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NEUROBIOLOGICAL MECHANISMS UNDERLYING THE COGNITIVE DEFICITS ASSOCIATED WITH THE NICOTINE WITHDRAWAL SYNDROME

TREBALL DE FI DE GRAU

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1. INTRODUCTION

Tobacco use is a global pandemic that affects approximately 1.2 billion people and produces a substantial health burden. With approximately 5 million tobacco-related deaths annually, tobacco smoking is the leading cause of preventable premature mortality in the world (Polosa and Benowitz, 2011). Tobacco smoke contains over 4000 chemicals, many of which have marked irritant properties, are known carcinogens and can enhance the addictive effects of nicotine (Berrendero et al., 2010). Death is primarily caused by lung and other cancers, coronary heart disease and stroke, and also by infectious diseases. Even though smokers know that quitting before the onset of tobacco-related disease can avoid the increased mortality risk, near 80% smokers attempting to quit will relapse within the first month of abstinence (Benowitz et al., 2009). More than half of smokers try to quit each year, and approximately one-third use medication to assist their quit attempt (Peto et al., 2000). There are currently three FDA-approved pharmacological treatments for nicotine dependence: nicotine replacement therapy (NTR), bupropion and varenicline (Polosa and Benowitz, 2011). However, among those treated with varenicline, 44% are abstinent at the end of treatment, and at 6 months, this number drops to 27%. Abstinence rates for combination NRT (e.g., patch plus inhaler) at 6 months are only slightly higher at 32% (Ray et al., 2009). Therefore, the need to investigate the neurobiological mechanisms involved in tobacco addiction is crucial in order to develop more effective treatments (Ashare et al., 2014).

Nicotine is the primary component of tobacco that maintains the smoking habit and develops addiction. Moreover, the reinforcing effects of nicotine are thought to be the primary reason why humans inhale tobacco smoke (Shoaib, 2006). When a person inhales smoke from a cigarette, nicotine is distilled from the tobacco and is carried in smoke particles into the lungs, where it is absorbed rapidly into the pulmonary venous circulation. It then enters the arterial circulation and moves quickly to the brain. Nicotine diffuses readily into brain tissue, where it binds to nAChRs, which are ligand-gated ion channels with a central cation pore composed of five subunits, which can either be homomeric or heteromeric. The nAChR complex is found in both the peripheral and central nervous system and 12 different neuronal nAChR subunits have been identified: α2-α10 and β2-β4 (Benowitz, N.L. 2009). The predominant nAChR contain α4 and β2 subunits and binds nicotine with high affinity (Gotti et al., 2009). This kind of nAChR is extensively present in several brain areas such as the ventral tegmental area, the nucleus accumbens, prefrontal cortex and hippocampus. When nicotine binds to the nAChR enhances dopamine transmission from the ventral tegmental area to the

nucleus accumbens, amygdala and prefrontal cortex, activating the reward system (Corrigall et al., 1992). The nAChRs play a modulatory role on the activity of multiple neurotransmitters, as they have been detected on presynaptic terminals, cell bodies and dendrites of many neuronal subtypes (Dajas-Bailador and Wonnacott, 2004). The activation of nAChRs by nicotine increases the release of most neurotransmitters (Berrendero et al., 2010). As a consequence, nicotine modifies a large number of physiological processes such as locomotion, nociception, anxiety, learning and memory, as well as produces several behavioral responses directly related to its addictive properties (Picciotto and Mineur, 2014)

Addiction is a complex behavioral phenomenon with causes and effects that range from molecular mechanisms to social interactions. Essentially, the process of nicotine addiction begins with molecular interactions that alter the activity and metabolism of the neurons that are sensitive to nicotine. Over time this alters the properties of individual neurons and circuits, and this leads to complex behaviors including dependence, tolerance, sensitization, and craving (Polosa and Benowitz, 2011).

Besides the rewarding properties of nicotine and the mechanisms involved in the long-term relapse to nicotine consumption, the manifestations of the nicotine withdrawal syndrome following tobacco cessation are another critical component of nicotine addiction. In humans, nicotine from tobacco induces stimulation and pleasure, and reduces stress and anxiety. Abstinence from smoking produces unpleasant physiological, affective, and cognitive withdrawal symptoms that peak within the first few days of nicotine deprivation (Hugues, 2007). Among them, cognitive impairments including impaired attention and working memory deficits are gaining attention as a crucial dependence phenotype and a target for development efforts (Ashare et al., 2014). Thus, studies in humans have shown that working memory deficits predict short-term smoking resumption following brief abstinence (Patterson et al., 2010). Moreover, the partial agonist α4β2 varenicline, which is used as treatment for tobacco addiction, prevents the abstinence-induced cognitive deficits also in humans (Loughead et al., 2010). The explanation about the relationship between these cognitive deficits and relapse is not clear at present, but deficits in prefrontal executive function observed during nicotine withdrawal could disrupt the motivation necessary to maintain abstinence (Ashare et al., 2014). According to the findings in humans studies, withdrawal from chronic nicotine results in learning deficits in rodent models, which could be evaluated by measuring several emotional symptoms such as increased anxiety, aversive effects and reward deficits (Gould and Leach, 2014).

As a whole, these data support the idea that the existence of cognitive deficits during nicotine withdrawal could be an important factor in the short-term relapse of tobacco consumption. Hence, understanding the behavioral and neurobiological substrates underlying the cognitive deficits associated with nicotine withdrawal is crucial for the treatment of nicotine addiction.

Recent studies have shown that the endocannabinoid system is involved in the common neurobiological mechanism underlying drug addiction. This system consists of cannabinoid receptors, endogenous ligands and several proteins responsible for their synthesis and degradation (Mechoulam et al., 2012). Two subtypes of cannabinoids receptors, CB_1 (CB_1R) and CB_2 (CB_2R) have been characterized. Both CB_1R and CB_2R are G protein-coupled receptors with quite different distributions in the central nervous system (CNS) and peripheral tissues. CB_1R is highly expressed in the CNS, while CB_2R is mainly localized in immune cells, although it is also expressed in brain neurons (Maldonado et al., 2013). The most relevant endogenous ligands for cannabinoid receptors are N-arachidonoylethanolamine (anandamide) and 2-arachidonoylglycerol (2-AG). These endocannabinoids are synthesized on demand, mainly postsynaptically and act as retrograde messengers regulating the presynaptic release of neurotransmitters (Maldonado et al., 2006). Anandamide (AEA) and 2-AG are produced from cell membrane lipids via different biosynthetic pathways. Anandamide acts as a partial agonist at both CB_1R and CB_2R. 2-AG is the most abundant endocannabinoid in the CNS and activates both CB_1R and CB_2R (Maldonado et al., 2013).

