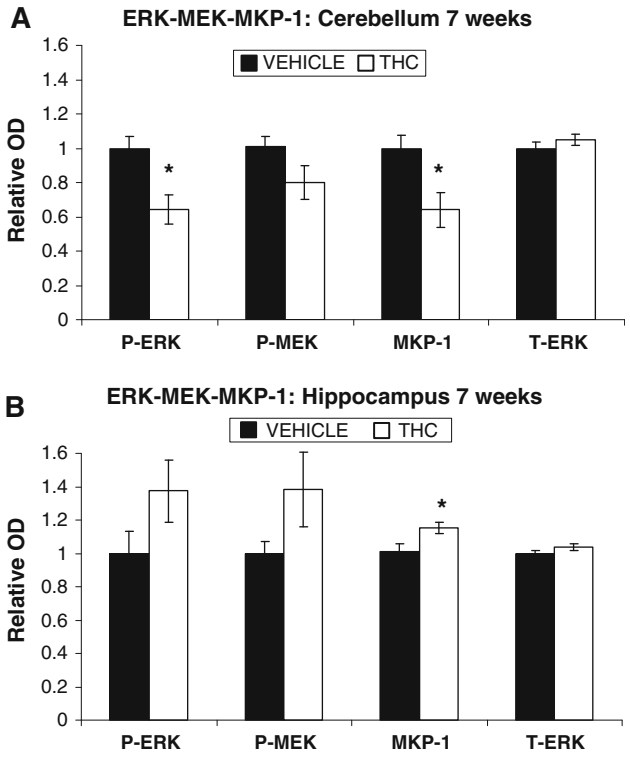

Fig. 5 An ultra-low dose of THC induces long-term changes in phosphorylated ERK2 (P-ERK), phosphorylated MEK (P-MEK) and MKP-1 in the cerebellum (**a**) and hippocampus (**b**), 7 weeks after its single injection. Mice ($n = 8$ per group) were injected with either THC (0.002 mg/kg) or vehicle, and the levels of P-ERK2, P-MEK, MKP-1 and total ERK2 (T-ERK2) in the cerebellum and hippocampus were determined by densitometric analysis of Western blots, 7 weeks following the treatment. The optical density of the bands in each film was standardized to the average of controls in the same film. Results (relative OD) are presented as mean ± SEM. *Asterisk* indicates a significant difference ($p < 0.05$, t test)

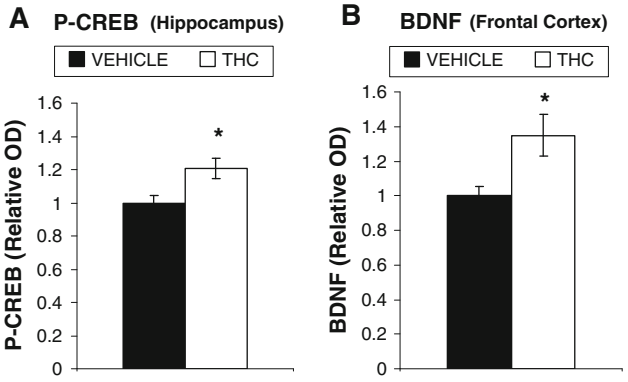

Fig. 6 An ultra-low dose of THC induces long-term changes in phosphorylated CREB (P-CREB) in the hippocampus (**a**) and in BDNF in the frontal cortex (**b**). Mice were injected with either THC (0.002 mg/kg in vehicle) or vehicle, and the levels of P-CREB and BDNF in the hippocampus and the frontal cortex were determined by densitometric analysis of Western blots, 7 weeks following the treatment. The experiments were performed 4 times for pCREB and 5 times for BDNF, each experiment included 8 THC-treated mice and 8 controls. In each of the experiments, there was a nonsignificant elevation in pCREB in the hippocampus and in BDNF in the frontal cortex. The histogram presents the combined results of 32/40 THC-treated mice and their 32/40 matched controls. Results (relative OD) are presented as mean ± SEM. *Asterisk* indicates a significant difference ($p < 0.05$, t test)