The endocannabinoid system regulates a range of physiological processes, including memory, anxiety, and nociception. Thus, endocannabinoids facilitate extinction of aversive memories, and naturally occurring cannabinoids, such as delta-9-tetrahydrocannabinol (THC), impair learning and memory in humans and in several animal models (Busquets-Garcia et al., 2011; Marsicano et al., 2002). Several studies support the view that the endocannabinoid system represents a new candidate for the control of drug rewarding properties. This system participates in the primary rewarding effects of cannabinoids, nicotine, alcohol and opioids, through the release of endocannabinoids in the ventral tegmental area. The endocannabinoid system also participates in the common mechanisms underlying relapse to drug-seeking behavior by mediating the motivational effects of drug-related environmental stimuli and drug re-exposure (Maldonado et al., 2006).

During the past decades, different empirical studies have documented that several neurological disorders are characterized by disruptions in dendritic spine shape, size or number. Dendritic spines are small protrusions from neuronal dendrites that form the postsynaptic component of most excitatory synapses in the brain. They play critical roles in synaptic component of most excitatory synapses in the brain (Calabrese et al., 2006). Since changes in spine shape and size are correlated with the strength of excitatory synapses, spine morphology directly reflects spine function. Perturbed spines are likely to have diverses functional effects such as synapse loss or aberrant signaling and plasticity, resulting in significant clinical manifestations (Fiala et al., 2002). Thus, studying spines structure and function and unveiling the specific mechanisms that regulate spine formation and morphology is essential for understanding the cellular changes that underlie learning and memory in normal and pathological conditions (Bellot et al., 2014).

2. OBJECTIVES

- Set up of a behavioral model to evaluate the cognitive deficits associate with the nicotine withdrawal syndrome.

- Evaluate the involvement of the endogenous cannabinoid system in the memory impairment observed during nicotine withdrawal.

- Measure changes in synaptic plasticity related to the cognitive deficits of nicotine abstinence.

Our general objective is to provide a better understanding of the mechanisms underlying nicotine withdrawal-cognitive deficits. These new data can contribute to the identification of new targets with potential usefulness for the treatment of nicotine dependence in humans.

3. METHODS

Animals

Experiments were performed in male C57BL/6J mice (Charles River) and in male CB_1 knockout mice. Wild-type C57BL/6J mice (8-10 weeks old) were used as a control. Mice were housed four per cage in a temperature (21± 1 ºC) -and humidity (55± 10 %)-controlled room with a 12-h/12-h light /dark cycle (light between 08:00 to 20:00). Mice were exposed to a normal cycle (lights on 08:00 A.M) and the experiments took place during the light phase. Mice had ad libitum access to food and water. The observer was blind to treatment in all the experiments. Animal procedures were conducted in accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and approved by the local ethical committee (CEEA-IMAS-UPF) (Comitè Ètic d'Experimentació Animal-Institut Municipal d'Assistència Sanitària-Universitat Pompeu Fabra).

Drugs

(-)-Nicotine hydrogen tartrate salt [(-)-1-methyl-2(3-pyridyl) pyrrolidine] (Sigma, Madrid, Spain) was dissolved in physiological saline (0.9% NaCl) and administered at the dose of 25 mg/kg/day by using subcutaneously implanted osmotic minipumps. Nicotine dose was calculated as (-)-nicotine hydrogen tartrate salt. Mecamylamine hydrochloride (2 mg/kg) (Sigma) was dissolved in physiological saline and administered by subcutaneous route (s.c) in a volume of 10 ml/kg body weight. The CB_1 antagonist rimonabant (1 mg/kg) (kindly provided by Sanofi-Aventis Recherche) was diluted in 5% ethanol, 5% cremophor-EL, 90% saline and administered intraperitoneally (i.p).

Nicotine treatment and withdrawal

Nicotine dependence was induced by using osmotic minipumps (Model 2002, Alzet®, Cupertino, CA, USA) (Figure 1). Minipumps were implanted while mice were anesthetized via isoflurane; a small incision was made on the lower back of the mouse, the pump was implanted, and the wound was closed with surgical staples. Minipumps were filled with saline or nicotine solutions and delivered a constant subcutaneous flow in rate of 0.5 µl/h during 14 days. Nicotine concentration was adjusted to compensate for differences in mice body weight. Thus, the average-weighed mice received a dose of 25 mg/kg/day of nicotine. On the 14^{th} day after minipump implantation, nicotine withdrawal syndrome was precipitated by the administration of the nicotinic receptor antagonist, mecamylamine (2mg/kg, s.c).

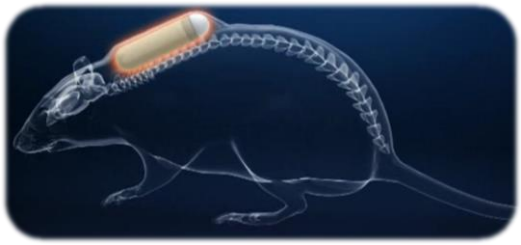

Figure 1
Schematic representation of the implantation of osmotic minipumps.

Object recognition test

This test provides the basis for the study of a wide range of cognitive and neuropsychological issues in rats and mice. Object recognition tasks exploit the natural propensity of rodents to spontaneously explore novel objects over familiar objects and consists of three phases: habituation, familiarization or training and test phase.

Object recognition memory was evaluated in the V-maze, made of black plexiglas with two corridors (30 cm long x 4.5 cm wide) set in V with a 90° angle, and 15 cm high walls (Figure 2). A camera mounted above the open field arena was used for recording and observation of behavior.

Figure 2
Schematic representation of the object recognition test.

1. Set up of the model to reveal the memory impairment during nicotine abstinence

On the 13th day after the implantation of nicotine minipumps delivering saline or nicotine, mice were habituated to the V-maze for 9 min. During this time each animal is allowed freely exploring the V-maze in the absence of objects. The animal is then removed from the arena and placed in its holding cage. On the 14th day, mice were placed back into the open field arena for 9 min and allowed to explore two identical objects placed in the corners of the maze (training phase). Nicotine withdrawal was

precipitated 20 min after training by the injection of mecamylamine (2 mg/kg) allowing us to test the influence of the withdrawal in memory consolidation. On the 15th day, the test phase, mice were again placed in the V-maze for 9 min, where the familiar object used the day before was replaced by a novel object. During this phase, mice were scored for exploratory behavior (the total time spent exploring each of the two objects, novel, Tn and familiar, Tf was counted) via observation on a television monitor using hand-held stop watches. Object exploration was defined as a mouse having its nose directed toward the object within approximately less than 2 cm. Climbing or sitting on objects was not scored as object exploration. Objects were made out of different materials (marble or plastic) and shapes, and had been tested in previous experiments to show no preference or dislike for control mice. A discrimination index was calculated as the difference between the time spent exploring either the novel or familiar object divided by the total time exploring the two objects **D.I = [Tn-Tf] / [Tn+Tf]** (Figure 3). A higher discrimination index is considered to reflect greater memory retention for the familiar object.