Discussion

Preconditioning is a phenomenon where a minor noxious stimulus protects from a subsequent insult. In our previous

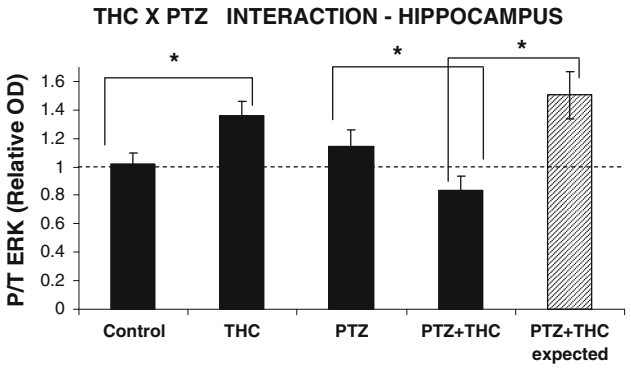

Fig. 7 The interactive effect of THC and PTZ on ERK2 in the hippocampus 7 weeks after treatment. The experiments were performed twice, each time with 8 mice per group. The experiments showed the same results, and the histogram presents the combined results of 16 mice per group. The control group was injected with vehicle and 24 h later with saline; the THC group was injected with THC (0.002 mg/kg in vehicle) and 24 h later with saline; the PTZ group was injected with vehicle and 24 h later with PTZ (60 mg/kg in saline); and the THC + PTZ group was injected with THC and 24 h later with PTZ. The levels of phosphorylated ERK2 (P-ERK) and of total ERK2 (T-ERK) in the hippocampus were determined by densitometric analysis of Western blots, 7 weeks following the treatment. "Expected PTZ + THC" (stripped column) was determined by combining the effects of THC and of PTZ in single mice in the two groups. Results are presented as mean ± SEM. *Asterisk* indicates a significant difference ($p < 0.05$, *t* test)

study we showed that an ultra-low dose of Δ^9-tetrahydrocannabinol (THC) that by itself induced a minor cognitive deficit protected against a more severe cognitive damage caused by the epileptogenic agent pentylenentetrazole (PTZ) (Assaf et al. 2011). In order to test how general the neuroprotective effect of THC was, we employed, in the present study, various insults that act through different mechanisms and investigated the protective effect of THC. We found that the ultra-low dose of THC protected the mice from cognitive deficits that were caused by pentobarbital-induced deep anesthesia, by carbon monoxide (CO)-induced hypoxia and by the aminergic modifying drug 3,4 methylenedioxymethamphetamine (MDMA; "ecstasy"). As depicted in Fig. 1, when THC was applied 24 h before the anesthetic barbiturate pentobarbital, mice were protected from the cognitive damage created by pentobarbital itself. Though THC did not induce any detectable cognitive damage in this specific experiment, the protection was still evident, implying that a considerable damage by itself was not necessary for inducing the compensatory mechanism(s) that led to neuroprotection. When THC had been applied 48 h before CO (Fig. 2), a toxic gas that binds to hemoglobin and prevents the effective distribution of oxygen to the brain, the cognitive damage caused by CO was abolished. In this experiment, THC by itself also caused a mild cognitive deficit. Surprisingly, mice that were treated with both insults (THC before CO) behaved not only better than CO-treated mice but even better than the THC-treated mice. It appears as if each insult activated compensatory mechanisms that protected against the preceding or the following insult. In this regard it is worth mentioning that pre- and postconditioning share common signaling pathways (Hausenloy and Yellon 2009) and that each insult can protect against a completely different type of another insult ("cross-conditioning") (Wada et al. 1999; Lin et al. 2009a, b). An alternative explanation assumes that a single minor insult just primes the endogenous compensating system, while the second insult activates it. Thus, two consecutive insults are required in order to fully activate the endogenous protective mechanism(s). A similar situation, where an amnesic drug (morphine) protected from the amnesic effect of another drug (lipopolysaccharide), was recently described (Rostami et al. 2012).