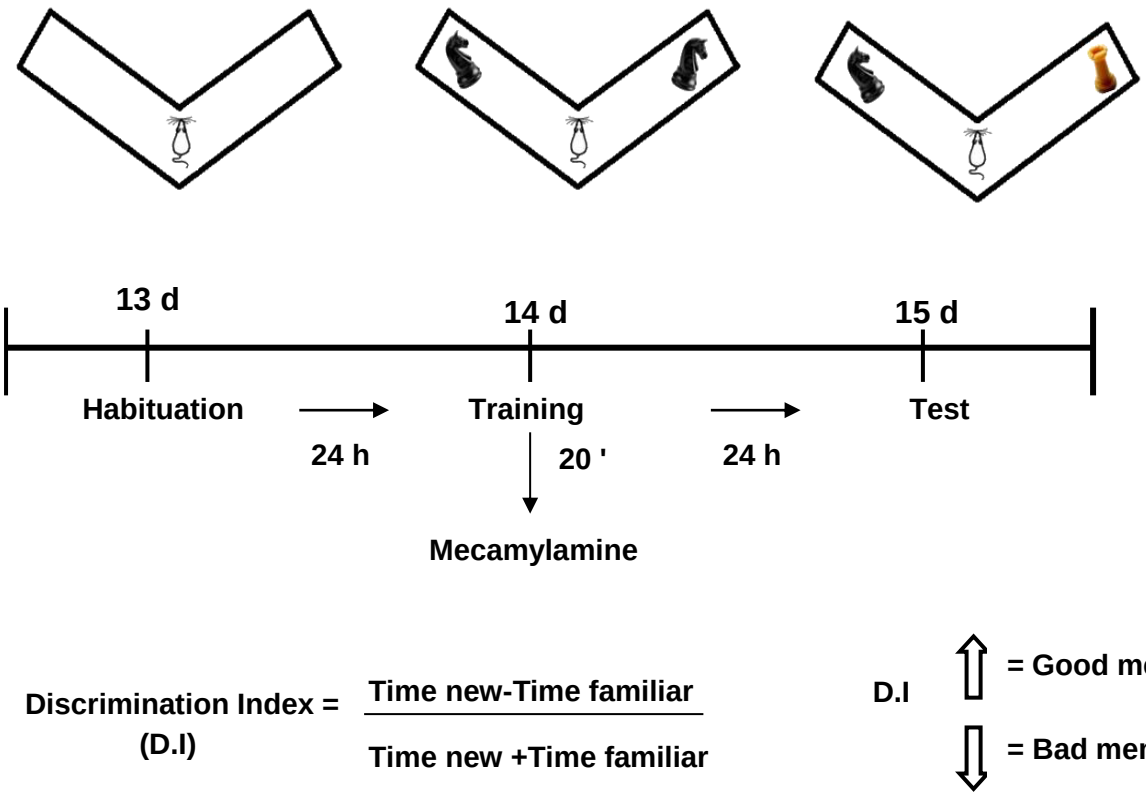

Figure 3
Schematic representation of the object recognition test model used to reveal the memory impairment during nicotine abstinence.

2. Effects of the chronic nicotine treatment in memory consolidation

To evaluate the possible effects of the chronic nicotine treatment on memory we performed the object recognition test before precipitation of the nicotine withdrawal syndrome. On the 11th day after the implantation of nicotine minipumps delivering saline or nicotine, mice were habituated for 9 min to the V-maze. On the 12th day, mice were placed back in the maze for 9 min where two identical objects were presented as training. On the 13th day mice were again placed in the V-maze for 9 min, where the familiar object used the day before was replaced by a novel object and the total time spent exploring each of the two objects (novel, Tn and familiar, Tf) was computed (Figure 4). A discrimination index was calculated as previously described.

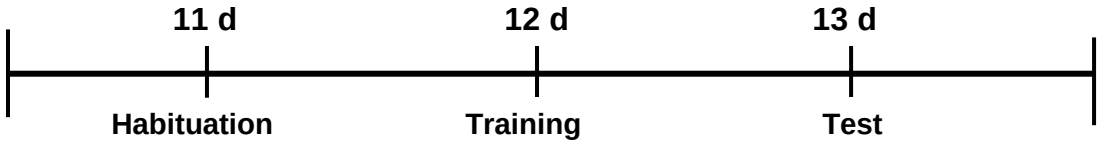

Figure 4
Schematic representation of the object recognition test model used to study the effects of the chronic nicotine treatment in memory cnsolidation.

3. Duration of the cognitive deficit associated with nicotine withdrawal

The duration of the cognitive effects associated with nicotine withdrawal on object recognition memory was evaluated in the V-maze 2, 4 or 8 days after the precipitation of withdrawal using different cohorts of animals for each experiment. On the 13th day after minipumps implantation, mice were placed in the V-maze for habituation. On the 14th day, mice were placed back in the V-maze for training, where two identical objects were positioned at the end of the corridors. Nicotine withdrawal was precipitated 20 min after training by the injection of mecamylamine (2mg/kg, s.c). 1, 3 or 7 days after withdrawal precipitation mice were placed in the V-maze for training, where two identical objects were positioned at the end of the corridors. The next day, which was the 2nd, 4th or 8thday after precipitation of withdrawal, animals were placed in the maze where the familiar object used the day before was replaced by a novel object. The total time spent exploring each of the two objects (novel, Tn and familiar, Tf) was computed and the discrimination index (D.I.) was calculated as follows: D.I. = [Tn-Tf] / [Tn+Tf].

4. Effects of the CB_1 antagonist rimonabant in the memory impairment associated with the nicotine withdrawal syndrome

Based on the same protocol as previously described, on the 13th day mice were habituated, on the 14th day took place the training phase and on 15th day the test phase. To evaluate the possible involvement of the CB_1 cannabinoid receptor on the memory impairment associated with the nicotine withdrawal syndrome, we injected rimonabant immediately after training, 20 min before the precipitation of the nicotine withdrawal with mecamylamine. A similar experiment was performed in CB_1 knockout mice.

Rimonabant (1mg/kg) was also chronically administered during 4 days to evaluate if this administration can prevent the memory deficit observed 4 days after the precipitation of withdrawal. For this purpose we performed the same habituation (13d) and training phase (14d) as before. The days 15th, 16th and 17th we injected rimonabant or vehicle and finally on 18th day we performed the test phase (Figure 5).