When THC was applied 24 h before and after MDMA, a drug that potentiates the activity of serotonin, norepinephrine and dopamine, the cognitive damage was abolished and the mice behaved as the controls (Fig. 3). Similar to the CO experiment, the mice that received both THC and MDMA behaved better than single drug-treated mice, implying a cross-protection between THC and MDMA as well. In this experiment it was required to treat the mice with THC both before and after MDMA in order to achieve an effective protection. Indeed, an additive effect of pre- and postconditioning treatments was previously demonstrated for both neuroprotection (McMurtrey and Zuo 2010) and cardioprotection (Sato et al. 2007).

Our behavioral experiments demonstrate that preconditioning with an ultra-low dose of THC is not specific for a certain type of damage, but exerts a more general neuroprotective effect. Cannabinoids have been previously found by others to protect the brain from different insults. It should be emphasized that all previous studies employed much higher doses of the cannabinoid agent (1–10 mg/kg) that were administered either immediately before (up to 60 min) (Nagayama et al. 1999; van der Stelt et al. 2001a, b; Hayakawa et al. 2007; Tourino et al. 2010) or immediately after (up to 210 min) (Shouman et al. 2006; Hayakawa et al. 2007) the insult. When the injection of the cannabinoid was delayed by 1 h (Panikashvili et al. 2001) or by 8 h (Shouman et al. 2006), it failed to protect the brain. These studies are in a sharp contrast to our current and previous (Assaf et al. 2011) results, where a long-term protection by an ultra-low dose of THC was observed when the drug was applied 1–7 days before or 1–3 days after the insult. The conventional neuroprotective properties of cannabinoids are attributed, among other factors, to their ability to suppress voltage-gated calcium channels (Mackie and Hille 1992) and consequently to attenuate the release of glutamate (Shen et al. 1996). The modulation by

cannabinoids of other mechanisms, such as the inhibition of NO synthesis (Hillard et al. 1999) and the inhibition of the release of the pro-inflammatory cytokine tumor necrosis factor alpha (TNFα) (Facchinetti et al. 2003) was also suggested. Other cannabinoid actions that may contribute to their direct neuroprotective effects include the induction of hypothermia (Leker et al. 2003) and vasodilatation (Wagner et al. 2001), and their anti-inflammatory effects (Maresz et al. 2007; Zhang et al. 2007; Fernandez-Ruiz et al. 2008). All these studies pointed to the acute protective features of cannabinoids that were dependent on the presence of the drug in a high enough concentration close to the time of insult. We believe that the long-term (24–48 h) neuroprotective effect of the ultra-low dose of THC that was demonstrated in our current study was dependent on the activation of long-lasting compensatory mechanisms, as had been previously suggested for other preconditioning treatments (Hausenloy and Yellon 2009). It is not clear yet what was the immediate target of THC that mediated its long-term protective effect. Nevertheless, in our previous study (Senn et al. 2008) we found that SR141716A completely blocked the long-lasting deteriorating effect of the ultra-low dose THC, suggesting the involvement of CB1 cannabinoid receptors.

The long-term protective effect of the ultra-low dose of THC, as well as the induction of cognitive deficits that lasted for up to 5 months (Tselnicker et al. 2007; Amal et al. 2010), led us to look for long-term biochemical changes in the brain that may underlie these behavioral outcomes. Extracellular signal–regulated kinase (ERK) is considered an important mediator of both preconditioning and post-conditioning (Gidday 2006; Hausenloy and Yellon 2006; Hausenloy and Yellon 2009; Pignataro et al. 2009). In our previous study (Senn et al. 2008) we found the activation of ERK in the cerebellum 24 h after the injection of an ultra-low dose of THC to mice. This delayed activation differed from the previously reported acute effect of the regular high doses (1–15 mg/kg) of THC that caused a rapid pERK elevation (10–30 min after injection) which then declined (Derkinderen et al. 2003; Rubino et al. 2004). In the current study we found that a single injection of the ultra-low dose of THC induced long-lasting changes in ERK activity in various brain regions. Seven weeks after the injection, ERK activity was elevated in the frontal cortex and the hippocampus and suppressed in the cerebellum. The modulation of ERK activity coincided with a parallel modulation of its activating enzyme MEK and its inactivating enzyme MKP-1, indicating the activation of the entire ERK signaling system in the hippocampus and its suppression in the cerebellum. It was previously suggested that the suppression of ERK phosphorylation, rather than its stimulation, is related to the protective effect of cannabinoids against kainite-induced neuroinflammation (Zhang and Chen 2008).

The differential effect of THC on different brain regions was also manifested by the finding that different downstream proteins were activated in different brain regions 7 weeks after the injection of THC. In the hippocampus we found a significant elevation of phosphorylated cAMP response element–binding protein (CREB) and no changes in the level of brain-derived neurotrophic factor (BDNF), while in the frontal cortex we found no changes in phosphorylated CREB, but a significant elevation in BDNF. Both these proteins were previously found to be acutely (15–60 min) activated by the conventional (1–15 mg/kg) doses of THC (Derkinderen et al. 2003; Rubino et al. 2004). Furthermore, both proteins are considered to be involved in the mechanism of preconditioning. Thus, a mild ischemic insult used for preconditioning was found to elevate BDNF in the hippocampus (Truettner et al. 2002) and to prevent the decline in BDNF that was caused by lethal ischemia to the brain (Lee et al. 2008). Similarly, activation of CREB was found in neonatal rat brains when ischemic preconditioning was introduced to protect against hypoxic–ischemic brain injury (Lee et al. 2004; Lin et al. 2009a, b). The protective role of these two proteins may be attributed to their participation in long-term memory formation and synaptic plasticity (Yin and Tully 1996; Lipsky and Marini 2007; Cunha et al. 2010). The relevance of each of these proteins to the long-lasting behavioral effects of the ultra-low dose of THC awaits further research.

In order to further investigate whether the long-term effect of THC on ERK activity is related to its protective properties, we searched for possible biochemical interactions between THC and one of the insulting agents (PTZ). We found that THC and PTZ, when applied separately, elevated ERK phosphorylation in the hippocampus; hence, we expected to receive an even greater elevation when applying the two drugs together. However, when THC was applied 24 h before PTZ, there was no additive effect; on the contrary, the elevation of pERK disappeared, and its levels were similar to those in the control group (Fig. 7). This biochemical interaction resembled the behavioral interaction, where the cognitive effects of THC and PTZ failed to appear when both drugs were applied to the same mice [see Fig. 1 in (Assaf et al. 2011)], and as was also seen with the other insults in the present study (see Figs. 2, 3).

In summary, we have demonstrated that a single injection of an ultra-low dose of THC can protect the brain from different insults that induce cognitive deficits.

When a chronic insult was employed (a daily application of MDMA), it was necessary to re-administer THC. The protective effect of THC lasted for many hours and provided an expanded therapeutic time window. These findings suggest that low doses of THC may be used to protect the brain not only against acute damage but also against

chronic insults or even against neurodegenerative diseases. The behavioral effects of THC coincided with a long-lasting modulation of the ERK signaling system and its downstream proteins BDNF and CREB in different brain regions. It is suggested that a treatment with such an extremely low dose of THC has a potential to provide safe, long-term neuroprotection, without the undesired psychotropic effects of the conventional doses of the cannabinoid drug, and without inducing downregulation of cannabinoid receptors that may interfere with the protective effects of conventional doses of cannabinoid drugs (Zhang and Chen 2008).

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