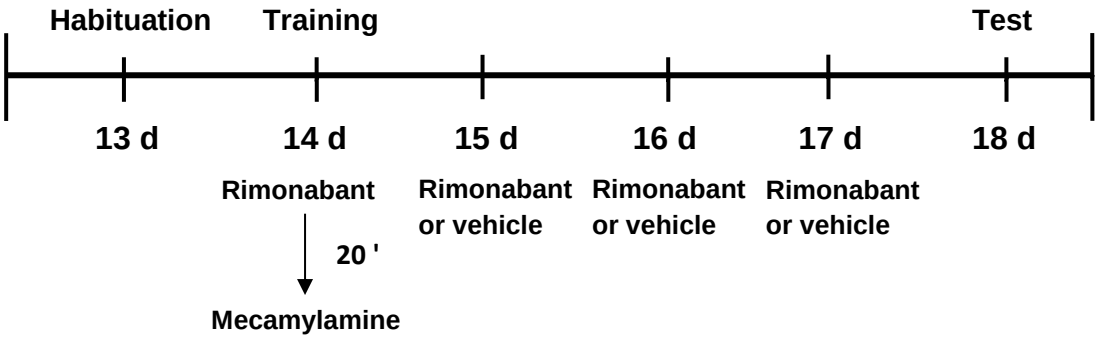

Figure 5
Schematic representation of the object recognition test model used to study the effects of the CB_1 antagonist rimonabant in the memory impairment associated with the nicotine withdrawal syndrome

Endocannabinoid quantification

Tissues were extracted 10 min after mecamylamine-precipitated nicotine withdrawal. A half right or left brain were homogenized on ice with a glass homogenizer in 1 mL 0.02 % TFA (pH 3.0). Aliquots of brain homogenate were used for anandamide (AEA) and 2-arachidonoylglycerol (2-AG) analysis. Aliquots were processed and analyzed with the liquid chromatography-mass spectrometry (LC-MS)/MS system. The endogenous concentrations of AEA and 2-AG were calculated based on the response of the deuterated analogues, as previously reported (Busquets–Garcia et al., 2011).

Changes in synaptic plasticity in neurons from the hippocampus and the prefrontal cortex

The possible duration of the memory impairment during nicotine abstinence can be related to changes in synaptic plasticity. These possible modifications in synaptic plasticity were evaluated by measuring changes in the morphology of dendritic spines. Dendritic spines are actin-rich protrusions from the dendritic shaft, considered to be the locus where most synapses occur, as they receive the vast majority of excitatory connections in the central nervous system (CNS) (Bellot et al., 2014).

The tissue was obtained 4 days after the precipitation of nicotine withdrawal when the memory deficit was still present. Mice will be deeply anesthetized by intraperitoneal injection (0.2 ml/10 g body weight) of a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg) prior to rapid intracardiac perfusion, delivered with a peristaltic pump with paraformaldehyde (PFA). Brains were quickly removed from the skull and postfixed in PFA for 10 min. Brain coronal sections (100µm) containing the hippocampus and the prefrontal cortex can be obtained by using a vibratome (Leica VT 1000 S, Nussloch, Germany) and kept in PBS 0.1 M until they are processed for fluorescent labeling. Brain slices were labeled by ballistic delivery of fluorescent dye DiI (Molecular Probes, Eugene, OR, USA) using a gene gun apparatus (Helios Gene Gun System, Bio-Rad, Deutschland) as described previously (Grutzendler et al., 2003) and postfixed with PFA for 4 hours at room temperature to further preserve structures and to allow the diffusion of the dye DiI. Sections were place on microscope gelatin-coated slides and coverslipped with mounting medium (Mowiol). Then, images were acquired with confocal microscope (Zeiss LSM 510, Germany) with and oil immersion lens (63x) to analyze dendritic spine density and structure. Distal dendrites (from secondary dendrites to terminal dendrites) and with a minimum of 20 µm per dendrite were analyzed by using IMARIS analysis software (Guegan et al., 2013).

Spines are typically categorized into different groups (Figure 6). Based on anatomical studies of fixed brain tissue, three main categories were established: the most common are the thin spines (long neck and smaller bulbous heads, <0.6 µm in diameter); mushroom spines (constricted neck and large bulbous head, >0.6 µm in diameter); and stubby spines (similar head and neck widths). Other approaches using confocal microscopy have enabled the distinction of additional spine categories. Filopodia-like spines are transient protrusions mostly found on developing spines and characterized by the absence of the spine head. Spines can also be branched, which have elongated spine necks with two or more spine heads (Bellot et al., 2014).

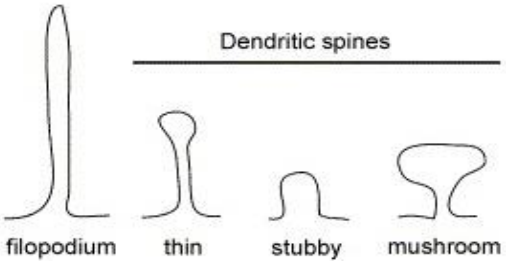

Figure 6
Schematic representation of the classification of dendritic spines.

Data analysis

The data were analyzed using two-way ANOVA with nicotine and pre-treatment/ genotype as the between factors of variation. Subsequent post-hoc analysis was used when required. Student T-test was used when comparing two experimental groups.

4. RESULTS

Mecamylamine-precipitated nicotine withdrawal results in memory impairment

Studies in humans have shown that abstinence from smoking is associated with difficulty in concentration, impairments in attention, deficits in learning and memory, and disrupted cortical plasticity. Deficits in learning and other cognitive processes are major symptoms of nicotine withdrawal and are strongly associated with relapse. Nicotine re-exposure reverses this negative effect of tobacco abstinence suggesting that relapse after smoking cessation may occur to ameliorate this cognitive impairment. Therefore we investigated whether memory consolidation was impaired during nicotine withdrawal in mice by using the object recognition task. Mice were examined in the object recognition task 24 h after the precipitation of nicotine withdrawal with mecamylamine (2mg/kg, s.c). A significant reduction in the discrimination index was observed in mice chronically treated with nicotine compared to saline-treated animals ($P < 0.01$) (Figure 7a). Moreover, we evaluated the duration of the memory impairment caused by nicotine abstinence. For this purpose, we performed the object recognition test 2, 4 or 8 days after the precipitation of withdrawal using different cohorts of animals. A significant difference in the discrimination index was found 2 and 4 days after the precipitation of nicotine withdrawal between mice chronically treated with nicotine or saline (1, 2 and 4 days, $P< 0.01$). We found no significant difference between animals treated with nicotine or saline 8 days after the precipitation of withdrawal (Figure 7b).

To ensure that the memory impairment observed during nicotine abstinence only corresponded to the withdrawal precipitation, we evaluated the effect of the chronic nicotine treatment on memory. We performed the object recognition task after 13 days of the minipump implantation, when the minipump was still releasing nicotine or saline (Figure 7c). There was no significant difference between animals treated with nicotine or saline ($P> 0.05$).

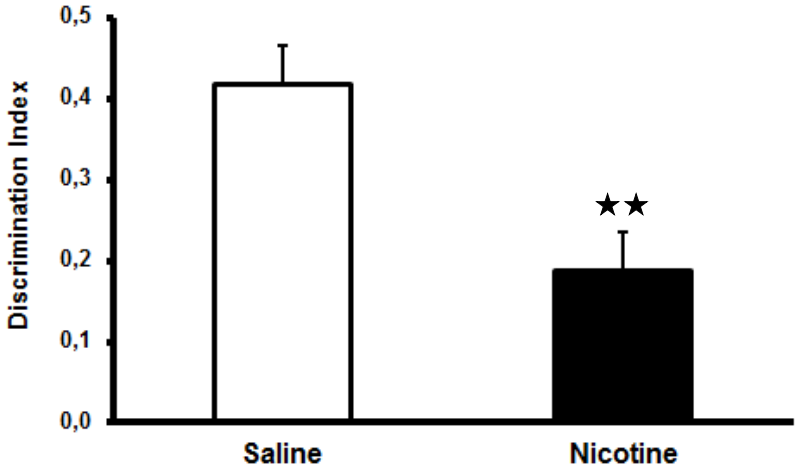

Figure 7a

Mecamylamine-precipitated nicotine withdrawal impairs memory consolidation in the object recognition task. Discrimination index values in mice implanted with minipumps delivering nicotine (25 mg/kg/day, during 14 days) or saline (n= 9-12 mice per group). Data are expressed as mean ± S.E.M. ★★ P< 0.01 (nicotine versus saline).

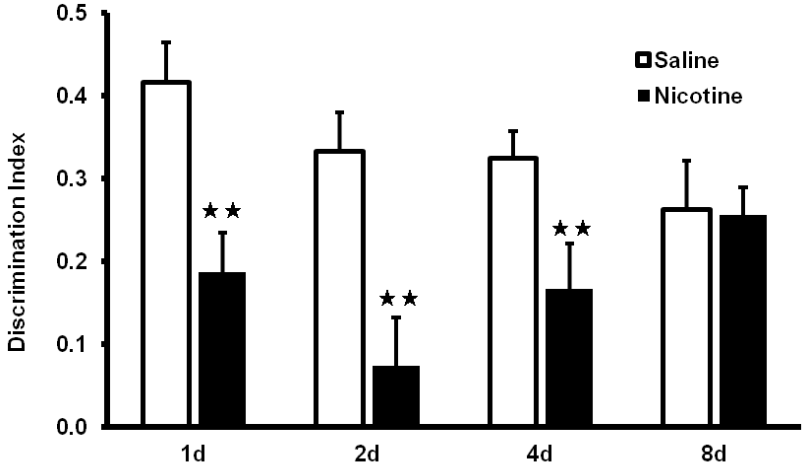

Figure 7b

The cognitive deficit was still present 4 days after the precipitation of nicotine withdrawal. After 8 days of withdrawal, animals recovered from the cognitive impairment. Discrimination index values in mice implanted with minipumps delivering nicotine (25 mg/kg/day, during 14 days) or saline (n=9-12 mice per group). Data are expressed as mean ± S.E.M. ★★ P< 0.01 (nicotine versus saline).

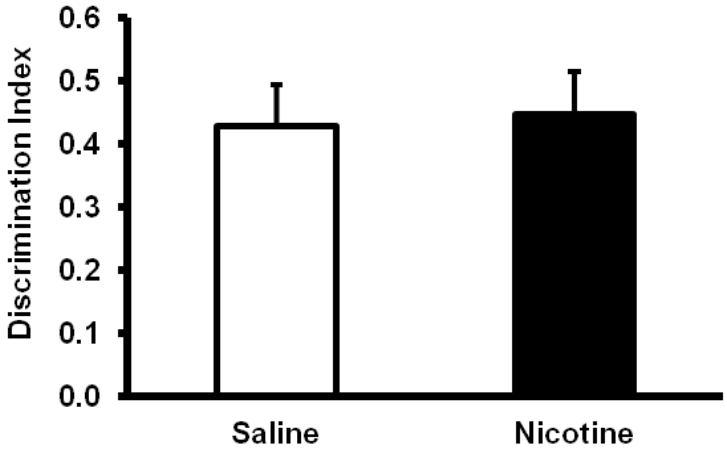

Figure 7c
Chronic nicotine treatment has no effect in memory. Discrimination index values in mice implanted with minipumps delivering nicotine (25 mg/kg/day, during 14 days) or saline (n=6-7 mice per group). No significant differences were found between treatments.

CB_1 cannabinoid receptors are involved in the memory impairment associated with the nicotine withdrawal syndrome

During the last decade it has become evident that endogenous ligands for cannabinoid receptors, or endocannabinoids, are released in an activity-dependent manner in areas of the brain crucial for memory processing, such as the hippocampus, the amygdala and the pre-frontal cortex. In fact, impairment of cognition and memory is perhaps the most pervasive alteration induced by acute exposure to THC or to synthetic cannabinoids (Clarke et al., 2008).

Based on the fact that activation of the CB_1 receptor impairs memory formation and consolidation, we wondered whether the activation of the cannabinoid receptors could be involved in the memory impairment observed during nicotine withdrawal. Rimonabant (1mg/kg) or vehicle were administered immediately after training, 20 min before the precipitation of the nicotine withdrawal syndrome with mecamylamine. Rimonabant treatment was able to block the cognitive deficit of the nicotine withdrawal (Figure 8a). Thus, two-way ANOVA revealed a significant interaction between nicotine treatment and rimonabant pre-treatment [$F_{(1,24)}$ = 10.78, $P< 0.01$]. Subsequent post-hoc analysis confirmed a blockade of the memory deficit when rimonabant was administered ($P< 0.01$). To corroborate the implication of the CB_1 cannabinoid receptor in this cognitive deficit, we carried out a similar experiment using CB_1 knockout mice (Figure 8b). Two-way ANOVA revealed a significant interaction between nicotine treatment and genotype [$F_{(1,21)}$ = 6.809, $P < 0.05$]. Post-hoc analysis showed an abolishment of the memory impairment in mice lacking CB_1 receptors ($P< 0.05$).

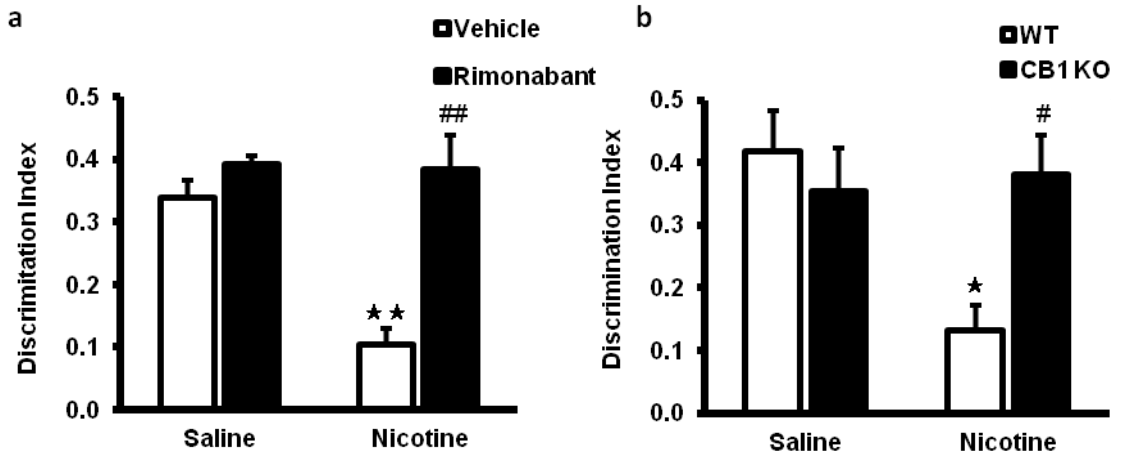

Figure 8

CB_1 receptors mediate the cognitive deficits induced by the precipitation of nicotine withdrawal. (a) Discrimination index values in the object recognition test of mice pretreated with rimonabant (1 mg/kg, i.p) 20 min before the precipitation of the nicotine withdrawal (n=5-12 mice per group). Data are expressed as mean ± S.E.M.; ★★ P< 0.01 (compared to chronic saline treatment), # # P< 0.01 (comparison between pretreatments). (b) Discrimination index values during precipitated nicotine withdrawal in wild-type and CB_1 knockout mice (n=5-6). Data are expressed as mean ± S.E.M. ★ P< 0.05 (compared to chronic saline treatment), # P< 0.05 (comparison between genotypes).

2-Arachidonoylglycerol levels are increased after the precipitation of the nicotine withdrawal syndrome

To corroborate the hypothesis that an activation of cannabinoid receptors is involved in the cognitive deficits associated with nicotine withdrawal and considering that CB_1 receptors are activated by endogenous cannabinoids such as 2-AG and AEA, we measured endocannabinoids levels (2-AG and AEA) in brain homogenates 10 min after the precipitation of nicotine withdrawal by using (LC-MS)/MS. Student T-test showed an increase in the levels of 2-AG (P< 0.05), but not AEA (Figure 9), in whole brain homogenates 10 min after precipitation of withdrawal.

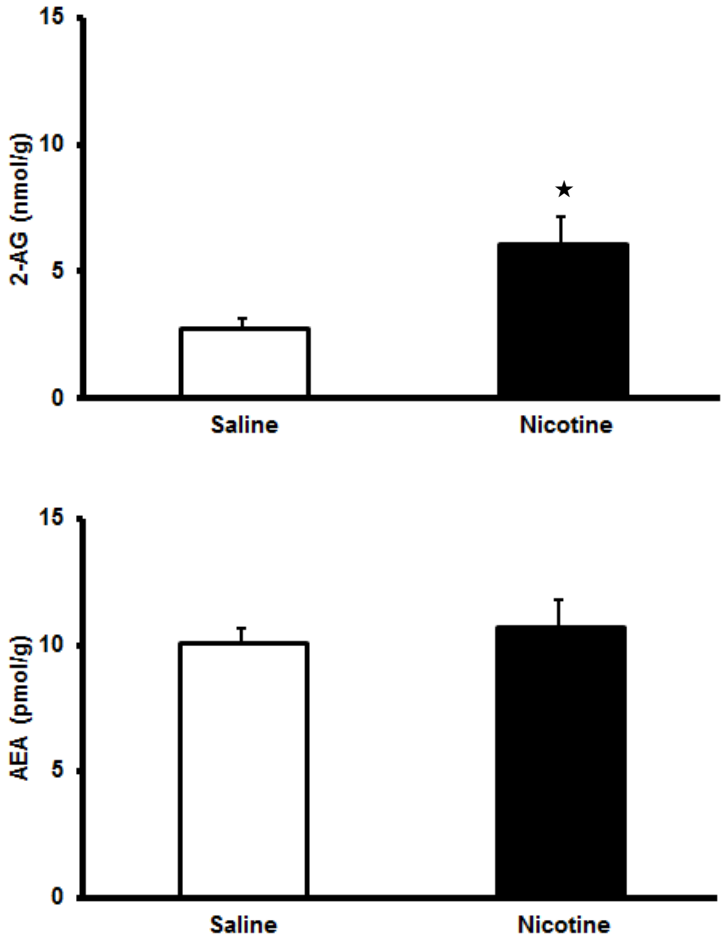

Figure 9

Brain concentrations of 2-arachidonoylglycerol (2-AG) and anandamide (AEA) 10 min after the precipitation of nicotine withdrawal by using the liquid chromatography–mass spectrometry. AG levels are increased during nicotine abstinent (n=8 mice per group). Data are expressed as mean ± S.E.M. ★ P< 0.05 (compared to chronic saline treatment).

CB_1 cannabinoid receptors are involved in changes in synaptic plasticity underlying the memory impairment observed 4 days after the precipitation of withdrawal

As previously show, the cognitive deficit associated with nicotine withdrawal last at least 4 days. We wondered if a chronic administration of rimonabant (1mg/kg) during 4 days, can prevent the deficit 4 days after the precipitation of withdrawal. Two way ANOVA revealed a significant interaction between nicotine treatment and rimonabant pretreatment [$F_{(1,39)}$ = 8.75, P<0.01]. Subsequent post-hoc analysis confirmed that chronic treatment with rimonabant was able to prevent the memory deficit (P<0.05) (Figure 10).

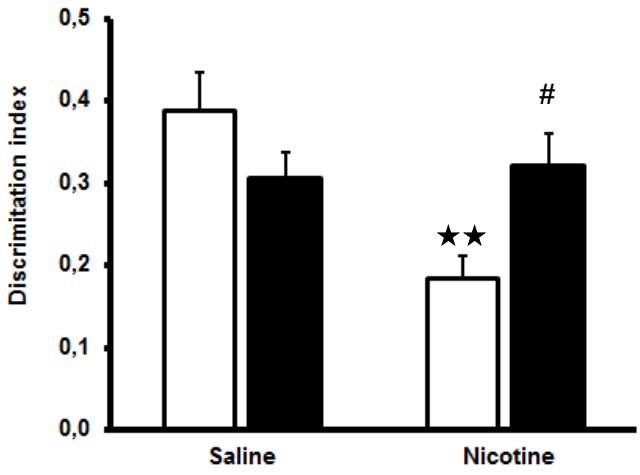

Figure 10

Chronic treatment with rimonabant was able to block the cognitive deficit 4 days after the precipitation of withdrawal. Discrimination index values in the object recognition test of mice pretreated with rimonabant (1mg/kg, i.p) during 4 days (n=11-13 mice per group). Data are expressed as mean ± S.E.M.; ★★ P< 0.01 (compared to chronic saline treatment), # P < 0.05 (comparison between pretreatments).

The possible duration of the memory impairment during nicotine abstinence can be related to changes in synaptic plasticity. These possible modifications in synaptic plasticity were evaluated by measuring changes in the morphology of dendritic spines by using the Diolistic labeling with the fluorescent dye DiI. Hipoccampal and prefrontal cortex dendritic spines density and morphology were evaluated 4 days after the precipitation of withdrawal. There were no significant changes in the total spine density in both the hipoccampus and the prefrontal cortex (Figure 11). Dendritic spines differ in shape and area depending if they are inmature or mature spines. Typical classification refers to thin spines as inmature while stubby and mushroom are considered as mature spines. We observed changes in the morphology of dendritic spines in the hipoccampus of nicotine abstinent mice. Interestingly, chronic administration with rimonabant reversed these changes in plasticity. Thus, two-way ANOVA showed a significant interaction between nicotine treatment and rimonabant pretreatment [$F_{(1,26)}$ = 7.48, P<0.05]. Animals treated chronically with nicotine have a decrease in the density of mature mushroom spines (P<0.01). Subsequent post-hoc analysis showed that this decrease can be blocked with rimonabant pretreatment (P<0.01) (Figure 12). No differences were found in mature or inmature spines in the prefrontal cortex of nicotine abstinent animals (data not shown).

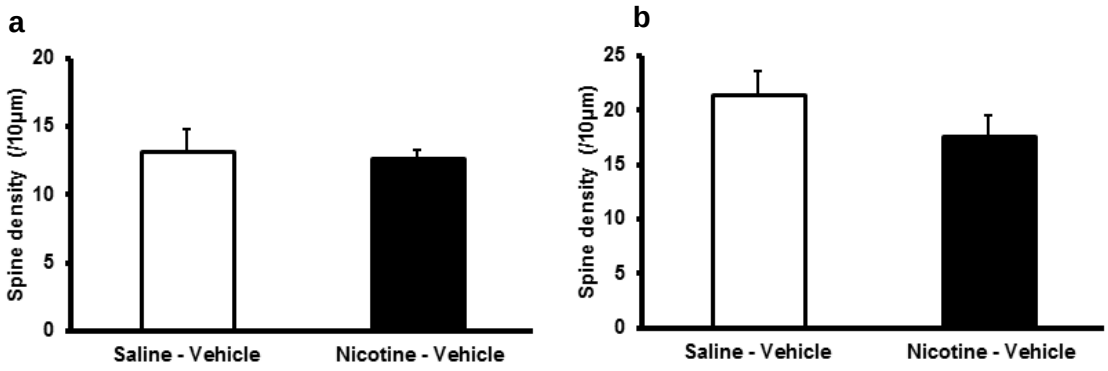

Figure 11

Spine density of hipoccampal (a) and prefrontal cortex dendritic spines (b). No significant differences were found between groups in the hippocampus (n= 7-8 mice per group) and the prefrontal cortex (n= 4 mice per group).

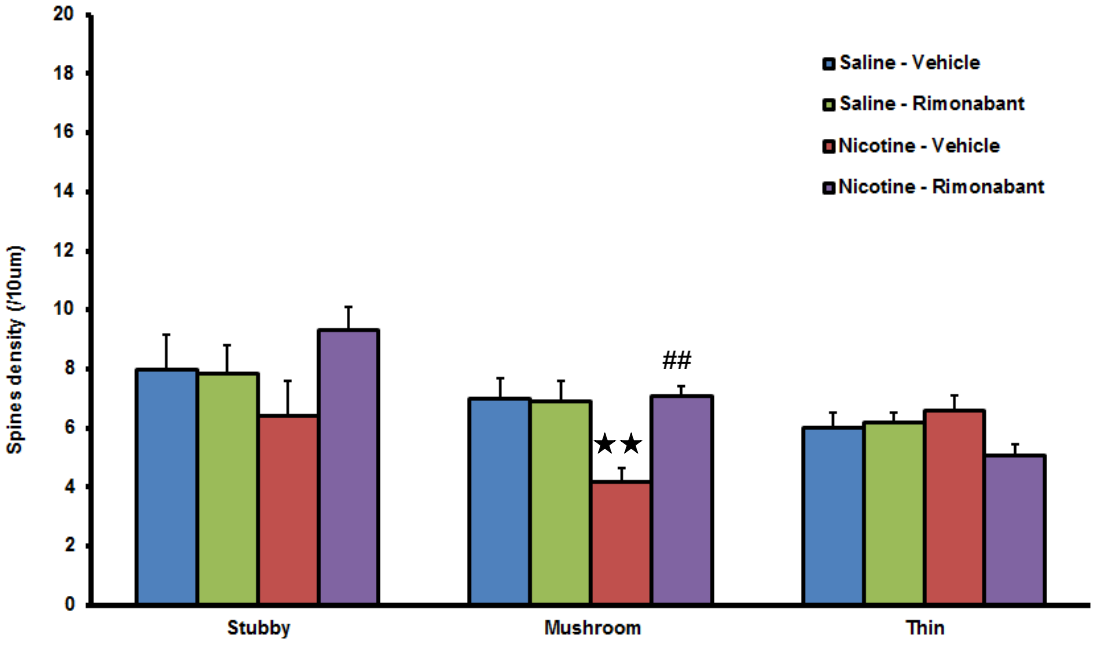

Figure 12

Animals treated chronically with nicotine have a decrease in the spine density of mushroom spines. Spine density values of mice treated chronically with nicotine or saline and pretreated with rimonabant or vehicle. Data are expressed as mean ± S.E.M.; ★★ $P < 0.01$ (compared to chronic saline treatment), # # $P < 0.01$ (comparison between pretreatments).

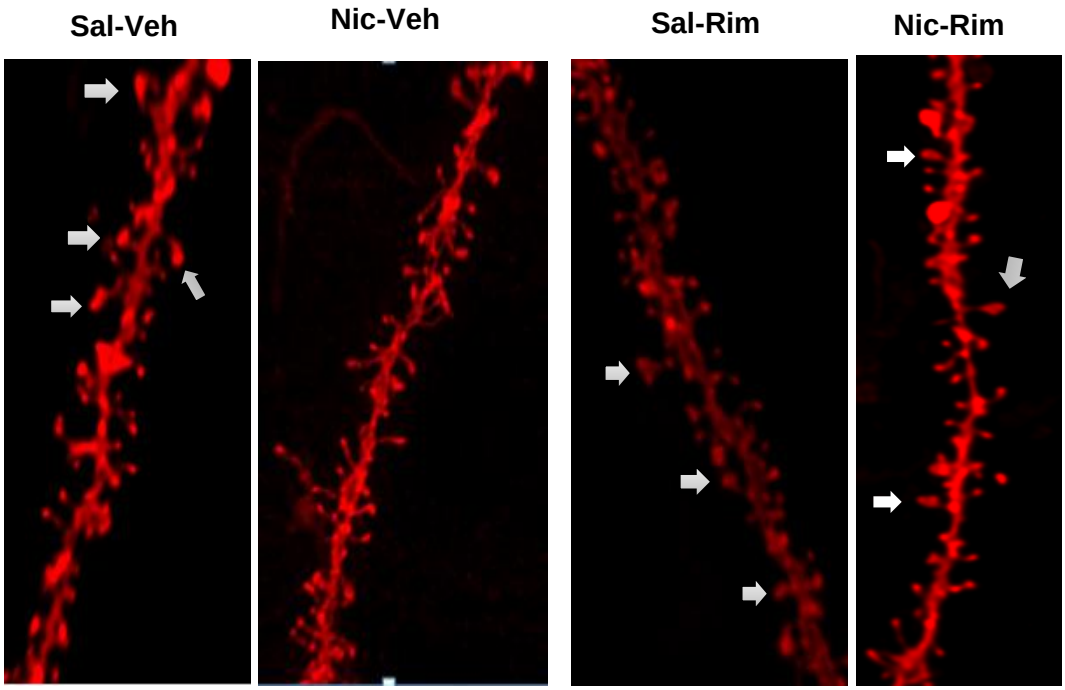

Figure 13
Representative images of dendritic spines from hipoccampus in the different experimental groups, 4 days after precipitation of withdrawal (n= 7-8 mice per group). Arrows indicate mature mushroom dendritic spines.

5. DISCUSSION

Our results demonstrate that precipitation of nicotine withdrawal by the nicotinic antagonist mecamylamine results in a cognitive deficit that is still present even four days after the precipitation of the withdrawal syndrome. Also, we show that this cognitive deficit is not present after a chronic nicotine treatment, corroborating that the memory impairment is specifically associated with nicotine abstinence. We reveal that CB_1 cannabinoid receptors are involved in the memory impairment associated with the nicotine withdrawal syndrome. Moreover, we observe an increase of AG levels after the precipitation of the nicotine withdrawal syndrome, demonstrating the involvement of the endocannabinoid system in this deficit. Finally we describe that the memory impairment is related to changes in synaptic plasticity in the hipoccampus. We observe a decrease in the density of mature dendritic spines in the hipoccampus of abstinent nicotine mice which could be related to the cognitive deficits observed in these animals.

Tobacco use is a major public health challenge that leads to millions of preventable deaths every year. Despite this, tobacco consumption is common throughout the world. Nicotine produces a biological dependence that makes quitting difficult, near 80% smokers attempting to quit will relapse within the first month of abstinence (Benowitz et al., 2009; Lerman et al., 2007). Abstinence from smoking produces a range of withdrawal symptoms, including impaired attention and working memory (Jacobsen et al., 2005; Mendrek et al., 2006). Relapse after smoking cessation may occur to ameliorate the cognitive impairment because nicotine re-exposure reverses this cognitive deficit (Davis et al., 2005; Myers et al., 2008; Loughead et al., 2010). Furthermore, Patterson et al. (2010) demonstrated that poor cognitive performance during nicotine abstinence predicted more rapid smoking resumption. These data support the idea that the existence of cognitive deficits during nicotine withdrawal could be an important factor in relapse of tobacco consumption (Ashare et al., 2014). In the present study, we observed that precipitation of nicotine withdrawal produces a cognitive deficit in the object recognition task, in agreement with previous results that showed that withdrawal from chronic nicotine treatment results in learning deficits in the contextual fear conditioning task in mice (Davis et al., 2005). The duration of the memory deficits was of at least four days after precipitation of withdrawal. A similar duration has been found in human studies, where the memory impairment was present 3 days after the last cigarette consumption (Patterson et al., 2010). This could indicate that long-term changes are needed to maintain the cognitive deficit.

The endocannabinoid system has been reported to participate in a range of physiological processes such as drug addiction and memory (Maldonado et al., 2013; Marsicano et al., 2002). Moreover, it has been described that activation of the CB_1 receptor in the hippocampus impairs memory formation and consolidation (Wise et al., 2009; Clarke et al., 2008). Therefore we studied the possible role of the endocannabionoid system in the cognitive deficit of the nicotine withdrawal syndrome due to the important function of this system in memory processes (Marsicano et al., 2008). The administration of the CB_1 receptor antagonist, rimonabant prevented the memory impairment produced by the nicotine withdrawal, even 4 days after the precipitation of this syndrome. A similar result was observed in CB_1 knockout mice confirming a crucial role of this receptor in this process.

CB_1 receptors are activated by endogenous cannabionoids such as 2-AG and AEA (Busquets-Garcia et al., 2011; Maldonado et al., 2006), which could be involved in the memory impairment observed during nicotine withdrawal. We observed a significant increase in brain 2-AG levels in nicotine-treated mice during nicotine withdrawal, although no significant differences were observed in AEA levels. In contrast with these data, fluctuation in levels of AEA, but not in 2-AG, has been recently reported in several rat brain regions during spontaneous nicotine withdrawal (Cippitelli et al., 2011).

The duration of the cognitive deficit suggests that the mechanism underlying the memory impairment implies long-term neurobiological changes that can be reversed with the administration of rimonabant. We evaluated the existence of possible changes in synaptic plasticity by measuring changes in synaptic plasticity by measuring the morphology and density of dendritic spines in the hipoccampus and prefrontal cortex of nicotine abstinent mice. This study has focused on hippocampal dendritic spines which contain a dense array of molecules involved in postsynaptic signalling and synaptic plasticity. Thereby they are thought to undergo structural changes associated with learning and memory (Bellot et al., 2014)

Myriad psychiatric and neurological diseases are characterized by spine loss or morphological disruption (Blanpied and Ehlers., 2004). Spines become malformed or lost in epilepsy, stroke, trauma, schizophrenia, dementia, major depression, normal aging, and chronic substance abuse (Fiala et al., 2002; Glantz and Lewis., 2001). Because spine morphology is linked to synaptic function, altered spines in disease states are likely to have diverse functional effects (Van Spronsen and Hoogenraad, 2010). Cognitive deficits in neurological disorders are frequently characterized by aberrant spine structure and plasticity that are restricted to the critical brain regions affected by disease (Bellot et al., 2014). Chronic neurodegenerative diseases such as

Alzheimer disease and Parkinson disease often include spine loss or other dendritic changes in the early stages of disease progression (Selkoe, 2002). Although it is not known whether spine abnormalities are a direct cause of cognitive deficits or are secondary to some other event, a molecular understanding of spine function will shed light on the implications of these region-specific defects (Calabrese et al., 2006). For instance, neurodevelopmental disorders such as Down's syndrome or Fragile X syndrome (FXS) show a failure to convert filopodia to dendritic spines, leaving adult dendrites in an immature state, and thereby leading to synaptic dysfunction and learning and memory deficits (Busquets-Garcia et al., 2013; Van Spronsen and Hoogenraad, 2010).

In our study we evaluated hipoccampal and prefrontal dendritic spines density and morphology after 4 days after precipitation of withdrawal. Although there were no significant changes in the total spine density of hipoccampal and the prefrontal cortex, we observed changes the density of mature spines in the hipoccampus. Our results showed that there was a decrease of mature spines associated with the cognitive deficit. Interestingly this decrease was blocked with rimonabant pretreatment.

In summary, we identify the CB_1 cannabinoid receptors as a potential target to treat the cognitive deficits related to the nicotine withdrawal syndrome. Understanding the neurobiological mechanisms underlying the memory impairment associated with nicotine abstinence could be crucial for the treatment of nicotine addiction.

6. CONCLUSIONS

- We set up a behavioral model and demonstrated that precipitation of nicotine withdrawal by the nicotinic antagonist mecamylamine resulted in a cognitive deficit that is still present even four days after the precipitation of the withdrawal syndrome.

- We revealed that the endocannabinoid system is involved in the memory impairment associated with the nicotine withdrawal syndrome.

- We showed that there was a decrease of mature spines in the hipoccampus associated with the cognitive deficit. We also revealed that this decrease was blocked with rimonabant pretreatment.

In conclusion we identified the cannabinoid system as a target to treat the cognitive deficits associated with the nicotine withdrawal syndrome. These results could be of clinical interest due to the increasing evidence that indicate the memory impairment during withdrawal as an important factor in tobacco smoking.

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